# EFFECT OF KIWIFRUIT ACTINIDIN ON THE DIGESTION OF GLUTEN PROTEINS

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**ISURI ACHINTHA JAYAWARDANA**

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MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA
UNIVERSITY OF NEW ZEALAND

Riddet Institute
ADVANCING FRONTIERS IN FOOD SCIENCE

# Abstract

Gluten proteins are resistant to complete proteolysis by the human gastrointestinal tract (GIT) enzymes, due to their high proline- and glutamine-rich peptide sequences. Proline confers resistance to proteolysis by digestive enzymes, producing indigestible proline-rich peptides, some of which can trigger immunogenic reactions that are responsible for gluten-related health disorders such as coeliac disease, wheat allergy and gluten sensitivity.

At present, gluten-free diets (GFD) are the only promising therapy for gluten-related health disorders. However, maintaining a lifelong GFD is challenging. As an alternative therapy, gluten-specific enzymes to hydrolyse immunogenic peptides have shown promising results. Most of these are of microbial origin. Identification of natural alternative enzymes is desirable, with fruit-borne enzymes a possible solution. Actinidin, a cysteine protease found in most green kiwifruit (*Actinidia deliciosa*), is suggested as an effective exogenous enzyme, to be utilized in this category.

The objective of this PhD study was to evaluate the effect of actinidin on the digestion of gluten and gluten-derived immunogenic peptides in the GIT. The effectiveness of actinidin was tested using different *in vitro* GIT models and an animal (pig) preclinical model with purified gluten or whole wheat bread as sources of gluten, and purified actinidin or and fresh green kiwifruit as sources of actinidin. Analytical techniques such as free amino nitrogen determination, enzyme-linked immunosorbent assay and both targeted and untargeted mass spectrometry were used to determine the degree of hydrolysis (DH), R5 gluten epitopes and immunogenic peptides respectively.

Actinidin hydrolysed peptide bonds adjacent to proline residues in the 33-mer peptide, one of the most immunogenic gluten peptides. The gastric DH of gluten proteins was influenced by an interaction between pH and actinidin concentration ($P < 0.05$). Actinidin at a concentration of $\geq$ 2.7 U/mL and pH > 2 during hydrolysis was considered ideal for gluten hydrolysis. Actinidin increased ($P < 0.05$) the rate of acceleration of DH of gluten and reduced the amount of R5 epitopes present in the small intestine using a semi-dynamic *in vitro* GIT digestion model. Actinidin also accelerated the gastric hydrolysis of wheat proteins in whole wheat soda bread, which was reflected in a faster reduction of R5 epitopes in the gastric conditions and the rate of reduction ($P < 0.05$) of most of the immunogenic marker peptides present in the small intestine.

*In vivo*, the presence of dietary actinidin in the form of green kiwifruit significantly ($P < 0.01$) enhanced the gastric digestion of wheat proteins in whole wheat soda bread fed to pigs as a model of human GIT digestion. The amount of R5 epitopes was lower ($P < 0.01$) in the stomach, proximal and distal small intestine and terminal ileum of pigs fed diets containing green kiwifruit ($P <0 .05$). The number of immunogenic peptides in the proximal small intestine was low in the pigs fed green kiwifruit diet compared to that of the pigs fed yellow kiwifruit diet (control). In addition, a diet containing green kiwifruit markedly reduced ($P < 0.05$) the amount of seven gluten immunogenic marker peptides including the 33-mer peptide in the stomach chyme of pigs. Actinidin was able to survive peptic proteolysis and gastric pH conditions until 300 min postprandial in pigs.

Taken together, these results suggest that actinidin enhanced the rate of proteolysis of both purified gluten and gluten in a food matrix and reduced the amount of immunogenic gluten epitopes reaching the small intestine during GIT digestion *in vitro* and *in vivo*. Actinidin was able to reduce both the amount of and the time of exposure to immunogenic peptides in the small intestinal lumen, therefore it is a promising candidate to be considered in oral enzyme therapy for gluten-related health disorders.

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# List of Abbreviations

| | |
|---|---|
| ANOVA | Analysis of variance |
| ATIs | Amylase trypsin inhibitors |
| CBZ | N-α-CBZ-lys-p-nitrophenol hydrochloride |
| CD | Coeliac disease |
| CD4/CD8 | Cluster of differentiation 4/8 |
| DGP | Deamidated gliadin peptide |
| DH | Degree of hydrolysis |
| DM | Dry matter |
| DPP IV | Dipeptidylaminopeptidase IV |
| DQ | Dragon quest |
| ELISA | Enzyme-linked immunosorbent assay |
| GFD | Gluten-free diet |
| GS | Gluten sensitivity |
| HGS | Human gastric simulator |
| HLA | Histocompatibility leukocyte antigen |
| HMW | high molecular weight |
| HPLC | High-performance liquid chromatography |
| IELs | Intraepithelial lymphocytes |
| IgA | Immunoglobulin-A |
| IgE | Immunoglobulin-E |
| IL-15 | Interleukin-15 |
| LC/MS-MS | Liquid chromatography tandem mass spectrometry |
| LMW | Low molecular weight |
| MW | Molecular weight |
| OPA | O-phthalaldehyde |
| RQs | Research questions |
| SDS-PAGE | Sodium dodecyl sulfate–polyacrylamide gel electrophoresis |
| SGF | Simulated gastric fluid |
| SIF | Simulated intestinal fluid |
| tTG | Tissue transglutaminase |
| TCEP | Tris (2-carboxyethyl)-phosphine |
| UPEX | Universal prolamin and glutelin extractant |
| WA | Wheat allergy |
| WDEIA | Wheat-dependent exercise-induced anaphylaxis. |

# Publications

Jayawardana, I. A., Montoya, C. A., McNabb, W. C., & Boland, M. J. (2019). Possibility of minimizing gluten intolerance by co-consumption of some fruits–A case for positive food synergy? *Trends in Food Science & Technology*, *94*, 91-97.

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Jayawardana, I. A., Boland, M. J., Loo, T., McNabb, W. C., & Montoya, C. A. (Submitted to Food Research International). Actinidin reduces gluten-derived immunogenic peptides reaching the small intestine in an *in vitro* semi-dynamic gastrointestinal tract digestion model.

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**Conference abstracts and presentations**

Isuri Jayawardana, Kamran Rostami, C.A. Montoya, and Mike J. Boland. Actinidin Kiwifruit enzyme hydrolyses antigenic gluten peptides – a case for positive food synergy. Gastro 2020 Hybrid Meeting, Auckland, New Zealand. 11th November 2020 (poster presentation) – Award for paper of excellence under the category of Nutrition/General.

Isuri A. Jayawardana, Carlos A. Montoya, Mike J. Boland, Trevor Loo, Gill Norris, Warren C. McNabb. Actinidin eliminates the immunogenic gluten peptides rapidly during *in vitro* gastrointestinal digestion – preliminary evidence. 5th International Conference on "Food Structures, Digestion & Health", Rotorua, New Zealand. 30 September to 3 October 2019 (poster presentation).

Isuri Jayawardana, C.A. Montoya, Mike J. Boland, and Warren C. McNabb. Actinidin: an exogenous enzyme to digest and reduce gluten immunogenicity. 6th International Conference on Food Digestion, Granada, Spain. 2nd April to 4th of April 2019 (poster presentation).

# Chapter One

# 1. Introduction and Thesis Outline

This chapter provides an overview of the background, rationale, research goals and approach of the thesis.

## 1.1 Research Background

Cereals have been the principal component of human food staples for thousands of years. Wheat, corn, rice, barley, sorghum, millet, oats, and rye are the world's most popular cereals (Koehler & Wieser, 2013). Considering the global per capita intake, cereals supply up to 50% of the daily dietary energy requirement of humans (Amine et al., 2002). Although cereals do not contain as much protein as other foods such as legumes, they supply nearly half of the daily total protein requirement of humans (Lasztity, 1995; Young & Pellett, 1994). The protein content of cereals varies between and within species due to genotype, and phenotype. For instance, the protein content of wheat can vary from 6 to 20% (Caporaso et al., 2018). The cereal proteins are classified into four groups based on their solubility in different solvents: albumins, globulins, prolamins, and glutelins (known as the Osborne classification) (Doll, 1977). Among these proteins, prolamins and glutelins of wheat, rye, and barley, are collectively known as gluten proteins. Gluten is found globally in an array of food products since wheat is commonly used to prepare different foods (e.g., bread, pasta). For example, the global average per capita wheat consumption per year during 2004 – 2018 was 60 kg and in New Zealand, it was 80 kg (Bradauskiene et al., 2021).

Gluten proteins contain amino acid sequences that are resistant to gastrointestinal proteases, which results in the prolonged presence of indigestible peptides in the

gastrointestinal tract (GIT). This resistance is due to their high proline and glutamine content, and that the main GIT proteases are not able to hydrolyse peptide bonds of these amino acids. Some of these undigested gluten peptides are highly immunogenic in genetically predisposed individuals, triggering immunogenic reactions when those individuals consume gluten-containing foods. These responses are collectively known as gluten-related health disorders. These include allergic reactions (wheat allergy, (WA)), autoimmune responses (celiac disease (CD), dermatitis herpetiformis and gluten ataxia) and other possible immune-mediated diseases (gluten sensitivity (GS)) (Sapone et al., 2012). The prevalence of gluten-related disorders in different parts of the world appears to be diverse (Catassi et al., 2013; Sapone et al., 2012; Unalp-Arida et al., 2017). However, a proportion of the human population (0.3% – 0.6% globally, and 3% – 10% in most wheat-consuming populations) suffers from one of these disorders (Bai et al., 2013; Elli et al., 2015; Igbinedion et al., 2017; Zevallos et al., 2017).

Celiac disease and WA have been extensively studied, while GS is a relatively novel clinical entity, therefore there is a greater understanding of the pathogenesis of CD and WA compared to GS. Celiac disease, an autoimmune disorder, is a common disease in countries where the Caucasian population is predominant (e.g., Europe and North America), however, it is distributed worldwide (Gujral et al., 2012). The global prevalence of CD is around 1.4%, however, this could be between 0.05% and 2.6% in different parts of the world (Singh et al., 2018). It is estimated that the prevalence of CD in New Zealand is around 1.2% (Ho et al., 2021). Studies have confirmed a worldwide rising trend in CD prevalence. In New Zealand, a rising rate has been noted in children (Kho et al., 2015). The global prevalence of WA i.e., IgE-mediated allergy to gluten or other wheat proteins ranges from 0.33% to 1.17% (Cabanillas, 2020). The

prevalence of GS, which is neither CD nor WA, varies between 0.16% and 13% (Cabanillas, 2020). These figures suggest that gluten-related health disorders are a growing public health issue worldwide.

The only currently successful treatment for gluten-related health disorders is a strict gluten-free diet (GFD). The amount of gluten required to trigger an immune response in gluten-related health disorders varies among individuals, but in general, the reported information suggests between 10 and 100 mg per day (Hischenhuber et al., 2006). According to the Codex Alimentarius Commission, a product labelled gluten-free should contain less than 20 ppm or less than 100 ppm for products extracted from wheat, barley, or rye (e.g., purified wheat starch). Despite the importance and effectiveness of GFD, adherence to GFD remains a difficult goal for many patients for many reasons. For example, (i) most of the commercially available gluten-free products are less palatable (Zannini et al., 2012); (ii) gluten-free products are less available (Oyarzún et al., 2015), e.g., 83–88% fewer gluten-free white and brown breads and pasta products available compared to gluten-containing counterparts in the UK (Allen & Orfila, 2018); (iii) they are more expensive than their gluten-containing counterparts (Singh & Whelan, 2011), e.g., on average 183% more expensive than their wheat-based counterparts in the USA (Lee et al., 2019); (iv) gluten-free products are nutritionally poor (Jamieson et al., 2018) – e.g., they have lower dietary fibre and micronutrient contents than conventional products (Taetzsch et al., 2018).

Therefore, multidisciplinary research efforts are currently being carried out to find new treatments (strategies) to replace or supplement a GFD. These treatments include methods to reduce the intestinal permeability of immunogenic peptides (Gopalakrishnan et al., 2012; Leffler et al., 2012; Paterson et al., 2007: Kelly et al., 2013), the use of proteolytic enzymes of different origins to hydrolyse immunogenic

peptides in the diet (Matysiak–Budnik et al., 2005; Stepniak et al., 2006) and the use of different mechanisms to change the structure of immunogenic proteins to prevent toxicity (Pinier et al., 2012). However, most of these treatments are still under investigation, with more work required before they can be used as safe alternatives to GFD.

Among the methods studied, enzyme therapy to detoxify immunogenic peptides when wheat-containing products are consumed has been demonstrated to be one of the promising approaches. Exogenous enzymes can improve health by enhancing the digestion of gluten in the stomach and reducing or eliminating the gluten-derived immunogenic peptides in the small intestine to relieve symptoms (Wei, Helmerhorst, Darwish, Blumenkranz, & Schuppan, 2020). Most of the exogenous enzymes currently being researched are generated from microorganisms (Angelis et al., 2006; Matysiak–Budnik et al., 2005; Stepniak et al., 2006; Tye-Din et al., 2010), which are then extracted and concentrated. The long-term effects of ingesting these products have not been subjected to research. A natural, readily available enzyme is a timely requirement. Actinidin, a member of the cysteine proteases, from green kiwifruit *(Actinidia deliciosa*), has been identified as a potential enzyme in this category of treatments. Scientific evidence suggests that actinidin helps to degrade (disappearance in SDS-PAGE) or digest (degree of protein hydrolysis) gluten and gliadin peptides *in vitro* and *in vivo* (Kaur et al., 2010a, 2010b; Montoya et al., 2014; Rutherfurd et al., 2011). For example, when tested in rats, the consumption of actinidin in the form of freeze-dried kiwifruit led to a 3.2-fold increase in the gastric digestion of gluten (Montoya et al., 2014). Also, a greater gastric disappearance was observed in gel electrophoresis for different gluten fractions (e.g., ω-gliadin 61%, and three low molecular weight subunits (35 (73%), 37 (65%), and 41 kDa (71%) of glutenin) (Rutherfurd et al., 2011).

Consequently, it is hypothesised that consuming actinidin-containing kiwifruit will increase the gastric digestion of gluten proteins and therefore reduce or eliminate the occurrence of immunogenic gluten peptides in the small intestine, those are involved in gluten-related health disorders, specifically CD and GS. If this hypothesis can be proved, then it suggests that kiwifruit may reduce the risk of adverse effects from the unintentional (or intentional) consumption of small amounts of gluten in gluten-containing products, so that kiwifruit can be considered as an adjunct to a GFD. To test this hypothesis, in this PhD thesis, different *in vitro* and *in vivo* digestion models were used with different analytical techniques to understand the effect of actinidin on the digestion of gluten and the gluten immunogenic peptides in the GIT.

## 1.2 Overall goal and research questions

### Overall goal

To test the effectiveness of actinidin from kiwifruit in enhancing gastric and small intestinal digestion of gluten proteins and thereby reducing (or eliminating) the immunogenic gluten peptides involved in gluten-related health disorders.

### Research Questions (RQs)

Based on the above background and the overall goal, the following research questions were tested in four experimental chapters.

**RQ1:** Can actinidin hydrolyse proline-and glutamine-containing peptide bonds *in vitro*, as proline and glutamine are the main amino acids present in gluten epitopes and these are abundantly present in gluten proteins and immunogenic peptides?

**RQ2:** What is the optimum level of gastric pH and minimum level of actinidin concentration required to hydrolyse gluten and its immunogenic peptides *in vitro*?

**RQ3:** Is actinidin as good as commercially available gluten-hydrolysing enzymes in hydrolysing immunogenic gluten peptides *in vitro*?

**RQ4:** Can actinidin hydrolyse gluten and its immunogenic peptides during simulated semi-dynamic gastric and small intestinal digestion?

**RQ5:** Can actinidin hydrolyse gluten and its immunogenic peptides in a commonly consumed gluten-containing food (bread) during simulated dynamic gastric and small intestinal digestion?

**RQ6:** Can fresh kiwifruit hydrolyse gluten and its immunogenic peptides in a common gluten-containing food (bread) during gastric and small intestinal digestion *in vivo* and reduce the accumulation of these immunogenic peptides in the small intestine?

### 1.3 Research Approach

To answer the above research questions, this PhD research followed a structured plan in which the first part of the research was carried out with purified gluten proteins and peptides, including synthesised peptides, before moving on to a real food model. Primary studies were focused on investigating parameters influencing digestion, such as pH and actinidin concentration under simulated digestion conditions. In the second part of this PhD, the experiments were carried out with a common gluten-containing food model (whole bread). Finally, an *in vivo* study was conducted to understand the

kinetics of gluten and its immunogenic peptide digestion in the stomach and small intestine of a pig a model of human digestion. Therefore, this PhD research consisted of four experimental chapters (Figure 1.1).

A comprehensive literature review was documented covering all the aspects of the research topic. The objectives of this review were to understand: (i) the basics of cereal proteins and their structures, (ii) the structural relationship of cereal proteins to gluten-related health disorders, (iii) the pathology of different gluten-related health disorders, (iv) the current nutritional and non-nutritional strategies to reduce the immunogenicity of gluten proteins, and (v) the current knowledge on actinidin.

It was expected that the hydrolysis of gluten proteins would increase, and the amounts of immunogenic peptides would either be reduced or eliminated when actinidin is present in the digestion medium. This was first explored with purified proteins. In the first experimental chapter, *in vitro* static digestion experiments were conducted with purified actinidin and purified gluten peptides and proteins (33-mer peptide, wheat gluten and gliadin). The level of pH and the actinidin concentration required to achieve maximum hydrolysis of gluten peptides and minimum immunogenic peptides were determined to identify the best conditions for gluten digestion under *in vitro* conditions. The first experimental chapter provided some basic mechanistic understanding of the effect of actinidin on the digestion of gluten proteins and their resultant peptides. However, the static digestion model could not provide an understanding of dynamic variables such as the amount of immunogenic peptides released from the stomach into the small intestine over time. Therefore, the work described in the second experimental chapter used a semi-dynamic digestion model with purified gluten and purified actinidin powder to evaluate the effect of actinidin on the digestion of gluten and its immunogenic peptides under simulated gastrointestinal conditions. This model

mimicked dynamic parameters of gastric digestion such as gastric acidification and gastric emptying. This chapter provided a basic understanding of the ability of actinidin to hydrolyse gluten and gluten-derived immunogenic peptides.

The digestion of gluten proteins present in a food matrix is expected to be different from that of the purified gluten proteins because the gluten proteins undergo structural changes during food processing, and they interact with other nutrients creating a food matrix that could compromise the accessibility of proteases to their specific amino acid. In addition, digestion in the human body is a dynamic process, where intestinal digestion systematically follows gastric digestion. Therefore, the third experimental chapter was set up to evaluate the effect of actinidin on the digestion of gluten proteins and immunogenic peptides in a commonly consumed gluten-containing food, whole bread. A human panel was used for the oral processing of bread and a dynamic approach was used in the subsequent *in vitro* gastric digestions, where a human gastric simulator, which mimicked dynamic parameters of gastric digestion such as reduction of pH, temperature, gastric secretions, peristaltic mixing, and gastric emptying was used, while a static digestion model was used to simulate small intestinal digestion.

The digestion of gluten-containing foods under simulated gastric and small intestinal conditions is a simplistic model of complex processes in the human body, which are affected by different conditions such as gastric and small intestinal secretions, amount and type of enzymes secreted, physical disintegration of foods due to mechanical movements of the stomach and small intestine. Therefore, in the fourth experimental chapter, an *in vivo* digestion model was used to be able to map the digestion of gluten and its immunogenic peptides throughout the GIT. The pig model was used in this chapter as it is too early (risky) to test the effect of actinidin on digestion and immunogenicity of gluten-containing foods using a human panel, and it is very invasive

to collect contents throughout the entire GIT. Pigs were selected as an animal model as they have anatomical and physiological similarities of the digestive tract between the mouth and the terminal ileum to humans (Deglaire & Moughan, 2012). In addition, pigs are meal-eaters, which ensures that they are able to eat fresh kiwifruit and large samples can be collected for different analyses.

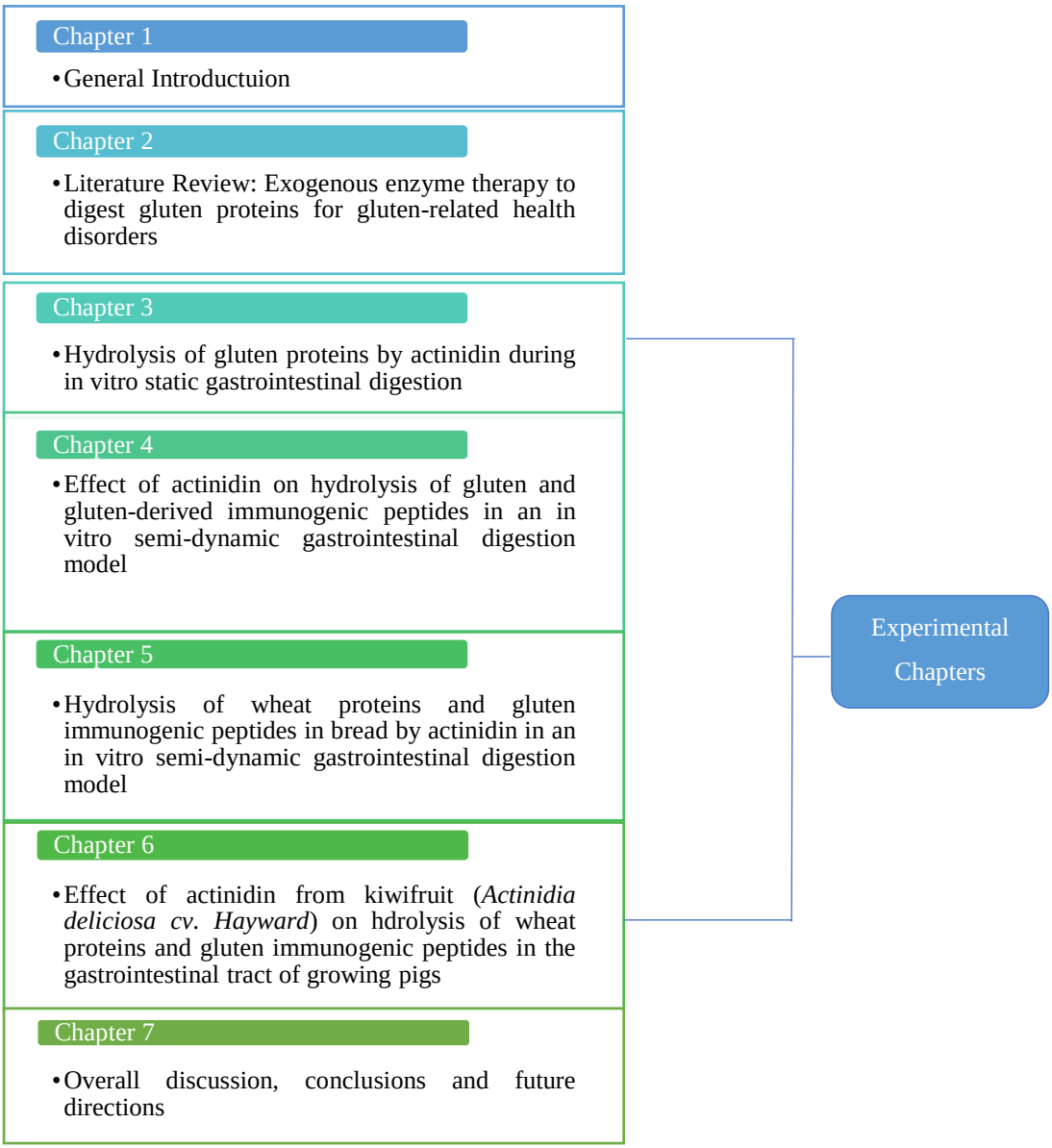


**Figure 1.1** Overview of the thesis structure

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# Chapter Two

## 2. Literature Review: Exogenous enzyme therapy to digest gluten proteins for gluten-related health disorders

Some parts of this chapter have been published as a commentary paper in Trends in Food Science & Technology:

Jayawardana, I. A., Montoya, C. A., McNabb, W. C., & Boland, M. J. (2019). Possibility of minimizing gluten intolerance by co-consumption of some fruits–A case for positive food synergy? *Trends in Food Science & Technology*, *94*, 91-97.

**Highlights**

- Gluten intolerance is a growing health problem worldwide.
- High proline content prevents complete digestion of gluten proteins.
- Some incompletely digested gluten peptides can trigger gluten intolerance disorders.
- Proline-specific exogenous proteases are one way of enhancing gluten digestion.
- Consumption of fruit containing proline-specific proteases is a possible partial solution.

**02.1 Abstract**

Gluten proteins are resistant to complete proteolysis by human gastrointestinal tract enzymes, due to their high proline and glutamine-rich peptide sequences, producing peptides that can trigger immunogenic reactions in sensitive individuals. These immunogenic reactions are responsible for gluten-related health disorders such as coeliac disease, wheat allergy and gluten sensitivity. At present, the prescription of a gluten-free diet (GFD) is the only acceptable therapy. However, maintaining a life-long GFD is not easy and gluten cross-contamination is a common problem in manufactured foods. New treatment strategies have been proposed, but they are not validated for safe human use. It is timely to recognize natural and readily available therapies to reduce gluten immunogenicity. Exogenous sources of proteolytic enzymes are being explored for their potential to accompany the human GIT proteases to digest gluten proteins. Actinidin, a cysteine protease found in kiwifruit (*Actinidia deliciosa*) is suggested as an effective exogenous enzyme, to be utilized in this category. The literature review section outlines the characteristics of gluten proteins and their resistance for GIT digestion, the pathogenesis of gluten-related health disorders and current alternative therapies for GFD including recent progress in these methods for replacing a GFD. The exogenous enzymes that are researched to aid in the digestion of gluten proteins, their effectiveness in digesting gluten proteins and relieving the gluten toxicity have also been reviewed. Finally, the currently available information on actinidin to be considered as a potential exogenous enzyme to digest gluten has been discussed.

**Keywords:** Actinidin, Exogenous enzymes, Gastric and small intestinal digestion of gluten, Gluten proteins, Gluten-related health disorders, Immunogenicity, Immunogenic peptides.

## 2.2 Introduction

Cereals have been identified as the principal component of human food staples for thousands of years (Koehler & Wieser, 2013). Considering the per capita intake, cereals supply up to 50% of the daily dietary energy requirement of humans (Amine et al., 2002). Although cereals do not contain as much protein as other foods such as legumes, they supply nearly half of the daily total protein requirement of humans (Lasztity, 1995; Young & Pellett, 1994). However, some cereal proteins also play a major role in stimulating hypersensitivity reactions in certain individuals causing chronic disease conditions.

The protein content of cereals varies between and within species due to genotype, and phenotype. For instance, the protein content of wheat can vary from 6 to 20% (Caporaso et al., 2018). The cereal proteins are classified into four groups based on their solubility in different solvents: albumins, globulins, prolamins, and glutelins (known as the Osborne classification) (Doll, 1977; Tan et al., 2011). Among these proteins, prolamins and glutelins of wheat, rye, and barley collectively known as gluten proteins, produce some peptides that are resistant to proteolysis during digestion. This resistance is due to their high proline and glutamine contents. The undigested gluten peptides can trigger immunogenic reactions in sensitive individuals. Studies have identified that oat prolamins in some oat varieties (Comino et al., 2011; Silano et al., 2014), and corn prolamins (Cabrera-Chávez et al., 2012) also produce immunogenic peptides during gastrointestinal digestion. These responses are collectively known as 'gluten-related health disorders'. These disorders can be classified into several types based on their pathologies: coeliac disease (CD), wheat allergy (WA) and gluten sensitivity (GS) (Sapone et al., 2012). The prevalence of gluten-related disorders in different parts of the world appeared to be diverse (Catassi et al., 2013; Sapone et al., 2012; Unalp-Arida

et al., 2017). However, a considerable proportion of the human population (0.3%–0.6% in general and 3%–10% in most wheat-consuming populations) suffers from one of these disorders (Bai et al., 2013; Elli et al., 2015; Igbinedion et al., 2017; Zevallos et al., 2017).

Coeliac disease and WA have been extensively studied, while GS is a relatively novel clinical entity, therefore there is a greater understanding of the pathogenesis of CD and WA compared to GS. However, the only currently successful treatment for gluten-related disorders is a GFD. Despite the importance and effectiveness of GFD, adherence to GFD remains a difficult goal for many patients for many reasons. Most of the commercially available gluten-free products are less palatable and less widely available than their gluten-containing counterparts. Some GFDs are not cost-effective and their 'gluten-free' label is sometimes questionable, as gluten cross-contamination may occur. Therefore, multidisciplinary research efforts are currently being carried out to find new treatment strategies in order to reduce gluten toxicity (Scherf et al., 2020). Such widely tested treatments include methods to reduce intestinal permeability of immunogenic peptides (Gopalakrishnan et al., 2012; Leffler et al., 2012), the use of proteolytic enzymes of different origins to hydrolyse immunogenic peptides in the diet (De Angelis et al., 2010; Di Cagno et al., 2004) and the use of different mechanisms to change the structure of immunogenic proteins to prevent toxicity (Pinier et al., 2012). However, most of these treatments are still under investigation, with more work required before they can be used as safe alternatives to GFD.

Among the methods studied, enzyme therapy to detoxify immunogenic peptides has been demonstrated to be one of the promising approaches. Actinidin, a member of the cysteine proteases, from green kiwifruit *(Actinidia deliciosa* cv. Hayward), has been identified as a potential enzyme that may be acceptable in this category of treatments.

Scientific evidence suggests that actinidin helps degradation (disappearance in gel electrophoresis) or digestion (degree of protein hydrolysis) of gluten and gliadin peptides (Kaur et al., 2010a, 2010b; Montoya & Hindmarsh, et al., 2014; Rutherfurd et al., 2011). However, there is a lack of information on whether actinidin is able to digest the immunogenic gluten peptides, which could help to reduce (or eliminate) their presence after consuming a meal containing gluten.

This review gives an overview of the cereal proteins, their immunogenicity during gastrointestinal digestion, gluten-related health disorders and their clinical course, diagnostics, complications, and treatments. Further, the alternative therapies for gluten-related health disorders and their effectiveness particularly highlighting the enzymatic therapies that have been identified so far to replace or supplement a GFD alongside their major outcomes and findings are discussed. Finally, a newly identified gluten-specific enzyme, actinidin, a cysteine protease largely present in kiwifruit (*Actinidia deliciosa*), has been proposed as a potential natural plant enzyme to be used in future enzymatic therapies to managing gluten-related health disorders.

### 2.3 Importance of cereals

Apart from serving as staples, cereals have a variety of other food and non-food uses such as in beverages, snack foods, nutraceuticals, etc. (Bietz & Lookhart, 1996; Rexen & Munck, 1984; Taylor et al., 2006). Therefore, there is a massive demand for cereals globally. According to the Food and Agriculture Organization (FAOSTAT, 2017), the total production quantity of cereal grains in the world in 2021 was about 2.8 billion tonnes. World cereal production is projected to increase to reach 3.0 billion tonnes by 2050 (OECD/FAO).

The nutritional significance of cereals to human wellbeing is well established. In all cereals, carbohydrates are the main nutrients representing up to 75% of the dry weight (Koehler & Wieser, 2013). In addition, cereals are a source of the B complex vitamins, vitamin E, iron, and trace minerals (Lasztity, 1999). However, the nutrient composition differs substantially between different types of cereals (Table 2.1).

Cereals can supply up to 50% of the daily dietary energy requirement of humans (Amine et al., 2002). Although cereals do not contain as much protein compared to sources such as legumes, cereals can supply up to 50% of the daily dietary protein requirement of humans (Lasztity, 1995; Young & Pellett, 1994). According to the FAO, globally, the protein quantity supplied by cereals in 2013 (latest data) was 31.8 g/capita/day whereas animal products altogether provided 32.1 g/capita/day (FAOSTAT, 2017).

**Table 2.1** Average nutrient composition of cereal grains (% dry matter basis).

| Cereal | Starch | Protein | Fibre | Lipid | Ash |
|---|---|---|---|---|---|
| Barley | 73.5 | 10.9 | 4.3 | 2.3 | 2.4 |
| Corn | 79.5 | 10.2 | 2.3 | 4.6 | 1.3 |
| Millets | 58.9 | 10.3 | 8.7 | 4.5 | 4.7 |
| Oats | 55.5 | 11.3 | 10.9 | 5.8 | 3.2 |
| Rice | 75.8 | 8.1 | 0.5 | 1.2 | 1.4 |
| Rye | 71.9 | 11.6 | 1.9 | 1.7 | 2.0 |
| Sorghum | 65.0 | 11.0 | 4.9 | 3.5 | 2.6 |
| Wheat | 71.9 | 12.2 | 1.9 | 1.9 | 1.7 |

Source: Lasztity, 1999.

## 2.4 Cereal proteins

The protein content of cereal grains varies (Table 2.1) between and within species due to genotype, and phenotype (e.g., growing conditions, nitrogen fertilization) conditions (Koehler & Wieser, 2013). In cereals, proteins are distributed throughout the grain, with varying concentrations in different parts of the grain. In general, the highest protein

concentration is located in the embryo (~30%), followed by scutellum (~27%) and the aleurone layer (~20%) while the endosperm contains the lowest concentration (~13%). However, in terms of quantity, the highest amount of protein is found in the endosperm since it is the largest part of the cereal grain (~83%) as shown in Table 2.2 for wheat (Lasztity, 1995).

Most of the endosperm proteins act as a store of amino acids to be used during germination and seedling growth. These proteins are known as storage proteins (Doll, 1977; Lásztity & Hidvégi, 2012). In wheat and barley grains, 80-85% of the total proteins are storage proteins (Lookhart & Bean, 2000). Storage proteins are of particular importance because they determine not only the total protein content of the seed but also its quality in terms of amino acid profile.

**Table 2.2** Protein content in major fractions of wheat grain.

| Fraction | Proportion (%) | Protein (g) |
|---|---|---|
| Whole grain | 100.0 | 7.6 |
| Endosperm | 82.5 | 5.2 |
| Aleurone | 7.0 | 1.4 |
| Pericarp | 8.0 | 0.3 |
| Embryo | 1.0 | 0.3 |
| Scutellum | 1.5 | 0.4 |

Source: Adapted from Shukla & Bushuk, 1975.

### 2.4.1 Amino acid composition of cereal proteins

Cereal proteins are considered to be low-quality proteins in terms of amino acid composition because all cereals are particularly limited in the essential amino acid lysine (Bressani et al., 1960; Hagan et al., 2003), which is important as a precursor for protein synthesis in humans (Tomé & Bos, 2007). Lysine content varies largely between cereals (Table 2.3). For example, rice and oats normally contain relatively high

amounts of lysine (Lasztity, 1995; Shewry, 2007), whereas wheat and corn have lower lysine contents (Shewry, 2007) compared to other cereals. Generally, based on a nitrogen requirement of 105 mg nitrogen/kg body weight per day (660 mg protein/kg body weight per day), the lysine requirement of an adult human is 30 mg/kg body weight per day (WHO, 2007).

Other essential amino acids such as threonine and tryptophan are also lower in cereals compared with amounts found in legumes and proteins of animal origin (Heldt & Piechulla, 2011; Young & Pellett, 1994). Therefore, cereals should often be complemented with legumes or any other sources of proteins to overcome the amino acid limitations. Through breeding and genetic engineering, attempts have been made to improve the content of essential amino acids in cereals. However, relatively limited success has been observed except for corn (Le et al., 2016; Ufaz & Galili, 2008).

Cereals are a rich source of glutamine, proline, and alanine. Cysteine, arginine, glycine, aspartate, and serine are also present in considerable amounts.

### 2.4.2 Classification of cereal proteins

Cereal proteins have been classified into broad groups based on different criteria (Figure 2.1) and they were the first food proteins to be characterised (Shewry et al., 1995). A detailed study of the classification of seed storage proteins dated back to the early 20$^{th}$ century. Osborne et al. (Osborne, 1907) categorised wheat proteins into four groups based on their solubility in different solvents, known as Osborne fractions: i.e., albumins, globulins, gliadins, and glutenins (Figure 2.2).

**Table 2.3** Essential amino acid profiles of selected whole cereal grains and adult essential amino acid requirements (g/100 g protein).

| Amino acid | Rice[a,b,c] | Wheat[a,b,c] | Corn[a,b,c] | Sorghum[c] | Millet[c] | Barley[a,b,c] | Rye[a,c] | Essential amino acid requirements[d] |
|---|---|---|---|---|---|---|---|---|
| Histidine | 2.4-2.8 | 2.0-2.3 | 2.7-2.8 | 1.7 | 2.3 | 2.1-2.3 | 2.2 | 1.5 |
| Isoleucine | 3.7-3.8 | 3.3-4.7 | 3.6-3.7 | 3.7 | 4.5 | 3.6-3.8 | 3.5-4.2 | 3.0 |
| Leucine | 8.2-8.7 | 6.7-6.8 | 12.4-13.6 | 13.1 | 10.3 | 6.5-7.0 | 6.2-6.6 | 5.9 |
| Lysine | 3.6-4.0 | 2.7-2.8 | 2.6-2.7 | 2.2 | 3.0 | 3.4-3.5 | 3.4 | 4.5 |
| Methionine | 2.1-2.3 | 1.2-1.7 | 1.8-1.9 | 1.2 | 1.8 | 1.6-2.6 | 1.4-1.6 | 1.6 |
| Phenylalanine | 4.8-5.4 | 4.5-4.9 | 4.8-5.1 | 4.8 | 4.9 | 5.1-5.2 | 4.5-4.8 | 3.8 |
| Tyrosine | 2.5-3.6 | 1.7-3.7 | 3.4-4.4 | 3.4 | 3.7 | 2.5-3.1 | 1.9-3.2 | |
| Threonine | 3.4-3.9 | 2.8-2.9 | 3.6-3.7 | 3.6 | 3.1 | 3.1-3.6 | 3.3-3.7 | 2.3 |
| Tryptophan | 1.1-1.3 | 1.1-1.5 | 0.6-0.7 | 1.2 | 1.4 | 1.4-1.9 | 1.1 | 0.6 |
| Valine | 5.5-5.8 | 4.4-4.5 | 4.8-5.3 | 4.5 | 5.3 | 4.9-5.4 | 4.8-5.2 | 3.9 |

Source: [a] Taylor et al., 2006; [b] FAO, 2017; [c] Comino et al., 2011; [d] calculated from WHO, 2007 and given for mean nitrogen requirement of 105 mg nitrogen/kg body weight per day. (660 mg protein/kg body weight per day).

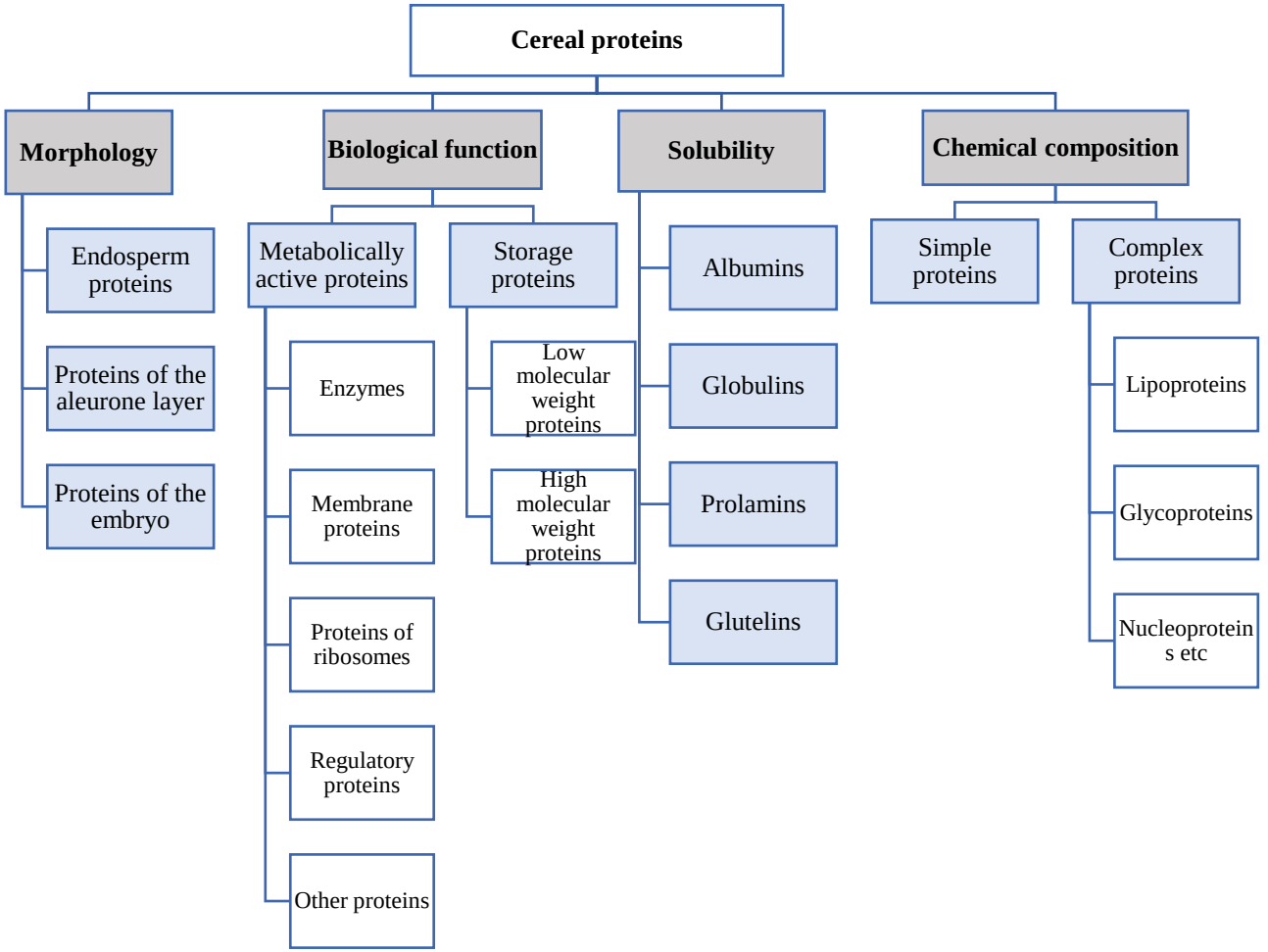



**Figure 2.1** Possible ways of categorising cereal proteins.

Source: Adapted from Lasztity, 1995.

The same classification has been used for other cereal proteins generally known as albumins, globulins, prolamins, and glutelins. The alcohol-insoluble fraction of wheat is called glutenin and the equivalent fractions from other cereals are known as glutelins (Koehler & Wieser, 2013; Shewry & Tatham, 1990) while alcohol-soluble fractions of cereal proteins (such as gliadins in wheat) are categorised as prolamins.

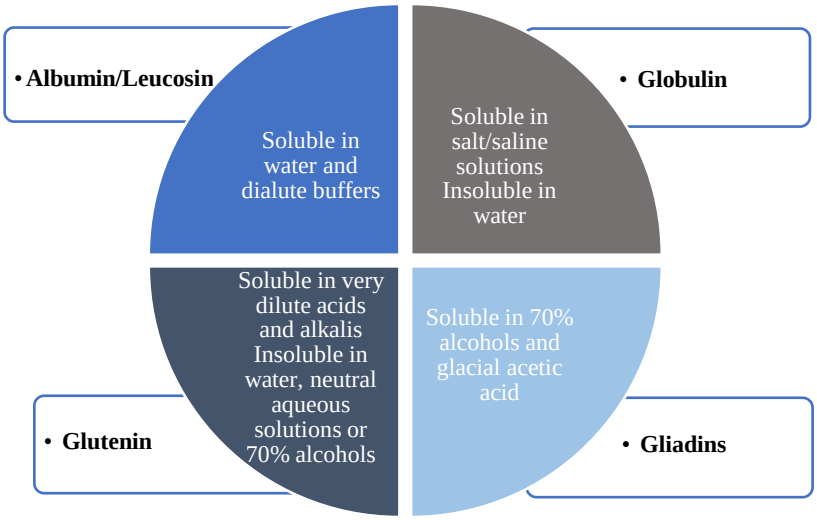



**Figure 2.2** Osborne fractions of wheat proteins.

Source: Adapted from Osborne, 1907.

Although Osborne fractionation is still widely used, slight changes have been made during the last 4-5 decades to the original classification. It was identified that Osborne classification is based only on the physicochemical properties of the proteins (Doll, 1977; Tan et al., 2011), and therefore some discrepancies occur between fractions. For instance, there are cereal proteins (e.g., lipoproteins) that do not belong to any of the Osborne fractions (Koehler & Wieser, 2013) and also the solubility of cereal proteins overlaps in these fractions (Gianibelli et al., 2001). Osborne classified glutelins based on their solubility in very dilute acids and alkalis. However, notable portions of

glutelins were found to be insoluble in dilute acids, and complete solubility of glutelins could be achieved by solvents containing a mixture of aqueous alcohols (Koehler & Wieser, 2013), which contradicts the Osborne fractionation. Further, prolamins and glutelins were thought to be in separate groups according to the Osborne classification. However, they are structurally and evolutionarily similar to each other, both containing high amounts of proline and glutamine (Shewry & Tatham, 1990). Therefore, in some Prolamins were defined on the basis of their solubility in aqueous alcohol solutions, and their insolubility in either water or dilute salt solutions classifications, these proteins have been considered together under prolamins (Miflin, 1983). When these two proteins (i.e., prolamins and glutelins) are considered under the same category, it is not reliable to describe prolamins as soluble only in aqueous alcohol solutions because glutelins are insoluble in aqueous alcohol solutions in their native state. Moreover, when these glutelins are at their reduced state, all of them are soluble in aqueous alcohol solutions. In the reduced state, one protein (γ prolamin of corn) is also soluble in water (Shewry, 1995). Therefore, the appropriate way of defining the solubility of prolamins when glutelins are included, is that they are insoluble in water or dilute salt solutions in their native state.

To overcome confusion, some authors have used different names to classify storage proteins. For example, Wieser and Koehler (Koehler & Wieser, 2013; Wieser, 1994; Wieser & Koehler, 2008) have categorised storage proteins according to related amino acid sequences and MWs into: (1) High MW (HMW) group; (2) medium MW group; and (3) low MW group (LMW). In another classification, protein superfamilies have been defined (Figure 2.3) based on amino acid sequences of proteins. Families whose members have low amino acid sequence identities but do share similar structural and functional features such as patterns of disulphide bonds, suggesting a probable common

evolutionary origin, are placed together in superfamilies (Breiteneder & Radauer, 2004; Cunsolo et al., 2012; Shewry, et al., 2002). It has been identified that most of the cereal proteins are falling into two major superfamilies: the prolamin superfamily and the cupin superfamily.

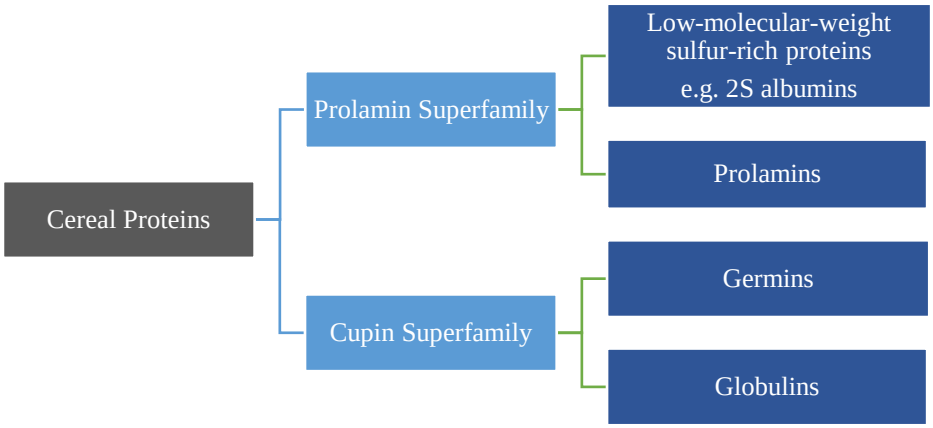



**Figure 2.3** Classification of cereal proteins into superfamilies.

Source: Adapted from Cunsolo et al., 2012.

### 2.4.2.1 Prolamins

Prolamins are found mainly in the seeds of grasses (Kreis et al., 1985) and they are the major storage proteins of most of the economically important cereals, except for oats and rice (Table 2.4) (Breiteneder & Radauer, 2004; Doll, 1977; González-Pérez & Arellano, 2009). The major storage proteins in oats and rice are globulins (Shewry, 1995). Prolamins are characterized by their high contents of proline and glutamine amino acids and their low contents of the essential amino acid lysine, together with threonine (specifically in wheat and barley) and tryptophan (in corn) (Shewry, 1995). Prolamins were defined on the basis of their solubility in aqueous alcohol solutions, and their insolubility in either water or dilute salt solutions in their native state.

**Table 2.4** Prolamin content (% of total protein in dry matter basis) of major cereals.

| Cereal | Prolamin name | Prolamin content | Reference |
|---|---|---|---|
| Barley | Hordein | 52 | Shukla & Bushuk, 1975 |
| Corn | Zein | 50-55 | Frey, 1973; Shukla & Bushuk, 1975 |
| Oats | Avenin | 10-16 | Krishnan & Coe Jr, 2001; Shukla & Bushuk, 1975 |
| Rice | Oryzin | 5-10 | Koehler & Wieser, 2013; Krishnan & Coe Jr, 2001; Muench et al., 1999; Shukla & Bushuk, 1975 |
| Rye | Secalin | 30-50 | Frey, 1973; Shukla & Bushuk, 1975 |
| Sorghum | Kafirin | 52-60 | Frey, 1973; Shukla & Bushuk, 1975 |
| Wheat | Gliadin | 45-69 | Breiteneder & Radauer, 2004; Frey, 1973; Shukla & Bushuk, 1975 |

Some prolamins are given specific names according to the species of origin, such as gliadin (wheat), hordein (barley), zein (corn), secalin (rye), kafirin (sorghum) and avenin (oat) (Table 2.4) (Frey, 1973; González-Pérez & Arellano, 2009). Notable differences such as amino acid composition and MWs between the above prolamins exist.

The *Triticeae* is a grass tribe, which includes barley, wheat, and rye. These cereals contain closely related groups of prolamins. Shewry and Tatham (Shewry & Tatham, 1990) classified the prolamins in *Triticeae* into three groups based on their amino acid sequences and evolutionary relationships: sulfur-rich, sulfur poor, and HMW prolamins (Figure 2.4). In this classification, glutelins and prolamins are considered as one group.

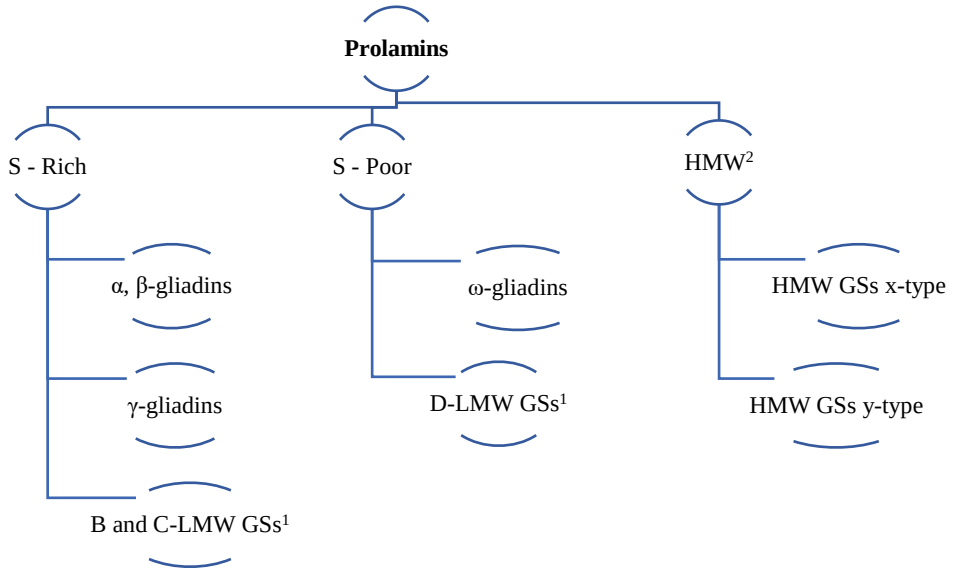



**Figure 2.4** Classification of prolamins based on the amino acid sequences and evolutionary relationship

[1]B-, C- and D-LMW GSs, polymeric B-, C- and D-type low molecular weight glutenin subunits; [2]HMW, high molecular weight; Source: Adapted from Cunsolo et al., 2012

For ease of understanding, this review will consider cereal storage proteins classified based on their original solubility characteristic (i.e., Osborne classification) as it is the most common classification in reported studies. The following sections describe the prolamins of wheat, barley, rye, corn, sorghum, oats, and rice.

#### 2.4.2.1.1 Wheat gliadins

Wheat gliadins are the aqueous alcohol-soluble fraction of the wheat storage protein, which corresponds to the prolamins of other cereals. Gliadins contain mainly single polypeptide chains (monomeric) that are associated with hydrogen bonding and hydrophobic interactions (Shewry, et al., 1986), having LMWs (30-80 kDa (Lafiandra & MacRitchie, 1997)) compared to glutenins, which are larger (> 500 kDa (Wieser, 2007)). Originally gliadins have been classified into four groups based on electrophoretic mobility: α-, β-, γ-, and ω-gliadins by Jones et al. (Jones et al., 1959).

Later, studies conducted using other techniques including amino acid sequence and amino acid composition have shown that some gliadins can be separated into further groups and some gliadins (α- and β- gliadins) can be considered under one group: ω5-, ω1,2-, α/β-, and γ-gliadins (Wieser, 2007). However, some reports still use the old classification (α-, β-, γ-, and ω-gliadins) or some have used α- and β-gliadins together under α-gliadins without mentioning β-gliadins. Gliadins can also be divided into S-rich (α-, β-, and γ-gliadins) and S-poor (ω-gliadins) based on the cysteine content as shown in Figure 2.4.

Within gliadin groups, ω-gliadins are characterized by the highest contents of glutamine, proline, and phenylalanine, with few or no residues of sulphur-containing amino acids i.e., cysteine and methionine (Shewry et al., 1986) (Table 2.5). Among the gliadins, ω5-gliadins have higher MWs than ω1,2-gliadins (Wieser, 1996), and α/β-gliadins generally have the lowest MWs while α/β- and γ- gliadins are found to be the most prevalent types. Studies related to the amino acid sequences of gliadins have reported that the ω-gliadins consist almost entirely of repetitive sequences rich in glutamine and proline while α/β and γ-gliadins have different N- and C-terminal domains. The N-terminal domain of α/β and γ-gliadins are repetitive and rich in glutamine, proline, phenylalanine, and tyrosine (Wieser, 2007) while the C-terminal domains are non-repetitive, have less glutamine and proline than the N-terminal domain, and they are rich in cysteine. An example of the repetitive amino acid sequence of each gliadin type is given in Table 2.5.

**Table 2.5** Partial amino acid composition (g per 100 g protein) and molecular weight of wheat gliadins.

| Characteristic | Gliadins | | | |
|---|---|---|---|---|
| | ω | | α/β | γ |
| | ω5 | ω1,2 | | |
| Partial amino acid composition | | | | |
| Glutamine | 55.0-56.0 | 43.0-45.0 | 36.0-41.0 | 33.0-38.0 |
| Proline | 19.0-20.0 | 25.0-27.0 | 15.0-19.0 | 16.0-18.0 |
| Phenylalanine | 9.0-10.0 | 7.0-8.0 | 3.0-4.0 | 4.0-6.0 |
| Cysteine | 0 | 0 | 1.0-2.0 | 2.0-3.0 |
| Methionine | 0-0.1 | 0-0.1 | 0.9-1.2 | 0.9-1.7 |
| Tyrosine | 0-1.0 | 1.0-2.0 | 3.0-4.0 | 0-1.0 |
| MW (kDa) | 66.0-79.0 | 55.0-65.0 | 32.0 | 38.0-42.0 |
| Proportion in total gluten (%) | 3.0-6.0 | 4.0-7.0 | 28.0-33.0 | 23.0-31.0 |
| Repeated amino acid sequence[1] | PQQQF | PQQPFPQQ | QPQPFPQQPYP | QPQQPFP |

[1]Standard single-letter codes for amino acids: F, phenylalanine; P, proline; Q, glutamine; Y, tyrosine; MW, molecular weight; Source: Adapted from Shewry et al., 1986; Wieser, 2007; Wieser, 1996.

#### 2.4.2.1.2 Barley hordeins

Hordeins are the prolamins found in barley. The mature seed contains about 3-4% (dry weight) hordeins (Fincher, 2010). Like gliadins, hordeins are rich in glutamine and proline and particularly low in lysine (Table 2.6). Based on their electrophoretic mobilities and amino acid compositions, barley hordeins are divided into B-, C-, D-, and γ-groups. The B- and C-hordeins are the major types comprising up to 70-80% and 10-20% respectively. B- and γ-hordeins (30-50 kDa) are rich in sulfur-containing amino acids while C-hordeins (44-80 kDa) are poor in sulfur-containing amino acids. D-hordeins (60-90 kDa) are known as HMW hordeins.

**Table 2.6** Partial amino acid composition (g/100 g protein) in whole hordeins and different hordeins fractions.

| Amino acid | Whole hordeins[a] | Hordein fractions[b, c] | | | |
|---|---|---|---|---|---|
| | | B | C | D | γ |
| Glutamine | 34.7 | 30.0 | 37.0 | 26.0 | 28.0 |
| Proline | 23.0 | 19.0 | 29.0 | 11.0 | 17.0 |
| Phenylalanine + Tyrosine | 5.9 + 2.4 | 7.3 | 9.4 | 5.5 | 7.7 |
| Glycine | 2.5 | 2.9 | 0.6 | 16.0 | 3.1 |
| Leucine | 6.9 | 8.0 | 8.6 | 4.1 | 7.0 |
| Valine | 4.6 | 6.2 | 0.3 | 4.1 | 7.3 |
| Lysine | 0.5 | 0.5 | 0.2 | 1.1 | 2.8 |

Adapted from [a] Lásztity & Hidvégi, 2012; [b] Koehler & Wieser, 2013; [c] Shewry, 1993.

#### 2.4.2.1.3 Rye secalins

The alcohol-soluble storage proteins (prolamins) of rye are called secalins, which account for about 30-50% of the total protein of the mature seed (Shewry et al., 1983; Shukla & Bushuk, 1975). Four major groups of secalins have been identified based on their mobility in gel electrophoresis (Shewry et al., 1983): HMW-secalins (100 kDa or greater), γ-40k-secalins (40 kDa), γ-75k-secalins (75 kDa), and ω-secalins (50 kDa). These groups are structurally related to prolamins present in barley and wheat. Nearly half of the total storage proteins are γ-75k-secalins, followed by γ-40k-secalins (24%), ω-secalins (17%), and HMW-secalins (7%) (Gellrich et al., 2003).

HMW-secalins contain high contents of glutamine, glycine, and proline (Koehler & Wieser, 2013). Similar to ω-1,2-gliadins of wheat and C-hordeins of barley, nearly 80% of the total amino acids in ω-secalins are glutamine, proline, and phenylalanine (Table 2.7). These three proteins are categorised as sulfur-poor prolamins where they do not contain or contain negligible amounts of cysteine and methionine. γ-75k secalins also have high amounts of glutamine and proline.

**Table 2.7** Partial amino acid composition (g/100 g protein) in different secalin fractions.

| Amino acid | Secalin fraction | | | |
|---|---|---|---|---|
| | **HMW** | **ω** | **γ-40k** | **γ-75k** |
| Glutamine | 34.0 | 40.0 | 34.0 | 38.0 |
| Proline | 12.0-15.0 | 29.0 | 18.0 | 22.0 |
| Phenylalanine | 0.6 | 7.0 | 4.5 | 6.3 |
| Tyrosine | 4.0 | 1.6 | 1.0 | 1.6 |
| Glycine | 18.0-20.0 | 0.6 | 2.4 | 1.6 |
| Leucine | 3.2-3.7 | 4.4 | 7.4 | 4.8 |
| Valine | 1.5-1.8 | 1.8 | 4.7 | 5.3 |
| Lysine | 0.2 | 0.3 | 1.3 | 1.0 |
| Cysteine | 1.3 | 0.0 | 2.7 | 1.6 |

HMW, high molecular weight; Adapted from Koehler & Wieser, 2013; Tatham & Shewry, 1991; Tatham & Shewry, 1995.

#### 2.4.2.1.4 Prolamins of other cereals

Prolamins of other cereals such as rice, corn, and sorghum have less glutamine and proline content compared to prolamins from wheat, barley, and rye. Corn prolamins were classified in many ways based on the solubility and the MWs by different research groups. These classifications are sometimes confusing since some of them have considered glutelin fraction of the corn storage proteins together with prolamins and given the name zein. Esen (Esen, 1987) suggested a classification based on the solubility of corn proteins with 2-propanol: i.e., α-zein (10 kDa and 21-25 kDa, high leucine (20%) and phenylalanine (6%)), β-zein (17-18 kDa, high methionine (10%) and tyrosine (8%)), and γ-zein (27 kDa, high proline (25%) and histidine (8%)). The same author also suggested that the LMW zeins found in the α-zein fraction could be categorised separately as δ-zein. This classification was confirmed based on electrophoretic mobility by another research group (Shewry, 1996), categorising zein proteins into four groups: α-zein, β-zein, γ-zein, and δ-zein. Except for α-zeins, others are only extracted when reducing agents are present in aqueous alcohols. α-zeins are

the major subclass (71-85% of total zeins), followed by γ-zeins (10-20%), β-zeins (1-5%) and δ-zeins (1-5%) (Esen, 1987).

Kafirin, the alcohol-soluble prolamin protein fraction in sorghum endosperm, makes up about 50% of the total grain protein and it is classified into three groups based on MW, solubility, and structure: α-kafirin (23 kDa), β-kafirin (16 kDa and 18 kDa), and γ-kafirin (28 kDa) (Shull et al., 1991). These proteins are rich in glutamine and non-polar amino acids such as leucine and alanine. Rice prolamins are about 4-5% of the grain (Hoogenkamp et al., 2017; Shukla & Bushuk, 1975). MWs of prolamin fractions of rice are between 12 kDa and 17 kDa which are rich in glutamine, alanine, glycine, and arginine. Avenins in oats have also been classified into α-, β-, γ- and δ-avenins based on the electrophoretic mobility of their polypeptides with MWs ranging from 20-34 kDa (Lasztity, 1995). These polypeptides contain a high amount of glutamine (36.1%), proline (~10%), leucine (10.3%) and valine (5.5%) (Lasztity, 1995; Peterson, 2016).

#### 2.4.2.2 Glutelins

Glutelins are the protein fractions from cereals that are soluble in very dilute acids and alkalis, but insoluble in water, neutral aqueous solutions or 70% alcohols. However, when a reducing agent (e.g., mercaptoethanol, dithiothreitol) is present, all the glutelins are soluble in aqueous alcohols. Due to their innate low solubility, glutelins are difficult to extract and most of the time, it may not be possible to extract glutelin fractions without contaminating prolamins (Shewry, 1993).

Wheat glutelins are specifically termed glutenins. Shukla et al. reported that 16% of the total grain protein is glutenins (Shukla & Bushuk, 1975). However, some authors have reported a higher glutenin content; around 40-45% of the total grain protein (Biesiekierski 2017; Veraverbeke & Delcour, 2002). These differences in glutenins

could be due to the varietal differences and the different levels of nitrogen fertilisation during the crop growing period (Herbert & Werner, 1998). The amino acid composition of glutenins is similar to that of gliadins in terms of glutamine and proline but they contain higher amounts of glycine compared to gliadins. Glutenins consist of subunits, which are usually divided into two classes according to their MWs: HMW-glutenin subunits (HMW-GS) and LMW-glutenin subunits (LMW-GS). The MWs of HMW-GS range from 60-136 kDa based on the mobility of gel electrophoresis (Lindsay & Skerritt, 1999; Shewry et al., 1986). HMW-GS represent approximately 10% of the total seed storage proteins and they contain a high amount of glycine (14.8%), glutamine (32.6%), and proline (~12.8%) (Field et al., 1982) (Table 2.8). Based on the number of cysteine molecules at the N-terminal domain of the HMW-GS, they have been classified into two groups: x (three cysteine molecules) and y (five cysteine molecules) (Figure 2.5). The C-terminal domain of all the HMW-GS contains only one cysteine. They have also been referred to as HMW prolamins in some reports since they can be partially extracted in aqueous alcohols.

In general, the LMW-GS represent about one-third of total seed storage proteins and 60% of glutenins (Bietz, 1973; Long et al., 2005). Most of the LMW-GS are purified from the gliadin fraction rather than glutenins. Therefore, LMW-GS are also known as HMW gliadins or aggregated type gliadins (Shewry et al., 1986). LMW-GS were originally classified as B (42-51 kDa), C (30-40 kDa), and D (55-70 kDa) subunits based on differences in mobility on gel electrophoresis. They are also considered under the sulfur-rich prolamins family because of the presence of a high amount of cysteine as shown in the partial amino acid composition of glutenin fractions in Table 2.8.

**Table 2.8** Partial amino acid composition of wheat glutenin fractions (g/ 100 g protein).

| Amino acid | Glutenin fraction | |
|---|---|---|
| | HMW | LMW |
| Cysteine | 0.4-1.5 | 2.7 |
| Methionine | 0.4 | 0.6 |
| Phenylalanine | 0.3-1.1 | 4·7 |
| Tyrosine | 11.0 | 1.0 |
| Glutamine | 34.0-39.0 | 38.0 |
| Proline | 13.0-16.0 | 15.0 |
| Glycine | 14.0-20.0 | 3.3 |

HMW, high molecular weight; LMW, low molecular weight; Source: Shewry et al., 1986; Wieser, 2007.

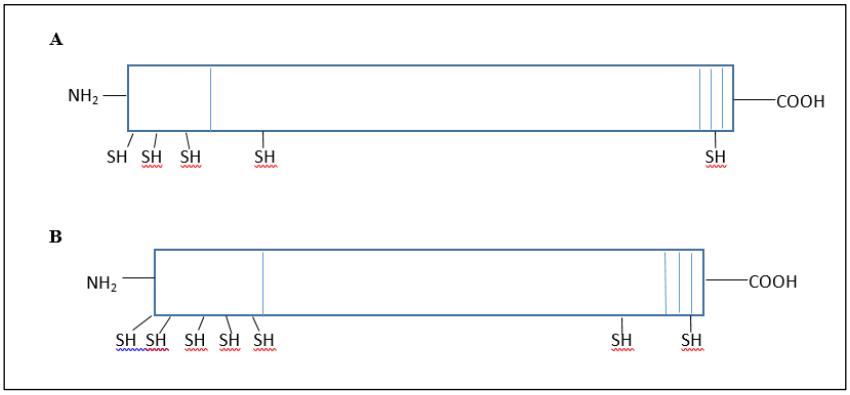



**Figure 2.5** Schematic diagram of general X (A) and Y (B) types of high molecular weight glutenin subunits.

SH, typical positions of cysteine residues; Source: Adapted from Shewry et al., 1992.

In barley and rye, about 22% and 30-50% of total proteins are glutelins respectively (Shukla & Bushuk, 1975). The amino acid composition of glutelin fractions of barley and rye is similar to that of wheat, having a high amount of glutamine and proline (Table 2.9). In sorghum, 32% of the total proteins are glutelins (Shukla & Bushuk, 1975). They also contain a high amount of glutamine, proline, in addition to leucine, and alanine (Table 2.8). Corn glutelins are about 39% of the total proteins whereas, in oat, glutelins account for less than 10% of the total protein (Lasztity, 1995; Shukla & Bushuk, 1975).

The glutelins of rice are known as oryzenins and they are the most important protein fraction in rice storage proteins (Lasztity, 1995), comprising ~80% of the total proteins (Shukla & Bushuk, 1975). The MWs of the oryzenins are high ranging from 45-150 kDa (Hoogenkamp et al., 2017). When cleaved, oryzenins form two major polypeptide subunits which are classified as α, or acidic, and β, or basic subunits with apparent MWs of 30-39 kDa and 19-25 kDa, respectively (cited in Champagne, 2004). In addition, oryzenins are classified into two subfamilies, i.e. A type and B type, based on primary amino acid sequence comparisons (cited in Takaiwa et al., 1991). The amino acid content of oryzenins is similar to that of glutelins from other cereals but contains comparatively higher lysine content and lower glutamine and proline contents (Table 2.9).

#### 2.4.2.3 Albumins and globulins

According to Osborne's solubility characteristics, albumins are soluble in water and globulins are insoluble in water but soluble only in dilute salt solutions. These proteins are present throughout the grain, mainly in the aleurone layer, bran, and germ of the cereal and limited amounts are present in the endosperm of certain cereals (Koehler & Wieser, 2013). The concentration of albumin and globulin proteins in cereal grains varies widely (Table 2.10). Particularly in oats, globulins (avenalins) account for about 70–80% of the total protein and represent their major storage proteins (Shewry & Halford, 2002).

**Table 2.9** Amino acid composition (g/100 g protein) and molecular weights (MWs) of glutelins of cereals.

| Characteristic | Wheat | Barley | Rye | Sorghum | Rice | Corn | Oat |
|---|---|---|---|---|---|---|---|
| Amino acid composition | | | | | | | |
| Lysine | 1.7 | 2.2 | 2.0 | 3.1 | 5.6 | 3.0 | 4.5 |
| Histidine | 2.0 | 1.9 | 1.3 | 3.1 | 4.0 | 2.5 | 2.9 |
| Arginine | 3.3 | 3.2 | 1.7 | 5.9 | 10.3 | 4.1 | 9.5 |
| Aspartic acid | 3.0 | 4.2 | 2.7 | 9.0 | 8.7 | 7.1 | 8.0 |
| Threonine | 3.1 | 3.8 | 2.7 | 4.8 | 3.3 | 3.4 | 3.7 |
| Serine | 5.2 | 5.7 | 5.3 | 5.3 | 6.1 | 5.9 | 4.5 |
| Glutamine | 40.9 | 25.9 | 33.7 | 29.0 | 18.1 | 20.0 | 26.5 |
| Proline | 13.1 | 14.1 | 16.6 | 14.8 | 8.3 | 10.9 | 6.2 |
| Glycine | 5.8 | 6.3 | 7.2 | 5.3 | 4.6 | 4.5 | 4.3 |
| Alanine | 3.0 | 5.1 | 3.4 | 9.4 | 5.0 | 6.2 | 5.1 |
| Valine | 4.8 | 6.5 | 9.4 | 5.5 | 6.0 | 5.1 | 8.8 |
| Methionine | 1.8 | 1.3 | 1.0 | 1.4 | 1.0 | 2.4 | 2.7 |
| Isoleucine | 3.6 | 4.2 | 2.3 | 4.0 | 4.4 | 3.2 | 4.6 |
| Leucine | 7.4 | 8.2 | 5.2 | 12.4 | 10.0 | 12.0 | 9.4 |
| Tyrosine | 4.1 | 2.7 | 2.3 | 3.2 | 2.9 | 4.6 | 4.6 |
| Phenylalanine | 4.4 | 4.3 | 3.4 | 4.9 | 5.8 | 4.9 | 6.9 |
| Tryptophan | 1.6 | 1.1 | 0.9 | - | 1.1 | 0.8 | 1.9 |
| Cysteine | 2.7 | 0.5 | 2.5 | 1.2 | 1.3 | 3.4 | 2.4 |
| Name given | Glutenins | Hordenins | Secalinins | Glutelins | Oryzenins | Zeanins | Glutelins |
| MW (kDa) | 30-136 | ~50 | 70-90 | 20-67 | 45-150 | 21-26 | 9 |
| % Total protein | 16-40 | ~23 | 30-50 | ~32 | ~80 | ~39 | ~5 |

Source: Lasztity, 1995; Lásztity & Hidvégi, 2012; Shukla & Bushuk, 1975; Takeda et al., 1970; Hoogenkamp et al., 2016; Shewry et al., 1978; Klose & Arendt, 2012; Alves et al., 2017.

The cereal globulins have been classified into two groups based on their sedimentation coefficients: 7S globulins (vicilin-type) and 11S globulins (legumin-type) (Shewry, 1995). The albumins present in cereals are known as 2S albumins based on the sedimentation coefficient (cited in (Moreno & Clemente, 2008)). The specific roles played by albumins and globulins in cereals are not properly understood. However, it has been identified that most proteins of the albumin and globulin fractions act as enzymes and enzyme inhibitors (Koehler & Wieser, 2013), apart from their role as seed storage proteins. They also serve in promoting water absorption during germination of the seed (cited in Shukla & Bushuk, 1975). In addition, it has been found that albumins can also play a protective role in plants against fungal attacks (Agizzio et al., 2006).

The albumin and globulin proteins usually have a better essential amino acid profile than prolamins and glutelins (Shukla & Bushuk, 1975). However, there are differences in amino acid composition between the two proteins. For example, albumin fractions contain high levels of cysteine and globulins are deficient in cysteine (Shewry, 1995; Youle & Anthony, 1981).

**Table 2.10.** Composition (g/100 g protein) of albumin and globulin in some cereals.

| Cereal | Albumin | Globulin |
|---|---|---|
| Rice | 5.0 | 10.0 |
| Oat | 1.0 | 78.0 |
| Barley | 13.0 | 12.0 |
| Wheat | 3.0-5.0 | 10.0 |
| Rye | 5.0-10.0 | 5.0-10.0 |
| Corn | 4.0 | 2.0 |
| Sorghum | 8.0 | 8.0 |

Source: Shukla & Bushuk, 1975.

## 2.5 Immunogenic cereal proteins

### 2.5.1 Gluten proteins

Generally, the term 'gluten' is used for gliadins and glutenins in wheat. It is believed that approximately equal fractions of gliadins and glutenins are present in wheat gluten (Figure 2.6). In addition to wheat gliadins and glutenins, prolamins of barley (hordeins), rye (secalins), and oat (avenins) are collectively referred to as gluten.

Studies have indicated that in wheat, all major gliadin protein subgroups (α-, β-, ω-, and γ-gliadins) can produce immunogenic peptides during digestion (Ciclitira et al., 1984; Wieser et al., 1982). However, among the wheat gliadins, A-gliadin (Figure 2.7), a small group of α-gliadins, has been shown to produce the strongest immunogenic effects (de Ritis et al., 1988; Sturgess et al., 1994). *In vivo* and *in vitro* studies have confirmed that A-gliadin peptides in the amino acid sequence such as residues l-30, 1-127, l-55, 31-49 (19-mer), 31-55, 57-73, 128-246, 202-220, and 206-217 can produce immunogenic reactions in sensitive individuals (Anderson et al., 2000; de Ritis et al., 1988; Ellis et al., 1998; Mantzaris & Jewell, 1991; Sturgess et al., 1994).

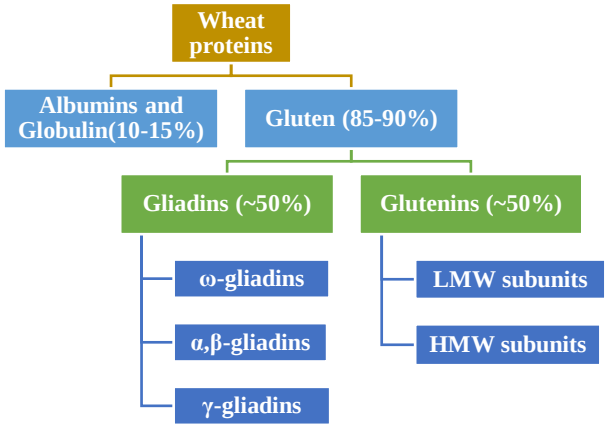



**Figure 2.6** Composition of wheat gluten proteins.

LMW, low molecular weight; HMW, high molecular weight; Source: Wieser, 2007; Biesiekierski, 2017.

```
         10         20         30         40         50         60
VRVPVPQLQP QNPSQQQPQE QVPLVQQQQF LGQQQPFPPQ QPYPQPQPFP SQQPYLQLQP

         70         80         90        100        110        120
FPQPQLPYSQ PQPFRPQQPY PQPQPQYSQP QQPISQQQQQ QQQQQQQQQQ QQQIIQQILQ

        130        140        150        160        170        180
QQLIFCMDVV LQQHNIAHGR SQVLQQSTYQ LLQELCCQHL WQIPEQSQCQ AIHNVVHAII

        190        200        210        220        230        240
LHQQQKQQQQ PSSQVSFQQP LQQYPLGQGS FRPSQQNPQA QGSVQPQQLP QFEEIRNLAL

        250        260
QTLPAMCNVY IAPYCTIAPF GIFGTN
```

**Figure 2.7** Amino acid sequence of A-gliadin.

Source: Adapted from Sturgess et al., 1991.

In addition, the 33-mer peptide (residues 56–88) from α2-gliadin, has been widely reported as one of the most immunodominant peptides (Qiao et al., 2004; Shan et al., 2002). It contains six overlapping copies of three DQ2-restricted T cell epitopes: PFPQPQLP, PYPQPQLPY, and PQPQLPYPQ (Figure 2.8).

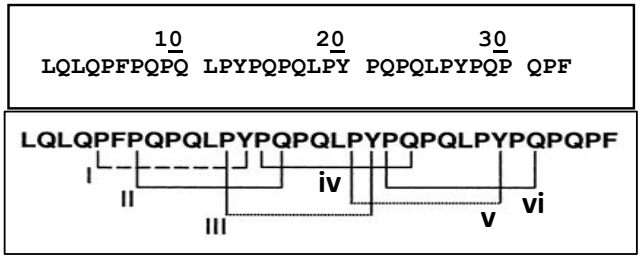



**Figure 2.8** The 33-mer and its epitopes.

Standard single-letter codes for amino acids: P, proline; L, leucine; F, phenylalanine; Y, tyrosine; Q, glutamine; Source: Adapted from Qiao et al., 2004.

Similar to wheat gliadins, secalins, and hordeins also produce immunogenic peptides (Table 2.11). For instance, similar immunogenic peptides to what α2-gliadin, α9-gliadin, α20-gliadin, γ1-gliadin, and γ2-gliadin have been found in secalins and hordeins (Vader et al., 2003; Wahab et al., 2016; Wieser & Koehler, 2008). Peptides in oat prolamins (avenins) in some oat varieties have also been identified as immunogenic (Comino et al., 2011; Silano et al., 2014).

**Table 2.11** List of major immunogenic peptides of cereal proteins.

| Peptide name/epitope | The sequence of amino acids | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| DQ2.5-glia-α1a | P | F | P | Q | P | E | L | P | Y |
| DQ2.5-glia-α1b | P | Y | P | Q | P | E | L | P | Y |
| DQ2.5-glia-α2 | P | Q | P | E | L | P | Y | P | Q |
| DQ2.5-glia-α3 | F | R | P | E | Q | P | Y | P | Q |
| DQ2.5-glia-γ1 | P | Q | Q | S | F | P | E | Q | Q |
| DQ2.5-glia-γ2 | I | Q | P | E | Q | P | A | Q | L |
| DQ2.5-glia-γ3 | Q | Q | P | E | Q | P | Y | P | Q |
| DQ2.5-glia-γ4a | S | Q | P | E | Q | E | F | P | Q |
| DQ2.5-glia-γ4b | P | Q | P | E | Q | E | F | P | Q |
| DQ2.5-glia-γ4c | Q | Q | P | E | Q | P | F | P | Q |
| DQ2.5-glia-γ4d | P | Q | P | E | Q | P | F | C | Q |
| DQ2.5-glia-γ5 | Q | Q | P | F | P | E | Q | P | Q |
| DQ2.5-glia-ω1 | P | F | P | Q | P | E | Q | P | F |
| DQ2.5-glia-ω2 | P | Q | P | E | Q | P | F | P | W |
| DQ2.5-glut-L1 | P | F | S | E | Q | E | Q | P | V |
| DQ2.5-glut-L2 | F | S | Q | Q | Q | E | S | P | F |
| DQ2.5-hor-1 | P | F | P | Q | P | E | Q | P | F |
| DQ2.5-hor-2 | P | Q | P | E | Q | P | F | P | Q |
| DQ2.5-hor-3 | P | I | P | E | Q | P | Q | P | Y |
| DQ2.5-sec-1 | P | F | P | Q | P | E | Q | P | F |
| DQ2.5-sec-2 | P | Q | P | E | Q | P | F | P | Q |
| DQ2.5-ave-1a | P | Y | P | E | Q | E | E | P | F |
| DQ2.5-ave-1b | P | Y | P | E | Q | E | Q | P | F |
| DQ2.2-glut-L1 | P | F | S | E | Q | E | Q | P | V |
| DQ8-glia-α1 | E | G | S | F | Q | P | S | Q | E |
| DQ8-glia-γ1a | E | Q | P | Q | Q | P | F | P | Q |
| DQ8-glia-γ1b | E | Q | P | Q | Q | P | Y | P | E |
| DQ8-glut-H1 | Q | G | Y | Y | P | T | S | P | Q |
| DQ8.5-glia-α1 | E | G | S | F | Q | P | S | Q | E |
| DQ8.5-glia-γ1 | P | Q | Q | S | F | P | E | Q | E |
| DQ8.5-glut-H1 | Q | G | Y | Y | P | T | S | P | Q |

glia-α, α-gliadin; glia-γ, γ-gliadin; glia-ω, ω-gliadin; glut-L, low molecular weight glutenin; glut-H, high molecular weight glutenin; hor, hordein; sec, secalin; ave, avenin; standard single letter codes for amino acids: A, alanine; E, glutamic acid; F, phenylalanine; I, isoleucine; L, leucine; P, proline; Q, glutamine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine; Source: Sollid, Qiao et al., 2012.

Previously, it was believed that wheat glutenins do not produce any immunogenic peptides during digestion. However, in 1999, a study concluded that only one peptide from HMW glutenins (residues 724–734) produces immunogenic reactions in sensitive individuals (van de Wal et al., 1999). The immunogenicity of this peptide was confirmed by using T-cell proliferation assays. This peptide is repetitively present in HMW glutenin molecules. Subsequently, several studies have confirmed that a few other HMW glutenin peptides are immunogenic (Molberg et al., 2003; W. Vader et al., 2002) (Table 2.11). However, to date, except for HMW glutenins, none of the other glutelins is reported to produce immunogenic peptides.

### 2.5.2 Other proteins

An *in silico* analysis confirmed that there are several peptides in corn prolamins that could be expected to trigger immunogenic reactions (Cabrera-Chávez et al., 2012). Amylase trypsin inhibitors (ATIs), also activate the innate immune system and immunogenic reactions in sensitive individuals via direct activation of the toll-like receptor 4 on myeloid cells (Cuccioloni et al., 2017; Junker et al., 2012; Schuppan & Zevallos, 2015; Yolanda et al., 2018; Zevallos et al., 2017). These inhibitors have been found in wheat, barley, rye, rice, maize, and finger-millet (Carbonero & García-Olmedo, 1999). Among these cereals, wheat, barley, and rye are reported to contain the highest concentrations of ATIs. For instance, ATIs account for ~2-4% of total wheat proteins (Dupont et al., 2011).

The globulins of domestic cereals have not been reported as immunogenic proteins. However, globulins (7S and 11S-12S) of *Brachypodium distachyon* were reported to produce immunogenic reactions in sensitive individuals (Gell et al., 2017). The primary storage protein of this cereal is globulins (Larré et al., 2010). *Brachypodium distachyon*

is a wild grass, which is identified as the closest wild relative of wheat and barley (Vogel et al., 2006).

Since gluten proteins seemed to be the key proteins causing immune responses, the rest of the review will consider mainly gluten proteins.

### 2.6 Production of immunogenic peptides during gastrointestinal tract digestion

Proteins undergo a complex series of hydrolyses in the digestive tract of humans, which are carried out by proteolytic enzymes originating in the stomach, pancreas, and small intestine. These proteolytic enzymes are responsible for the cleavage of the proteins into fragments (amino acids or di- and tri-peptides) that can be absorbed by the small intestine. Apart from these enzymes, microbial enzymes are also responsible for the hydrolysis of certain amounts of proteins. Gluten proteins undergo gastric and small intestinal digestions to produce immunogenic peptides, which will be discussed in the next sections.

#### 2.6.1 Gastric digestion of gluten proteins

Dietary protein digestion begins in the stomach with pepsins. The gastric glands of the stomach secrete hydrochloric acid and pepsinogens (the inactive proenzymes of pepsins). Hydrochloric acid can change pepsinogen into pepsin, the active form of the gastric protease. When a food bolus enters the stomach, the fundus and body of the stomach (Figure 2.9) act as a reservoir for food. Peristaltic waves in the antrum help to break large food particles by grinding and mixing with secreted enzymes and hydrochloric acid. The pylorus acts as a sieve and a pump for the selective emptying of small particles into the duodenum.

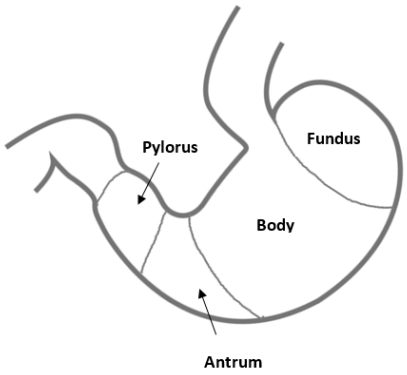



**Figure 2.9** Different regions of the human stomach.

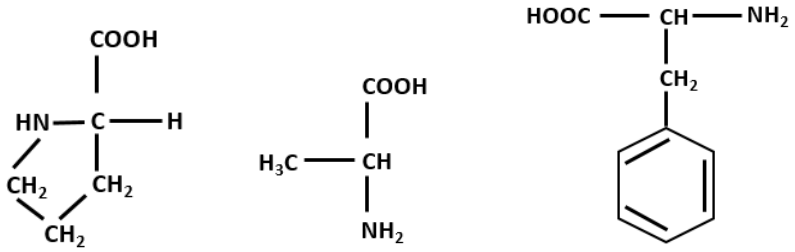
The action of pepsin is mediated in the acidic pH of the stomach in the range of 1.3 to 3 (Kong & Singh, 2008). However, the pH of the stomach chyme during the first postprandial minutes is closer to the pH of the diet (Malagelada et al., 1976 ), which indicates that the action of pepsin is limited during this time.

The amino acid sequences of gluten proteins are unusually rich in proline and glutamine. For example, gliadins contain 46% of glutamine and 17% of proline (Table 2.12) and hordeins contain 35% glutamine and 23% proline (Lásztity & Hidvégi, 2012). Proline is a nonpolar, aliphatic amino acid. It is the only amino acid where the side chain is connected to the protein backbone twice, forming a five-membered nitrogen-containing ring (Figure 2.10). The presence of a higher amount of proline in the amino acid chains of gluten proteins is problematic when comes to digestion because this structure of proline avoids the peptide chain from proteolytic attack by pepsin, thereby preventing the cleavage by pepsin.

**Table 2.12** Partial amino acid composition (g per 100 g protein) and molecular weight of wheat gliadins.

| Characteristic | Gliadin fractions | | | | Whole gliadin |
|---|---|---|---|---|---|
| | ω | | α/β | γ | |
| | ω5 | ω1,2 | | | |
| Partial amino acid composition | | | | | |
| Glutamine | 55.0-56.0 | 43.0-45.0 | 36.0-41.0 | 33.0-38.0 | 46.6 |
| Proline | 19.0-20.0 | 25.0-27.0 | 15.0-19.0 | 16.0-18.0 | 17.0 |
| Phenylalanine | 9.0-10.0 | 7.0-8.0 | 3.0-4.0 | 4.0-6.0 | 6.3 |
| Cysteine | 0 | 0 | 1.0-2.0 | 2.0-3.0 | 2.3 |
| Methionine | 0-0.1 | 0-0.1 | 0.9-1.2 | 0.9-1.7 | 1.8 |
| Tyrosine | 0-1.0 | 1.0-2.0 | 3.0-4.0 | 0-1.0 | 2.9 |
| MW (kDa) | 66.0-79.0 | 55.0-65.0 | 32.0 | 38.0-42.0 | |
| Proportion in total gluten (%) | 3.0-6.0 | 4.0-7.0 | 28.0-33.0 | 23.0-31.0 | ~50 |

MW, molecular weight; Source: Adapted from Shewry et al., 1986; Wieser, 2007; Wieser, 1996; Shukla & Bushuk, 1975.




(a) (b) (c)

**Figure 2.10** Structure of proline (a) vs other amino acids (alanine (b) and phenylalanine (c))

Pepsins are aspartic proteases that split peptide bonds between hydrophobic aromatic and large aliphatic amino acids, particularly cleaving peptide linkages either before or after phenylalanine, tyrosine, and leucine residues at the middle of each chain (Figure 2.11) (Herman et al., 2006; Moran, 2016). However, pepsin prefers to cleave peptide bonds particularly when several other aliphatic or aromatic amino acids are in the

sequence on either side of the cleaving bond (Moran, 2016). Gluten proteins lack aliphatic and aromatic amino acids in their polypeptide chains. The available aliphatic or aromatic amino acids in the polypeptide chain are usually followed by proline. For example, the repetitive unit of the α-gliadin: QPQPFPPQQPYP, (Q, glutamine; P, proline; F, phenylalanine; Y, tyrosine), has two proline units on either side of the phenylalanine. As shown in Figure 2.12, pepsin will not cleave the peptide bond between P1 and P1', when proline is present in positions P2 to P3'.

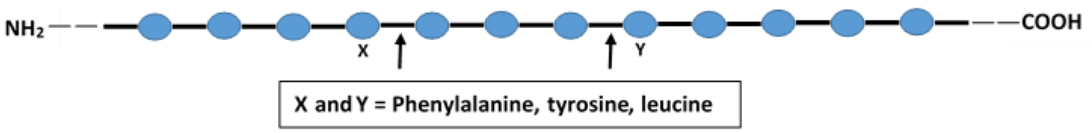



**Figure 2.11** Substrate specificity of pepsin.
Source: Adapted from Ganapathy et al., 2008.

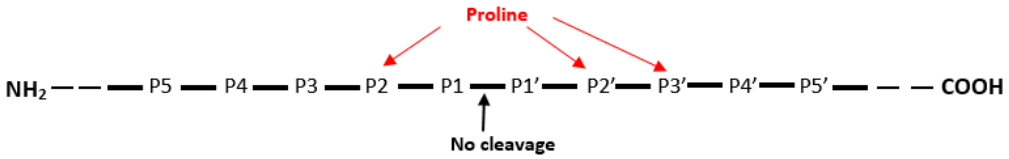



**Figure 2.12** Schematic representation of how proline affects the pepsin cleavage.
P1-5, P1'-5', amino acids in the polypeptide chain; Source: Adapted from Keil, 2012.

Poor gastric digestion of gluten proteins has been observed *in vivo*. In a rat study, the gastric digestion of whole gluten protein after around 5.5-7 h was only 4% when compared to 12% in whey protein isolate and 9% in beef muscle protein (Montoya & Hindmarsh, et al., 2014). The same research group determined the *in vivo* digestion of individual gluten proteins, by measuring the disappearance of individual intact proteins using a tricine-SDS-PAGE approach (Rutherfurd et al., 2011). This technique permits

semi-quantitative determination of degradability of protein within a food matrix and there may be proteins, which overlap with endogenous proteins giving overestimated values. The gastric degradability of high molecular weight wheat glutenin was reported around 42-46% and it was less than 40% for all low molecular weight glutenins. For ω-gliadin, 44.3% gastric degradability was observed. (Montoya & Hindmarsh, et al., 2014). These observations could be partially attributed to the fast stomach-emptying rate of gluten. A faster stomach-emptying rate means that there is less time for pepsin to digest gluten proteins. For example, in this reported study, the time to reach half-gastric emptying (T½) of wheat gluten (T½=157 min) was lower than other protein sources (T½=245–266 min for zein, beef muscle, and gelatine proteins) in rats.

Limited information is available for the *in vitro* gastric digestion of gluten proteins. Therefore, as a part of this review, an *in silico* gastric digestion was performed for certain gluten proteins to provide a better understanding of the digestion pattern with pepsin. Peptide sequences of gluten protein fractions were obtained from the protein databases: UniprotKB (Anonymous, 2018) and ProPepper (Juhász et al., 2015). The protein sequences were then subjected to an *in silico* digestion by pepsin using the online tool Peptide Cutter (Gasteiger et al., 2003) as described elsewhere (Dave et al., 2014). The pepsin cleavage was set to before or after phenylalanine, tyrosine, tryptophan, and leucine in the middle of the polypeptide chain at pH 1.3. The resulting peptides from different gluten protein fractions were further tested for their immunogenic effects using an online database: ProPepper (Juhász et al., 2015).

**Table 2.13** Cleavage pattern and release of epitopes from gluten protein fractions with pepsin *in silico*.

| Gluten protein | Number of amino acids | Number of peptide bonds cleaved by pepsin | Digestion % |
|---|---|---|---|
| HMW-GS | 848 | 68 | 8 |
| LMW-GS | 356 | 40 | 11 |
| α/β-gliadin | 286 | 49 | 17 |
| γ-gliadin | 327 | 41 | 12 |
| ω-5-gliadin | 439 | 60 | 13 |
| 75k γ-secalin | 455 | 43 | 9 |
| C-hordein | 105 | 6 | 6 |

HMW-GS, high molecular weight glutenin subunit; LMW-GS, low molecular weight glutenin subunit.

The *in silico* approach only considers the primary structure of the proteins therefore it is expected overestimated digestion values. Despite this, it is a useful tool to understand a wide range of digestions for different protein fractions. Results of the *in silico* pepsin digestion indicated that the pepsin digestion of gluten protein fractions is much lower (Table 2.13) than those observed using the SDS-PAGE approach described above, which could be mainly explained by the limitations of the latter approach. It is evident that *in silico* low digestion of gluten protein fractions is attributed to the amino acid sequence of their polypeptide chain. When the resulting peptides from different gluten protein fractions were further tested for their immunogenic effects, it was found that some of the resulting peptides contained probable epitopes inducing immunogenic reactions causing different gluten-related health disorders. Some of the identified epitopes are listed in Table 2.14.

Overall, the faster stomach-emptying rate and the poorer gastric digestion of gluten proteins means that a greater amount of undigested gluten peptides reach the small intestine, with probable epitopes inducing immune responses in sensitive individuals.

**Table 2.14** Some of the B cell and T cell epitopes obtained after *in silico* gastric digestion of different gluten protein fractions.

| Parent protein | Epitope | Cell type | Disease |
|---|---|---|---|
| HMW-GS | GQGQQGQQPGQGQQ | B cell | Allergy |
| HMW-GS | GQGQQPGQGQQGQQ | B cell | WDEIA |
| HMW-GS | QQPGQGQQ | B cell | WDEIA |
| LMW-GS | QQQPP | B cell | Allergy |
| α/β-gliadin | QQPFP | B cell | Allergy |
| γ-gliadin | QQPFP | B cell | Allergy |
| γ-gliadin | FPQQPQQPF | T cell | CD |
| ω-5-gliadin | QIPQQPQQF | T cell | CD |
| 75k γ-secalin | FPQQPQQP | T cell | CD |

HMW-GS, high molecular weight glutenin subunit; LMW-GS, low molecular weight glutenin subunit; CD, coeliac disease; WDEIA, wheat-dependent exercise-induced anaphylaxis.

### 2.6.2 Intestinal digestion of gluten proteins

After gastric digestion, the chyme reaches the small intestine where it is exposed to a range of pancreatic proteolytic enzymes in the duodenum (trypsin, chymotrypsin, elastase, and carboxypeptidases) and brush-border membrane peptidases (aminopeptidases, dipeptidylcarboxypeptidase, and dipeptidylaminopeptidase IV (DPP IV)) secreted by the enterocytes. These enzymes further cleave the polypeptide residues after gastric digestion based on their amino acid specificities (Table 2.15), under neutral pH conditions (Boland, 2016).

**Table 2.15** Enzyme specific amino acids of the stomach and pancreatic proteolytic enzymes.

| Enzyme | Enzyme-specific amino acids |
|---|---|
| Trypsin | Arginine, lysine |
| Chymotrypsin | Phenylalanine, tyrosine, tryptophan |
| Elastase | Alanine, leucine, glycine, valine, isoleucine |
| Carboxypeptidases | Lysine, arginine, phenylalanine, tryptophan, leucine |

Source: Erickson & Kim, 1990; Neurath et al., 1970.

Trypsin, chymotrypsin, and elastase are capable of hydrolysing the carboxyl end of peptide bonds in the middle of the proteins and polypeptides (Ganapathy et al., 2008). In contrast, carboxypeptidases (A and B) hydrolyse the terminal peptide bond on the carboxyl-terminal of polypeptides/proteins (Figure 2.13), releasing free amino acids from them.

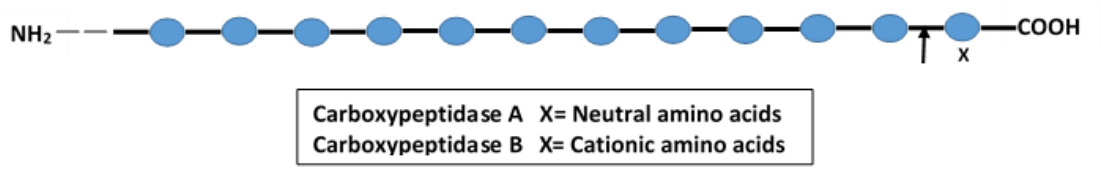



**Figure 2.13** Substrate specificity of carboxypeptidases.
Source: Adapted from Ganapathy et al., 2008.

The action of pancreatic enzymes on proteins in the intestinal lumen produces a mixture of oligopeptides and free amino acids. The peptidases associated with the brush-border membrane play a major role in hydrolysing these oligopeptides into a mixture of free amino acids, dipeptides, and tripeptides. The amino acid specificities of brush-border enzymes are such that the aminopeptidases hydrolyse the terminal peptide bonds at the amino-terminal of oligopeptides, which produce free amino acids. The dipeptidylcarboxypeptidase hydrolyses the peptide bond adjacent to the carboxyl-terminal peptide bond, while DPP IV hydrolyses the peptide bond adjacent to the amino-terminal releasing di-peptides (Figure 2.14). The substrate specificities of these enzymes are such that they generally cleave the peptide bonds associated with proline (Gray, 2010; Hausch et al., 2002), which means these enzymes are able to cleave proline present in the gluten peptides.

Similar to what occurred in the stomach, the digestion of proline-rich gluten peptides in the small intestine is limited. First, the activities of chymotrypsin and trypsin are limited for gluten peptides due to high proline contents.

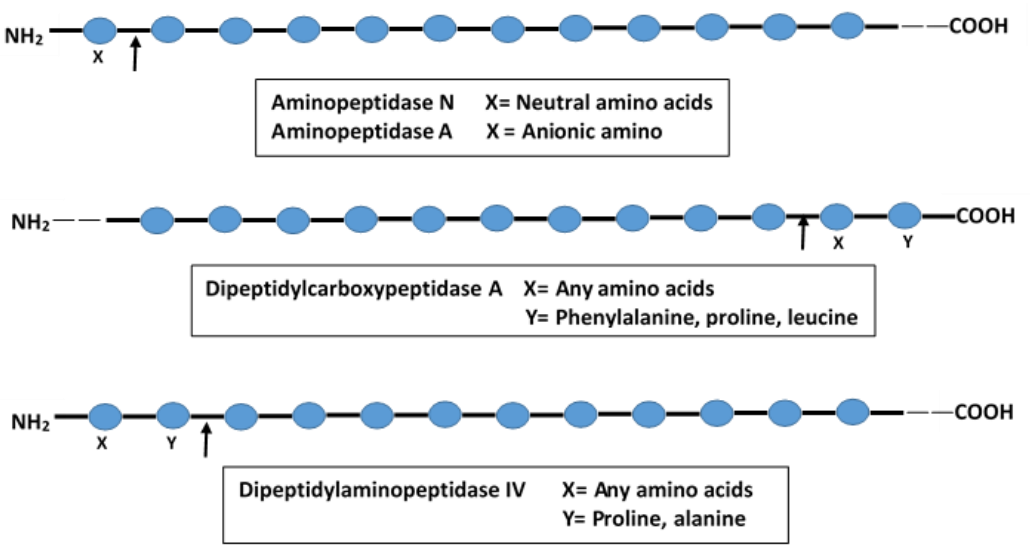



**Figure 2.14** Substrate specificities of peptidases associated with brush-border membrane.

Source: Adapted from Ganapathy et al., 2008.

As is the case with pepsin, the structure of proline may act as a disruptor, which prevents it from proteolytic attack by chymotrypsin and trypsin. Also, the specific amino acids cleaved by trypsin (lysine and arginine) are rare in gluten peptides (Scherf et al., 2016). Second, even though the brush-border membrane peptidases can hydrolyse proline-containing peptides, the amount of the enzyme may not be sufficient to hydrolyse a very high amount of proline-rich peptides in gluten proteins because gluten peptides are unusually proline-rich as mentioned above. The exposure time of the gluten peptides to these peptidases may not be sufficient to cleave all the gluten peptides available in a gluten-containing meal.

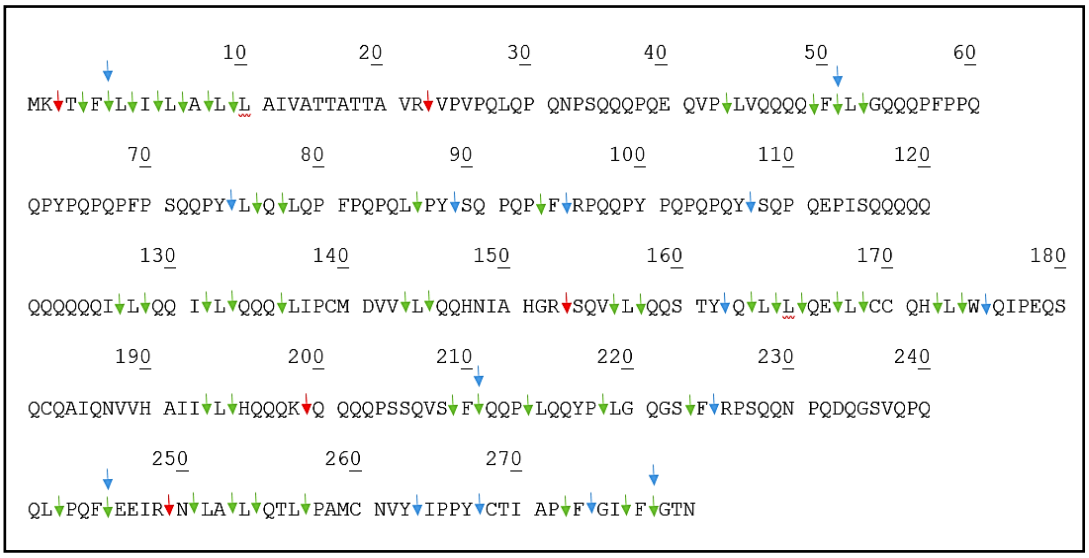
When gluten proteins were exposed *in vitro* to pepsin followed by pancreatin digestions, a number of undigested peptides have been identified (Prandi et al., 2017).

These peptides were well-known immunogenic peptides. One such peptide is 33-mer peptide. This peptide was remained intact even after exposure to rat brush-border membrane proteases for a prolonged period (~ 15 h) (Shan et al., 2002). This suggests that the time of exposure to the enzymes is not a limiting factor for the digestion of certain gluten peptides but not all.

Due to the scarce information on small intestinal digestion of gluten proteins, the same *in silico* digestion approach described above for pepsin was conducted with pancreatic and brush-border enzymes. Since α-gliadins are reported to be highly resistant to proteolysis, one of the α-gliadins (279 amino acids) was selected in this digestion. The amino acid sequence of the protein was obtained from UniprotKB (Anonymous, 2018). First, simultaneous *in silico* peptic, chymotryptic, and tryptic digestions were performed using the online tool Peptide Cutter (Gasteiger et al., 2003) (Figure 2.15A). After this *in silico* digestion, a manual prediction of proteolytic fragments by pancreatic elastase, carboxypeptidases, aminopeptidases, dipeptidylcarboxypeptidase, and DPP IV was performed for peptides with only more than three amino acids using the enzyme specificities of these enzymes (Figure 2.15B). It was assumed that amino acids, di- and tri-peptides are readily absorbed by the small intestine, and these do not need any further cleavage.

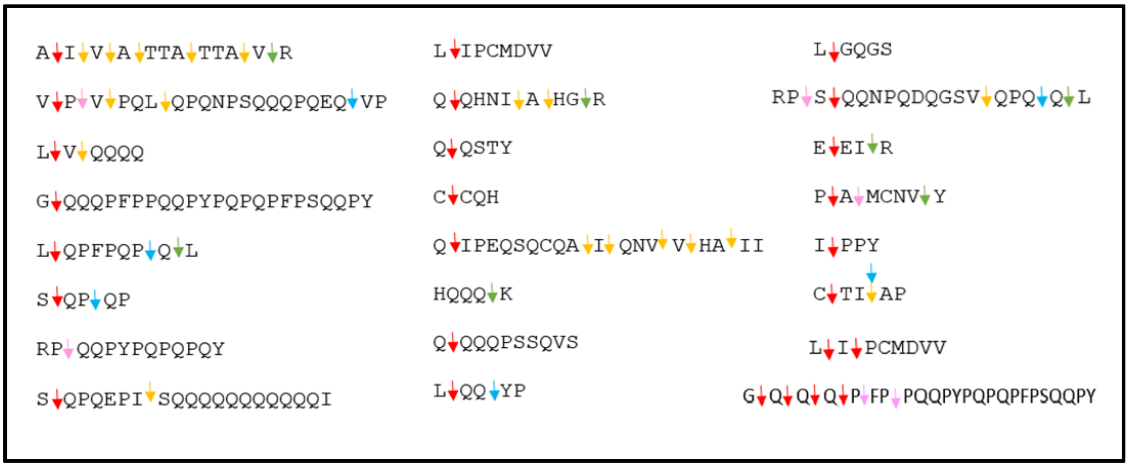
Pancreatic elastase cleavages were set after alanine, leucine, glycine, valine, and isoleucine in the middle of the peptide chain; carboxypeptidase A and B cleavages were set at the terminal peptide bond on the carboxyl-terminal of lysine, arginine, phenylalanine, tryptophan, and leucine; aminopeptidases cleavages were set at the terminal peptide bonds at the amino-terminal of alanine, glycine, isoleucine, leucine, valine, cysteine, serine, threonine, methionine, asparagine, and glutamine. Dipeptidylcarboxypeptidase cleavages were set at the amino-terminal of adjacent

amino acid when phenylalanine and proline are on the carboxyl-terminal end, while DPP IV cleavages were set at the carboxyl-terminal end of the proline and alanine when they are next to any amino acid of the amino-terminal of the polypeptide chain (Figure 2.14).




Positions of cleavage in the α-gliadin polypeptide by pepsin (↓), chymotrypsin (↓), and trypsin (↓)

A




Positions of cleavage by elastase (↓), carboxypeptidases (↓), aminopeptidases (↓), dipeptidylcarboxypeptidase (↓) and DPP IV (↓) in peptides having four or more than four amino acids resulted after peptic, chymotryptic, and tryptic digestions

B

**Figure 2.15** *In silico* simulated gastrointestinal digestion of α-gliadin with pepsin, chymotrypsin, and trypsin (A) and with elastase, carboxypeptidases, aminopeptidases, dipeptidylcarboxypeptidase and DPP IV (B).

The peptic, chymotryptic, and tryptic digestions resulted in twenty-four peptides with more than three amino acids. Even after the digestion with elastase, carboxypeptidases, aminopeptidases, dipeptidylcarboxypeptidase, and DPP IV, eighteen peptides were observed with more than three amino acids suggesting that the gluten peptides are incompletely digested in the gastrointestinal tract of humans. These peptides were further tested for their immunogenic effects using an online database: ProPepper (Juhász et al., 2015). Results revealed that most of the peptides resulted after *in silico* digestion with human gastric, pancreatic, and brush-border enzymes are epitopes trigging either B cells or T cells of the genetically predisposed individuals, causing CD (Table 2.16). The smallest epitope contained four amino acids.

**Table 2.16** B cell and T cell epitopes obtained after in silico gastric and small intestinal digestion of one specific α-gliadin.

| Epitope | Cell type | Disease |
|---|---|---|
| *After gastric digestion* | | |
| IPEQ | B cell | CD |
| FPPQQPYPQPQP | B cell | CD |
| FRPQQPYPQ | T cell | CD |
| FPPQQPYPQP | B cell | CD |
| QPFPPQQPYP | B cell | CD |
| PPQQPYPQPQPFPSQQPY | B cell | CD |
| QPFPPQQPYPQPQPFPSQQPY | B cell | CD |
| *After small intestinal digestion* | | |
| IPEQ | B cell | CD |
| PPQQPYPQPQPFPSQQPY | B cell | CD |
| QPFPPQQPYPQPQPFPSQQPY | B cell | CD |
| FPPQQPYPQP | B cell | CD |
| FPPQQPYPQPQP | B cell | CD |

CD, coeliac disease.

Recently Montoya et al. (Montoya CA, 2018) have shown that the amino acid digestibility in the small intestine is affected by the amount of digested protein entering the small intestine (i.e., the combination between the rate of digestion of protein in the stomach and stomach emptying). Highly digested proteins entering the small intestine

are digested and absorbed in the first half of the small intestine, while poorly digested proteins are digested and absorbed in the second half of the small intestine. This suggests that gluten proteins, which are poorly digested in the stomach, are digested in the second half of the small intestine and therefore, some gluten peptides may not be digested in the small intestine.

High ileal digestibility values of gluten proteins have been reported for various *in vivo* studies. For example, the true ileal digestibility of raw gluten was 97% in rats (Table 2.17). Similar observations were found for other gluten-containing cereals as shown in Table 2.17. This suggests that gluten proteins are readily digested when they reached the latter part of the small intestine. This may be due to the presence of proteases from sources other than the host such as microbial proteases that could digest gluten proteins.

**Table 2.17** True ileal nitrogen digestibility (%) of cooked and raw proteins of gluten-containing cereals in monogastric species.

| Cereal flour | Species | | Reference |
|---|---|---|---|
| | Pigs | Rats | |
| *Cooked (boiled)* | | | |
| Wheat | 71.0-86.3** | | (Sauer & Ozimek, 1986), |
| Gluten | - | 98.5[a] | (Fleming & Vose, 1979) (Metta & Johnson, 1959) |
| Rye | 73.0** (baked) | - | (Glitsø et al., 1998) |
| Barley | 69.2 (extruded) | - | (Fadel et al., 1988) |
| Oat | 84.0**(rolled) | 88.5 | (Bach Knudsen & Hansen, 1991; Rutherfurd et al., 2015) |
| *Raw* | | | |
| Wheat | 88.9 | | (Jondreville et al., 2001) |
| Gluten | - | 96.8 | (Rutherfurd et al., 2011) |
| Rye | 77.3 | | (Büchmann, 1979; Jondreville et al., 2001) |
| Barley | 75.9-82.7 | 66.0 | (Jondreville et al., 2001), (Moughan et al., 1984) |
| Oat | 83.6 | | (Pettersson et al., 1996), (Lin et al., 1987) |

** Apparent digestibility, [a] Faecal digestibility, % True ileal nitrogen digestibility = [(Nitrogen in the diet – (Nitrogen in the ileal digesta-Ileal endogenous nitrogen flow))/Nitrogen in the diet] x 100.

### 2.6.3 Microbial digestion of gluten proteins

Several gastrointestinal tract microbes are able to produce enzymes that can hydrolyse gluten. Glutamine endoprotease enzymes secreted by salivary microorganisms have been derived from the dental plaque of humans. These enzymes were found to be capable of digesting glutamine and proline-rich gluten proteins (Helmerhorst et al., 2010). *Rothia mucilaginosa* and *Rothia aeria* were identified as bacterial strains secreting glutamine endoproteases with high activity towards gliadin proteins. It was observed that half of the wheat gliadins were degraded, including immunogenic domains of gliadins, after ~30 min of incubation with these bacteria (Zamakhchari et al., 2011). However, these results were obtained after isolation of the bacteria from human dental plaque and incubation in a Petri dish with a very small amount of gliadin proteins (250 µg/mL). In a real situation, assuming an intake of two slides of white bread, the amount of gluten eaten could be much higher (~3-5 g) than this. In addition, mastication in the oral cavity lasts less than one min, so the time of exposure of these enzymes to gluten will not be sufficient to give considerable hydrolysis unless these bacteria or the enzymes can survive or be active in the stomach and the small intestine. Although the optimal activity of these enzymes is at neutral to basic pH conditions, they are active in a wide pH range (3-10) (Helmerhorst et al., 2010). It has also been found that these bacterial enzymes are reversibly inactivated at pH 3, and their activity could be fully restored when the pH is adjusted to pH 7 (Fernandez-Feo et al., 2013). This suggests that these enzymes can survive in the pH changes that occur in the stomach and the small intestine. Unfortunately, there is no sufficient *in vitro* data to know if these bacteria provide a considerable contribution to the digestion of gluten proteins in a normal diet.

Studies have also reported the presence of gluten-degrading bacteria in the upper small intestine of humans (Caminero et al., 2016). *Pseudomonas aeruginosa* and several *Lactobacillus* spp. isolated from the duodenum are able to degrade gluten peptides. In addition, colonic gluten-degrading bacteria have been isolated from human faeces. Most of the isolated strains belonged to the phylum *Firmicutes* mainly from the genera *Lactobacillus, Streptococcus, Staphylococcus,* and *Clostridium* (Caminero et al., 2014). These suggest that microbial enzymes are responsible, at least partially, for the degradation of gluten proteins in the gastrointestinal tract, which may have also contributed to the high true ileal digestibility values reported for gluten *in vivo.*

### 2.6.4 Other factors affecting the digestion of gluten proteins

Apart from the above-described reasons (amino acid sequence and protease specificity), the digestion of gluten proteins can be affected by various reasons such as the presence of anti-nutritional factors, the level of processing (e.g., grinding, heat treatment), the structure of the food material, etc. Cereals contain naturally occurring anti-nutritional compounds that reduce the digestion of proteins (Ahmed et al., 1991; Jansman et al., 1995; Reddy, 2002; Sarwar Gilani et al., 2012). Examples of such major anti-nutritional factors include phytates, alkylresorcinols, tannins, and trypsin inhibitors (Grela, 1996). The digestion of gluten proteins also varies with the level of exposure to heat (Duodu et al., 2003; Bender, 1972). Thermal processing could induce protein modifications, which result in the formation of compounds resistant to digestion (Gilani et al., 2005; Mauron, 1990; Oeste et al., 1987; Sarwar Gilani et al., 2012). For example, exposure to high heat can cause proteins to react with sugars, forming compounds that are resistant to digestion (Maillard compounds). Heating can also induce the formation of cross-links between protein molecules, making them unavailable for digestion or causing

incomplete digestion. On the other hand, heating can also be responsible for the inactivation of protease inhibitors, modifications in the structure of proteins, and the breakdown of food matrices, increasing protein digestion (Ejeta et al., 1987; Hamaker et al., 1986). The level of temperature and the method used to process the food (baking, extrusion etc.) could also affect the level of digestion in gluten proteins (Table 2.18).

**Table 2.18** *In vitro* degree of hydrolysis of gluten proteins with different heat treatments.

| **Heat treatment** | **Wheat product** | ***In vitro* pepsin digestion at pH 2 (time, activity, protein:pepsin)** | **Degree of hydrolysis %** |
|---|---|---|---|
| Raw | Wheat bread dough | 30 min, ~3,000 U/mg protein, 60 mg:0.2 mg | 17.0 |
| Baked at ~100 °C | Wheat breadcrumb | | 73.0 |
| Baked at ~180-200 °C | Wheat bread crust | | 43.0 |

Source: Hamaker et al., 1986.

These differences in the digestion of gluten proteins may also be related to the physical structure of the cereal grain that prevent the accessibility of digestive enzymes to proteins such as lipid-carbohydrate-protein interactions in the food matrix (Lang et al., 2015), the particle size of the foods being digested (Mandalari et al., 2018), and the presence of physical barriers like cell walls (Mandalari et al., 2008). Therefore, the action of lipases and carbohydrate degrading enzymes (typically amylases) also play an important role in breaking the food matrices and making the protein accessible to enzymatic breakdown. For instance, the addition of α-amylase in an *in vitro* digestion caused a rapid disappearance of larger polypeptides of gluten (40-55 kDa) compared to the control in SDS-PAGE gels (Smith et al., 2015).

Undigested and unabsorbed large peptides from the small intestine, enter the large intestine, where they are hydrolysed by bacterial peptidases (Macfarlane et al., 1986). The remaining undigested peptides are excreted with the faeces.

### 2.7 Immunogenicity of gluten proteins

The high resistance of gluten proteins to digest in the stomach creates a greater amount of undigested gluten peptides reaching the small intestine. These peptides can comprise epitopes with unique amino acid sequences such as those shown in Table 2.11. When these epitope containing peptides reach the first part of the small intestine, they activate either T cell or B cell receptors present in the intestinal mucosa of the genetically predisposed individuals (Ciclitira et al., 1984; Gass et al., 2007; Morón et al., 2008), to trigger immunogenic reactions. The mechanism of action of these immunogenic responses will be discussed later (Sections 2.8.8.1, 2.8.2 and 2.8.3).

Other than that, these immunogenic peptides can cross the intestinal epithelium as intact peptides and antigen-specific immune responses could be created. However, limited scientific evidence is available to confirm that the transport of larger intact macromolecules other than single, di- and tri-peptides across the intestinal endothelium of the healthy adult human. Nevertheless, it has been found that individuals, who are susceptible to immunogenic reactions, may possess increased intestinal paracellular permeability allowing larger peptides to pass through the epithelial barrier of the intestine, by modulating tight junctions of the small intestine. Zonulin, a protein known to regulate intestinal permeability reversibly, has been suggested to play a role in this modulation of tight junctions, enhancing intestinal permeability (Fasano, 2012). In addition, these peptides can cross the epithelial surface through disruptions in the tight

junctions, in individuals who have damaged intestinal epithelium due to various reasons (MacDonald & Monteleone, 2005).

### 2.8 Gluten-related health disorders

Celiac disease, GS, and WA have genetic, environmental, and immunological components that are involved in the development of these diseases. The only modifiable component, which is the environmental factor, is mainly the intake of gluten. Therefore, these diseases are collectively known as 'gluten-related health disorders' (Figure 2.16).

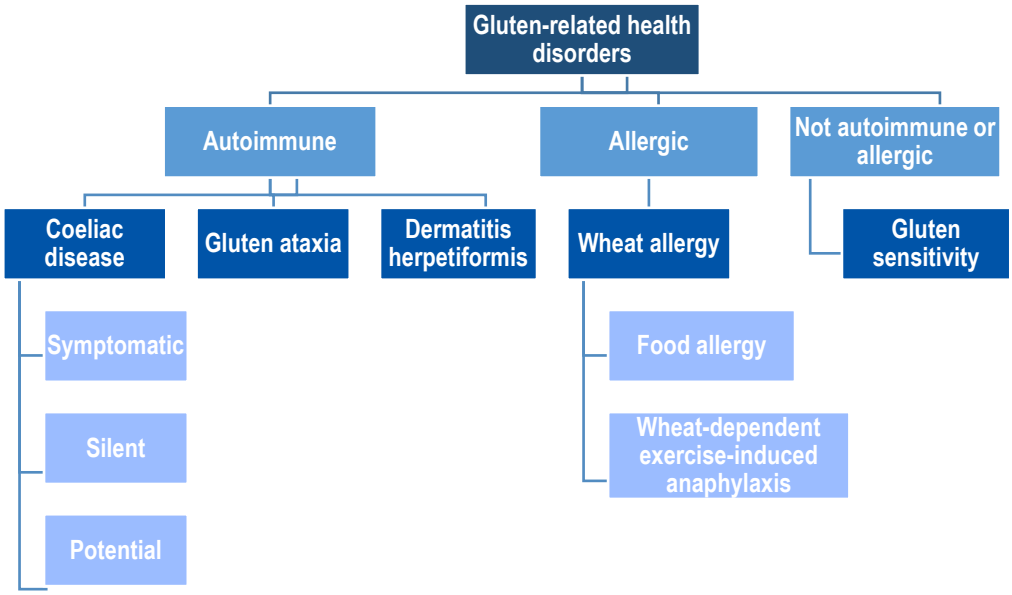



**Figure 2.16** Disorders related to gluten consumption.

Source: Adapted from Sapone et al., 2012.

#### 2.8.1 Coeliac disease

Coeliac disease, also known as coeliac sprue or gluten-sensitive enteropathy, is one of the most frequent food intolerances and an autoimmune disorder. The prevalence of CD in the population has been estimated to approximate 0.5-1% in different parts of the world, with some regions demonstrating a higher prevalence than others. For instance,

the prevalence of CD in adults is between 0.7% - 2.4% in Finland and Italy, whereas it is only 0.3% in Germany (Mustalahti et al., 2010) and 1.6% in Asia according to a recent meta-analysis (Singh et al., 2016). However, most of the CD cases remain undiagnosed (Catassi et al., 2007).

#### 2.8.1.1 Role of immunogenic peptides in disease pathogenesis

In the pathogenesis of CD, the role of the adaptive immune system is well understood (T-cell mediated). Histocompatibility leukocyte antigen (HLA) class II genes (HLA-DQ2 and HLA-DQ8) are the most important genetic factors for the CD. About 90% of the CD patients have HLA-DQ2 coded by DQA1*05/DQB1*02 genes, and the majority of the remaining patients are HLA-DQ8 coded by DQA1*03/DQB1*0302 genes (Zubillaga et al., 2002). The immunogenic gluten peptides are bound to either HLA-DQ2 or HLA-DQ8. These DQ2- or DQ8-peptide complexes are recognized by the $CD4^+$ T-cell receptors present in the intestinal mucosa of these individuals (Hausch et al., 2002; Scherf et al., 2016). This triggers an inflammatory response in the small intestine. T-cell responses were found to be enhanced by the influence of the tissue transglutaminase (tTG) enzyme. The tTG enzyme converts some specific glutamine residues in gluten peptides into glutamic acid, which results in a higher affinity of these peptides for HLA-DQ2 or HLA-DQ8 (Sollid, 2002). The tTG has been identified as the most notable autoantigen in CD (Dieterich et al., 1997).

In addition, these peptides can also bind to the human HLA class I genes and these complexes are recognised by the $CD8^+$ T cells present in the epithelium of the small intestine (Mazzarella et al., 2008). It has also been shown that the innate immune system plays a role in the pathogenesis of CD (non-T-cell mediated). Certain gluten epitopes (e.g., α-gliadin peptide 31–43) can also induce the secretion of interleukin-15 (IL-15)

by intestinal dendritic cells (Maiuri et al., 2003). This IL-15 secretion can lead to an increase in the number of intraepithelial lymphocytes (IELs) in the epithelial layer and IELs can cause epithelial cell destruction. This is one of the innate immune responses in the CD. Apart from adaptive and innate responses in CD, immunogenic peptides can also trigger reactions in mucosal IgA B cells (Mesin et al., 2012).

According to Sapone et al. (Sapone et al., 2012), the clinical spectrum of CD (Figure 2.16) includes symptomatic cases, silent and potential forms. Symptomatic cases include CD with intestinal features (e.g., diarrhoea, steatorrhea, vomiting, constipation, and gastroesophageal reflux disease and abdominal pain) or without intestinal features (e.g., anaemia, osteoporosis, and neurological disturbances) while silent forms are discovered after serological screening. In the potential forms of CD, even if the antibodies are detected during serological screening, no autoimmune response of the intestinal mucosa is present.

The CD predominantly affects the mucosa of the upper small intestine, and the effect is known as villous atrophy (Figure 2.17). The damage can vary from mild to a total absence of villi, crypt hyperplasia, and increased lymphocyte infiltration of the epithelium (Sollid, 2002). The atrophy of the villi negatively affects both the release of brush border enzymes and the absorption of nutrients. The decreased absorption of nutrients explains several of the common symptoms of CD such as weight loss, fatigue, vitamin, and mineral deficiencies such as anaemia, decreased bone mineral density, bone pain and fractures, dental enamel defects, and night blindness (Kaukinen et al., 2010).

Gluten ataxia is the term used to describe neurological manifestations attributed to CD. This can be defined as idiopathic sporadic ataxia with positive serum CD antibodies. These patients have serological evidence of CD, but they do not show any

gastrointestinal symptoms or evidence of small-bowel inflammation related to CD, but ataxia (Hadjivassiliou et al., 1998).

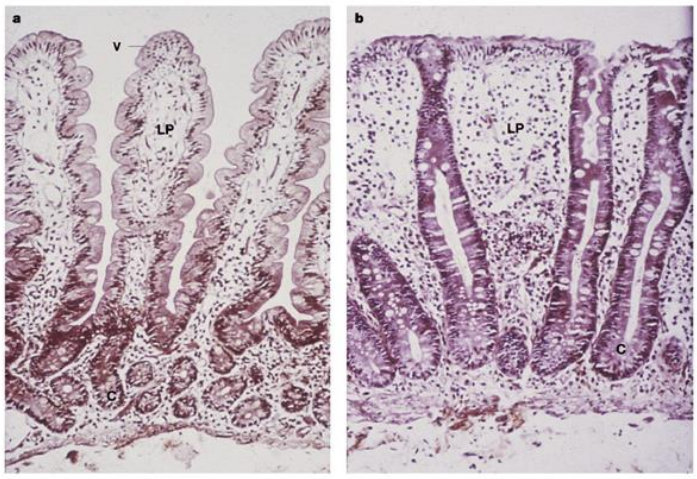



**Figure 2.17** Small intestinal mucosa of normal individuals and coeliac disease patients. a, histology of a normal small intestine showing (V) villi and (C) crypts; the (LP) lamina propria; b, the coeliac mucosa characterized by villous atrophy; Source: Sollid, 2002.

Dermatitis herpetiformis occurs in some patients with a genetic tendency for CD, and it is observed as a skin manifestation of CD (Salmi et al., 2011). Equivalent to CD, the immune reaction to dietary gluten is mediated by T-cell activation in the intestinal mucosa. However, the main autoantigens that develop CD and dermatitis herpetiformis are different but share common epitopes (Sárdy et al., 2002). This is the reason why only certain CD patients show dermatological symptoms. Gastroenterological symptoms in dermatitis herpetiformis are generally mild or absent. However, inflammatory small bowel changes can often be present (Sapone et al., 2012). Typical symptoms are intense itching and burning of the skin, especially on the elbows, upper forearms, buttocks, and knees while granular immunoglobulin-A deposits are present in the skin (Scherf et al., 2016).

### 2.8.2 Wheat allergy

The wheat allergy occurs due to immune-mediated reactions by the ingestion of immunogenic peptides in sensitive individuals who form specific immunoglobulin-E (IgE) antibodies against these peptides (Hischenhuber et al., 2006). The immunogenic peptides form cross-links with IgE antibodies and trigger the release of chemical mediators, such as histamine, platelet-activating factor, and leukotrienes (Cianferoni, 2016; Tanabe, 2008).

Therefore, in contrast to CD, WA develops immediate reactions followed by symptoms on the skin, the gastrointestinal tract, and the respiratory tract. Unlike CD, WA does not cause permanent gastrointestinal or other organ damage once the acute reaction has resolved.

Wheat allergy-related conditions include two types, food allergy and wheat-dependent exercise-induced anaphylaxis (WDEIA) (Figure 2.16). Food allergic responses include skin swelling, itching, urticaria, wheezing, impaired breathing, bronchial obstruction, bloating, abdominal pain, diarrhoea, and sometimes anaphylaxis (Scherf et al., 2016) after ingestion of foods made from wheat and other cereals. The WDEIA is a form of allergic reaction induced mainly by the ingestion of ω5-gliadins of wheat proteins and subsequent physical exercise (Morita et al., 2003). The typical symptoms associated with WDEIA are pruritus, urticaria, angioedema, dyspnoea, hypotension, gastrointestinal symptoms, and respiratory tract obstruction. In severe cases, anaphylactic shock can also be observed (Palosuo et al., 1999).

### 2.8.3 Gluten sensitivity

Gluten sensitivity or non-coeliac gluten sensitivity occurs in certain individuals, who are neither allergic (WA) nor autoimmune (CD) in nature. The patients with GS express

similar symptoms to CD upon ingestion of immunogenic peptides, but they do not show villous atrophy similar to CD (Catassi et al., 2013). Although about 50% of the GS patients have HLA-DQ2 and HLA-DQ8 encoding genes, which are markers of CD (Sapone et al., 2011), there is no correlation between the presence of these genes and the occurrence of GS. Further, it has also been reported that GS is triggered not only by gluten, but also by wheat amylase-trypsin inhibitors, fermentable oligo-, di- and mono-saccharides, polyol, and food additives such as glutamates, benzoates, sulphites, and nitrates (Volta et al., 2013).

Currently, the diagnosis of GS is done based on exclusion (Figure 2.18), as there are no specific blood tests for this condition. However, the diagnosis of GS should be confirmed by a double-blind, placebo-controlled gluten challenge (Carroccio et al., 2012). Common symptoms of GS include abdominal pain, bloating, diarrhoea, and weight loss together with a few extra-intestinal symptoms including headache, migraine, foggy mind, chronic fatigue, muscle pain, leg or arm numbness, anxiety, and depression shortly after ingestion of immunogenic epitopes (Czaja-Bulsa, 2015).

Knowledge regarding GS is still lacking, and many aspects of this disorder remain unknown. Therefore, future research is needed to generate more knowledge regarding GS. Table 2.19 compares the common characteristics of CD, WA, and GS.

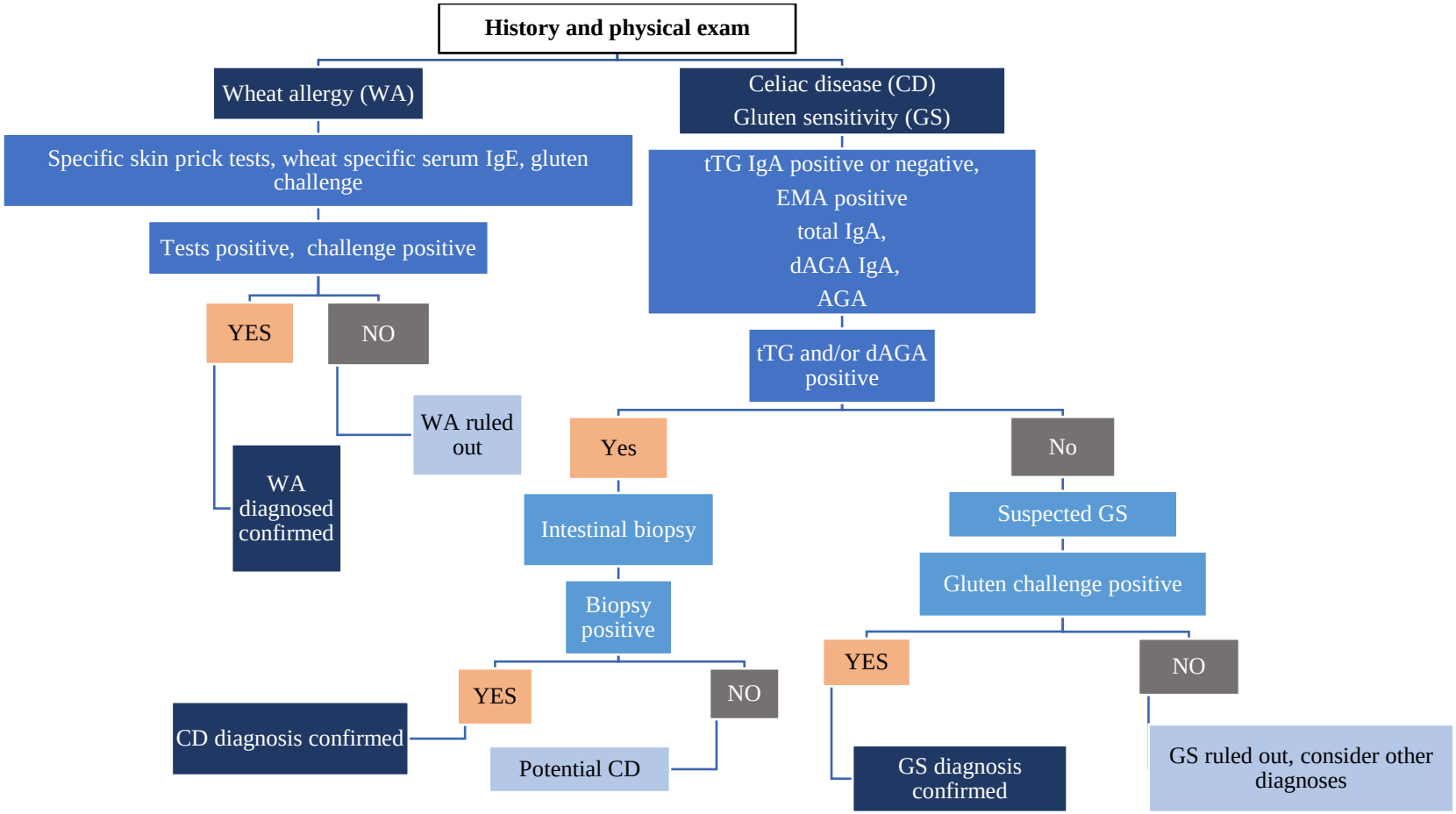



**Figure 2.18** Diagnosis of coeliac disease, gluten sensitivity and wheat allergy.

tTG, tissue transglutaminase; AGA, anti-gliadin antibodies; dAGA, deamidated anti-gliadin antibodies; EMA, endomysial antibodies; IgA, immunoglobulin-A; IgE, immunoglobulin-E; Source: Adapted from Sapone et al., 2012.

**Table 2.19** Common characteristics of CD, wheat allergy and gluten sensitivity.

| Characteristics | Coeliac disease | Wheat allergy | Gluten sensitivity |
|---|---|---|---|
| Morbidity | 1% | 1% | Unknown, possibly 0.6-6% |
| Immunity | Adaptive/innate | Allergic | Innate? |
| Genetic background | In 95% patients: HLA-DQ2 or HLA-DQ8 | In 100%: atopy | In 50%: HLA-DQ2 or HLA-DQ8 |
| Pathogenic mechanisms | Disturbances in the acquired immune response to toxic peptides depend on the combination of HLA-DQ2 and HLA-DQ8 | IgE-dependent reactions, immune response to toxic cereal peptides, especially wheat allergens | Unknown, probably the disturbances in the primary immune response to toxic peptides and other toxic compounds |
| Antibodies in serum | tTG, EMA, DGP, AGA primarily in the IgA class, less frequently in the IgG class | IgE for wheat<br>IgE for ω5-gliadin (in WDEIA)<br>In 25% of patients: IgG-AGA | In 50% of patients: IgG-AGA |
| Atrophy of duodenal villi | Present | May be present | Absent |
| Symptoms | Intestinal and extra-intestinal | Intestinal and extra-intestinal | Intestinal and extra-intestinal |
| Mortality | Increased | Increased in anaphylaxis | Unknown |
| Duration of GFD to be taken | Lifelong | The average of 6 years, individual; lifelong in anaphylaxis | Unknown |
| Onset of symptoms | Days-weeks | Minutes-hours | Hours-days |
| Complications | Long-term | Short-term | Long-term |

tTG, tissue transglutaminase; EMA, endomysium antibodies; DGP, deamidated gliadin peptide; AGA, antigliadin antibodies; WDEIA, wheat-dependent exercise-induced anaphylaxis; GFD, gluten-free diet; Source: Adapted from Czaja-Bulsa, 2015; Scherf et al., 2016.

## 2.9 Treatments to eliminate the disorders related to immunogenic peptides

Treatments to eliminate immunogenic reactions have mainly been researched on CD since CD is the most common gluten-related disorder reported to date. Therefore, this section mainly contains information reported for the CD. However, similar treatments could be applied to WA and GS.

### 2.9.1 Dietary treatments

A lifelong GFD is the only known medical treatment for CD and other gluten-related disorders (Haines et al., 2008). A GFD includes the complete elimination of wheat, barley, and rye products. Since immunogenic proteins have been found in cereals (corn, oat) other than wheat, barley, and rye, it is recommended to eliminate these cereals for patients with CD, WA, or GS, except for patients with WDEIA. When treating WDEIA, only wheat glutens are excluded from the diet.

The average Western diet contains roughly 10-40 g of gluten per day. For example, a slice of whole wheat bread (45 g with 8% protein) contains around 3 g of gluten and the amount of gluten in a serving of pasta (75 g with 11% protein) is roughly 8 g of gluten. Research studies have focused mainly to determine the minimum intake of gluten to prevent CD, but less attention has been given to determine the limit of gluten intake for WA and GS.

It has been reported that a daily intake of 50 mg of gluten has a definite effect on the mucosal histology of CD patients and even with 10 mg of gluten, observable mucosal changes have been reported (Akobeng & Thomas, 2008; Catassi, et al., 2007). Therefore, after years of research, it has been identified that the safe limit could be a daily intake of less than 10 mg of gluten for the CD. However, the level of gluten tolerance varies across individuals. Therefore, the proposed Codex Alimentarius limit

for gluten of less than 20 mg/kg for naturally gluten-free food (Codex Alimentarius Commission, 2003), is still under debate. The minimum daily gluten intake to develop WA has been reported to be higher than that for the CD. A study conducted by Moneret-Vautrin et al. (2003) with 38 children and 41 adults with wheat allergy reported that children respond to lower amounts of wheat protein than adults. Less than 1 g of wheat protein intake triggered allergic reactions in only 40% of the children, while 80% of the children developed allergic reactions to less than 2 g of wheat protein intake. However, 6% of the children developed an allergic reaction even with less than 10 mg of wheat protein intake. At least 1 g of wheat protein intake was required to trigger the allergic reactions in adults. A GFD leads to the complete disappearance of symptoms in most patients with GS. Further studies are still required to conclude a limit of intake of gluten in GS patients.

Despite the importance and effectiveness of GFD, adherence to GFD remains a difficult goal for many patients due to many reasons. Commercially available gluten-free products are less palatable and less widely available compared to their gluten-containing counterparts. Some GFDs are not cost-effective (Stevens & Rashid, 2008) and their 'gluten-free' label maybe sometimes questionable as gluten cross-contamination may occur since gluten is a common constituent in many foods (McDowall, 1970; Wieser, et al., 2021). Therefore, it is not easy to maintain a strict GFD. Therefore, many people inadvertently consume gluten-containing foods, which indicates the need for alternative therapies to GFD.

Many of the alternative therapies for GFD are still under investigation for safe use on patients who have gluten-related disorders. Since GFD is generally effective for clinical improvement in most patients, most of the alternative therapies identified do not replace GFD but are recommended as adjuncts to the GFD. Some of the therapies recognized

may not be cost-effective for long-term use. Additional studies are required to seek efficient, affordable, and safe alternatives for GFD.

Dietary treatments have been identified as a promising solution to eliminate CD, WA, and GS. Gluten-free sources such rice, sorghum, millets, corn, nuts, seeds (flax, chia, pumpkin) and tubers (arrowroot, tapioca, jicama, taro, potato) are possible alternatives to eliminate gluten from the diet (Jnawali et al., 2016).

Other than complete avoidance of gluten-containing foods, the use of alternative dietary therapies to degrade gluten present in diets have also been investigated. The use of proteolytic enzymes to hydrolyse immunogenic gluten peptides either before or after intake of gluten is one of the dietary alternative therapies that has gained attention from the scientific community due to its success in eliminating immunogenic gluten peptides (Table 2.20). Several enzymes have been identified and proven to elicit gluten immunogenicity, however, the safety of prolonged usage of these enzymes is yet to be confirmed.

**Table 2.20** Enzymatic treatments for gluten-related disorders.

| Compound/mode of delivery | Source | Comment | Efficacy to reduce immunogenicity | Reference |
|---|---|---|---|---|
| Prolyl endopeptidases (PEPs)/ Oral supplement | *Sphingomonas capsulate* | Moderately stable against the acidic gastric conditions and pancreatic protease action, stable toward both intestinal secretions and brush-border membrane peptidases, optimal activity at pH 7-8, poor activity against the 33-mer, prefers shorter peptides (e.g., 13-mer) *in vitro* and *in vivo.* | Limited. Clinical trials are yet to be done. | (Shan et al., 2004) |
| | *Flavobacterium meningosepticum* | Highly susceptible to pepsin hydrolysis, optimal activity at pH 7-8, better performance than *Sphingomonas capsulate* and *Myxococcus xanthus* in cleaving 33-mer, however, produces long intermediate peptides, which may still possess immunogenic properties *in vitro* and *in vivo*, prefers both shorter and longer peptides. | Limited. Clinical trials are yet to be done. | (Marti et al., 2005; Shan et al., 2004) |
| | *Myxococcus xanthus* | Stable toward acid exposure as well as pancreatic proteases, highly susceptible to pepsin hydrolysis, prefers shorter peptides (e.g., 13-mer), optimal activity at pH 7-8. | Limited. Clinical trials are yet to be done. | (Gass et al., 2005; Shan et al., 2004) |
| | *Aspergillus niger* | Stable and active at low pH (2-4), resistant to pepsin hydrolysis, complete disappearance of T-cell stimulatory peptides of gliadins and glutenins when present in a complex food matrix *in vitro*, effective in digesting a small amount of gluten (4 g) *in vivo*. | Effective but its efficacy *in vivo* with real gluten dose remained to be established. | (Montserrat et al., 2015; Salden et al., 2015) |
| Cysteine endoprotease and PEP/Oral supplement | ALV003 (EP-B2; a cysteine endoprotease from germinating barley seeds and a PEP from *Sphingomonas capsulate*) | Stable under stomach conditions, effectively digests gluten (2 g) in a complex food matrix *in vivo* and attenuates gluten-induced small intestinal mucosal injury and abolishes immune responses in patients with celiac disease. | Effective but its efficacy *in vivo* with real gluten dose remained to be established. | (Cavaletti et al., 2019; Lähdeaho et al., 2014) |

| | | | | |
|---|---|---|---|---|
| Bacterial endoprotease/Oral supplement | E40 endoprotease from soil bacteria acidophilic actinomycete *Actinoallomurus* A8 | Resistant to pepsin and trypsin proteolysis, and active in the acidic pH range 3 to 6. Effectively cleaves gliadin peptides and 33-mer peptide. | Only tested the effectiveness with gliadin and 33-mer peptide. Only at the preliminary trial stage. | (Cavaletti et al., 2019) |
| Lactic acid bacteria, bifidobacteria Pre-treatment of the flour with enzyme preparation prior to ingestion | Sourdough lactic acid bacteria: *Lactobacillus alimentarius* 15M, *L. brevis.* 14G, *L. sanfranciscensis* 7A and *L. hilgardii* 51B | Hydrolyzes the 33-mer peptide and 31–43 fragment of A-gliadin extensively during prolonged incubation *in vitro*, improves tolerance to 2 g of gluten *in vivo*. | Limited. Only done with few gliadin peptides and its efficacy *in vivo* with real gluten dose remains to be established. | (Di Cagno et al., 2004) |
| | Probiotic culture (VSL#3) containing *Streptococcus thermophilus*, *L. plantarum*, *L. acidophilus*, *L. casei*, *L. delbrueckii* spp. *bulgaricus*, *Bifidobacterium breve*, *B. longum* and *B. infantis* | Hydrolyzes completely the gliadin-derived epitopes 62–75 and 33-mer *in vitro*. | Limited. Clinical trials are yet to be done. | (De Angelis et al., 2006) |
| | Fungal proteolytic enzymes and lactic acid bacteria: *Enzymes from Aspergillus oryzae*, *A. niger* and *L. alimentarius* 15 M, *L. brevis* 14G, *L. sanfranciscensis* 7A, *L. hilgardii* 51B, *L. sanfranciscensis* LS3, LS10, LS19, LS23, LS38, and LS47 | Hydrolyzes the immunogenic epitopes including 33-mer and effectively hydrolyzes intact gluten *in vitro*. However, some glutenin polypeptides persisted after hydrolysis. | Limited. Clinical trials are yet to be done. | (De Angelis et al., 2010) |

The tTG enzyme has been identified as the most notable autoantigen in the CD. The tTG enzyme converts some specific glutamine residues in gluten peptides into glutamic acid (deamidation), which results in a higher affinity of these peptides for HLA-DQ2 or HLA-DQ8. Therefore, intake of tTG inhibitors with gluten to prevent the deamidation of gluten peptides has been identified as one of the dietary treatments to prevent the immunogenicity of gluten peptides. Such identified tTG inhibitors include 5-(S)-dihydroisoxazole, 2-[(2-oxopropyl) thio] imidazolium inhibitor/L682777, cystamine, aldehyde-bearing gluten peptide analogues and cinnamoyl triazole. However, it has been found that few gluten T-cell epitopes can be recognized by HLA-DQ2 or HLA-DQ8, without being deamidated by tTG2. Therefore, this method is not very effective in reducing gluten immunogenicity (Choi et al., 2005; Christophe et al., 2008; Øyvind et al., 2001; Siegel & Khosla, 2007; Siegel et al., 2007; Watts et al., 2006).

Other dietary treatments include oral administration of chemicals to reduce intestinal permeability to prevent the absorption of immunogenic gluten peptides. Some of the commercially available products are larazotide or AT-1001, and a synthetic hexapeptide derived from Zonula Occludens toxin of *Vibrio cholera*. These compounds inhibit the opening of tight junctions of the small intestine epithelial cells and prevent absorption of immunogenic peptides (Gopalakrishnan et al., 2012; Leffler et al., 2012; Paterson et al., 2007; Kelly et al., 2013). However, the effectiveness of these chemicals was demonstrated with a low amount of daily gluten intake (~2.5 g), which may not represent the gluten content in a real diet (10-40 g of daily gluten as described above). Also, these compounds only inhibit the paracellular route of gluten peptides absorption through tight junctions, which is not the only mechanism of gluten peptides absorption

(Heyman et al., 2012). Further, immune reactions can occur in the intestinal lumen without being absorbed by the small intestine.

Oral administration of chicken egg yolk immunoglobulin (IgY) antibody has been shown to neutralize gliadin in the gastrointestinal tract and prevent its absorption (Gujral et al., 2012; Gujral et al., 2015). Laying hens were immunized against a selected antigen (gliadin or gluten) and the resulting antibodies were collected from the egg yolks. This is an easy, cost-effective, and hygienic alternative to mammalian antibodies. However, further investigations with patients are required to prove the effectiveness of this approach.

A polymeric binder has been identified by Pinier et al. (2012), poly (hydroxyethyl methacrylate-co-styrene sodium sulfonate) [P(HEMA-co-SS)], which can bind with gluten in the gastrointestinal tract and thereby prevent degradation and triggering of immunogenic responses. They observed that this compound could reduce the digestion of wheat gluten and partially eliminate the production of peptides that induce immune responses. However, the experiments were done only with rats and intestinal biopsy specimens from CD patients. Human clinical trials are required to confirm the effectiveness as a treatment for CD, WA, and GS.

### 2.9.2 Non-dietary treatments

Scientists have considered the possibility of crop genetic alteration to produce varieties with less amount of gluten (or immunogenic peptides) (Carroccio et al., 2011; Spaenij–Dekking et al., 2005; van den Broeck et al., 2009). However, due to poor baking properties, the public refusal for genetically modified crops, contamination of genetically modified crops with gluten-containing crops grown nearby and

heterogeneous uncharacterised immunostimulatory epitopes in gluten, this method was considered less effective.

Peptide vaccination to modulate immune tolerance towards gluten has been identified as an effective and efficient method to prevent the immunogenicity of gluten peptides. Nexvax2® (mix of three peptides i.e., α- and ω-gliadins, and B-hordein) has been clinically proven with CD patients to be effective when gradual dose escalation was practised (Daveson et al., 2017; Goel et al., 2017). Continued clinical development of this potential therapeutic vaccine is still under investigation.

The effect of oral administration of *Lactococcus lactis* secreting a DQ8-restricted gliadin peptide (Huibregtse et al., 2009) and the effect of intranasally administered recombinant α-gliadin in HLA-DQ8 (Senger et al., 2003) on gluten-related immunogenic responses were studied using a transgenic mouse model. More work is needed to assess the effectiveness of these therapies on the spectrum of gluten-related health disorders. Immune modulation with *Necator americanus* (hookworm) is also currently under investigation (Croese et al., 2013; Daveson et al., 2011; McSorley et al., 2011) as a potential therapy for gluten immunogenicity.

Refractory CD patients do not respond to a GFD. Therefore, management often requires pharmacologic therapies, including treatment with steroids or immune-suppress chemicals such as azathioprine and cyclosporine (Rolny et al., 1999; Rubio-Tapia & Murray, 2010; Stuart & Gent, 1998; Vaidya et al., 1999). Other immune-suppress chemicals such as budesonide (Brar et al., 2007), infliximab (Costantino et al., 2008; Gillett et al., 2002), and prednisone (Goerres et al., 2003) have also been tested as a treatment for the refractory CD. It has also been shown that further dietary restrictions such as the exclusion of soy and milk could be considered in the management of refractory CD (Baker & Rosenberg, 1978; Ryan & Kelleher, 2000).

## 2.10 Natural ways of reducing gluten immunogenicity

Many alternative therapies to GFD explored so far are either synthetic or microbial originated. Long-term administration of these compounds into the human body is therefore questionable. In addition, the applicability of some of the alternative therapies is also uncertain. For example, although the immunogenic peptides generated during the digestion of gluten should be degraded before they reach the upper small intestine, many therapeutic enzymes identified for gluten degradation are inactivated in the acidic conditions of the stomach and could also be degraded by pepsin. On the other hand, the safety of long-term use of commercially available gluten-degrading enzymes is also challenging and most of them are recognized as ineffective in degrading immunogenic gluten peptides (Janssen et al., 2015). Therefore, finding a natural way of effective detoxification of gluten peptides is a timely requirement.

Other than EP-B2, a cysteine endoprotease from germinating barley seeds, natural enzymes have not been widely tested as therapeutic enzymes for gluten detoxification. Natural, readily available, and cost-effective enzymes should be explored in this context. Several natural cysteine proteases such as papain (EC 3.4.22.2) and caricain (EC 3.4.22.30) in papaya (*Carica papaya*), bromelain (EC 3.4.22.32) in pineapple (*Ananas comosus*) and actinidin (EC 3.4.22.14) in green kiwifruit (*Actinidia deliciosa*), are potential candidates to assist gluten digestion.

Digestion of gluten by the enzymes present in crude papain extract was observed in an early study (Messer et al., 1964). More recently, *in vivo*, and *in vitro* studies identified that papain (Li et al., 2016; Xue et al., 2019) and caricain (Buddrick et al., 2015; Cornell & Stelmasiak, 2011) are the enzymes mainly responsible for this effect. Of the two, caricain was more effective in removing the toxic effect of gliadin than papain (Cornell et al., 2010). Bromelain has also been shown to reduce the toxic effect of gluten when

incorporated into wheat flour (Tanabe et al., 1996). However, the effectiveness of bromelain in detoxifying gluten was less than that of papain (Pahlavan et al., 2016). 'Actinidin', a member of the cysteine proteases, found naturally in kiwifruit *(Actinidia deliciosa)*, has also been identified as a potential enzyme that helps to hydrolyse different dietary proteins including gluten proteins **(**Kaur & Boland, 2013; Kaur et al., 2010a, 2010b; Lewis & Luh, 1988; Montoya & Hindmarsh, et al., 2014; Montoya, Rutherfurd, et al., 2014; Rutherfurd et al., 2011). In this thesis, we propose actinidin as a candidate for detoxifying gluten.

### 2.11 Potential effects of actinidin intake

The protein content of fresh green kiwifruit ranges from 1-1.14% (USDA national nutrient database for standard reference, (Boland, 2013)) (Table 2.21). Among four proteins found in green kiwifruit, actinidin (synonym actinidain, EC 3.4.22.14) is the major protein comprising up to 40% of the total soluble proteins of the fruit **(**Boland & Moughan, 2013). According to Nishiyama and Oota (Nishiyama & Oota, 2002), about 2.9 mg/mL of actinidin is available in fresh green kiwifruit juice. However, different levels of actinidin have been reported in different cultivars of kiwifruit. For example, *Actinidia chinensis* cv. Hort 16A, the commercial yellow kiwifruit from New Zealand, does not have any detectable amount of actinidin (Nishiyama & Oota, 2002).

**Table 2.21** Nutrient composition of fresh green and gold kiwifruit.

| Nutrient | Units | Value per 100 g edible flesh | |
|---|---|---|---|
| | | ***Actinidia deliciosa* (green)** | ***Actinidia chinensis* (gold)** |
| Water | g | 83.07 | 83.22 |
| Energy | kcal | 61 | 60 |
| | kJ | 255 | 251 |
| Protein | g | 1.14 | 1.23 |
| Total lipid (fat) | g | 0.52 | 0.56 |
| Ash | g | 0.61 | 0.76 |
| Carbohydrate, by difference | g | 14.66 | 14.23 |
| Fiber, total dietary | g | 3 | 2.0 |
| Sugars, total | g | 8.99 | 10.98 |
| Vitamin C, total ascorbic acid | mg | 92.7 | 105.4 |
| Vitamin B1—thiamin | mg | 0.027 | 0.024 |
| Vitamin B2—riboflavin | mg | 0.025 | 0.046 |
| Vitamin B3—niacin | mg | 0.341 | 0.28 |
| Vitamin B5—pantothenic acid | mg | 0.183 | 0.5 |
| Vitamin B6—pyridoxine | mg | 0.063 | 0.057 |
| Vitamin B9—folic acid | mg | 25 | 34 |
| Choline | µg | 7.8 | 5.0 |
| Vitamin B-12 | mg | 0 | 0 |
| Vitamin A, RAE | µg | 4 | 4 |
| Vitamin A | µg RAE | 87 | 72 |
| Vitamin E (α-tocopherol) | IU | 1.46 | 1.49 |
| Vitamin E (γ-tocopherol) | mg | 0.03 | 0.01 |
| Vitamin E (δ-tocopherol) | mg | 0.00 | 0.01 |
| Vitamin K | mg | 40.3 | 5.5 |

RAE, Retinol activity equivalents; IU, International unit; Adapted from USDA national nutrient database for standard reference, Boland, 2013.

Actinidin was first characterised by Arcus (Arcus, 1959), as a proteolytic enzyme from the green kiwifruit and it was grouped in the class of plant thiol proteinases since it contains a thiol or sulfhydryl group that is responsible for the proteinase activity (McDowall, 1970). In 1972, a method to purify actinidin with tetrathionate and ethylenediaminetetraacetic acid was introduced by Boland and Hardman (Boland &

Hardman, 1972) and then later in the same decade, the structure and amino acid composition of actinidin were identified (Baker, 1977; Carne & Moore, 1978). The actinidin molecule consists of a single chain of 220 amino acids (Figure 2.19) and the MW and the isoelectric point of actinidin were identified as 23.5 kDa and pH 3.1 respectively (Carne & Moore, 1978). Although the physiological function of actinidin in kiwifruit is unknown, it is believed to play a role as a protecting protein against insects (Malone et al., 2005).

Actinidin is reported to be active over a wide range of pH ranging from 3-10.5 pH **(**Sun et al., 2016). However, when using 1% casein as the substrate, the highest activity (28.8 ± 1.7 U/g) was reported at pH 3 while the activity was reduced to 7.8 ± 0.6 U/g when the pH was 10.5. When gelatine was hydrolysed with actinidin, the optimum pH was reported to be 4-4.3 (Arcus, 1959) while it was 7.5-8 with collagen (Sun et al., 2016). This suggests that the optimum pH for actinidin depends on the hydrolysing substrate. In a pig study, Montoya, Rutherfurd, et al., (2014) observed that, when actinidin is consumed by pigs in the form of fresh green kiwifruit, after 3 h, the activity of actinidin (or protease) in stomach chyme, was greater (~90 μmol-o-nitrophenol/min per g of dry matter) than that present in the original diet which contained beef muscle proteins and green kiwifruit (36 μmol-o-nitrophenol/min per g of dry matter). This confirms that actinidin is more active in gastric pH conditions. However, the pigs fed the control diet (i.e., without actinidin which contained beef muscle proteins and yellow kiwifruit) had a protease activity of ~23 μmol-o-nitrophenol/min per g of dry matter in the stomach. Therefore, the authors mentioned that this increase of protease activity in the chyme for the actinidin-containing diet could be due to the activation of either pepsinogen or actinidinogen, pro-enzymes of pepsin and actinidin.

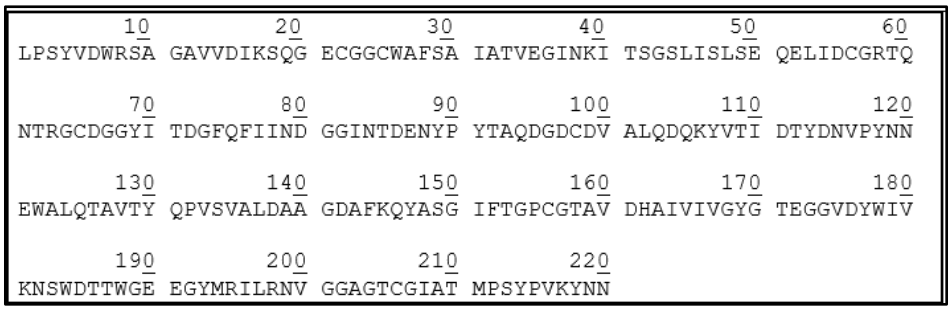



**Figure 2.19** Amino acid sequence of actinidin from kiwifruit.

Standard single-letter codes for amino acids: G, glycine; P, proline; A, alanine; V, valine; L, leucine; I, isoleucine; M, methionine; C, cysteine; F, phenylalanine; Y, tyrosine; W, tryptophan; H, histidine; K, lysine; R, arginine, Q, glutamine; N, asparagine; E, glutamic acid; D, aspartic acid; S, serine; T, threonine; Source: Carne & Moore, 1978.

## 2.12 Effect of actinidin intake on cereal protein digestion

Kaur et al. (Kaur et al., 2010a, 2010b) studied the *in vitro* gastric and intestinal digestion of wheat gluten, gliadin, and α-zein in the presence of actinidin in the form of fresh green kiwifruit extract. The SDS-PAGE patterns of the peptides obtained after gastric digestion revealed that there was no effect of actinidin on the digestion of either wheat gluten or gliadin. They did not observe any effect of actinidin on the gastric digestion of α-zein. However, they observed significantly enhanced digestion of gliadin (complete disappearance of SDS-PAGE bands for > 30 kDa peptides) and α-zein (~40%) when gastric digestion (pepsin + actinidin for 30 min) was followed with small intestinal digestion (pancreatin for 120 min).

Based on these results, the same research group conducted two studies *in vivo*, one to measure the total protein hydrolysis with actinidin (using o-phthalaldehyde method) and the other to evaluate the effect of actinidin on stomach emptying rate. Then in an attempt to determine which of the protein fractions from the cereals were digested in the gastrointestinal tract, they used an SDS-PAGE approach (Table 2.22). Montoya & Hindmarsh, et al., (2014) observed a 3.2-fold enhanced gastric protein hydrolysis of wheat gluten when actinidin was present in the diet. However, they did not observe any

significant influence of actinidin on the hydrolysis of zein, but they found that the presence of actinidin slowed the stomach-emptying rate only for the zein-containing diet (the half-gastric emptying times with and without actinidin were 266 min and 249 min respectively). Rutherfurd et al. (2011) observed that the gastric degradability (or disappearance in gel electrophoresis) of ω-gliadin (61%) and three LMW subunits (35 (73%), 37 (65%), and 41 kDa (71%)) of glutenin were significantly higher in rats fed with the actinidin-containing diet compared to the same diet without actinidin. However, the gastric degradability of high molecular weight subunits of glutenin was not different between both diets. At the end of the small intestine (terminal ileum), the disappearance for all the gluten peptides was > 95% for both diets, except for one B glutenin subunit of low molecular weight, which had a small (> 2.3% units) but significant increase in ileal degradability when actinidin was present in the diet. The presence of actinidin in the diet did not have any effect on the gastric or ileal digestion of α-zein in rats.

**Table 2.22** Effect of actinidin on digestion of cereal proteins.

| **Cereal protein** | ***In vitro* data*** | ***In vivo* data**** |
|---|---|---|
| Gastric digestion [1,2] | | |
| Wheat gluten | No Significant effect | Significant effect on LMW-GS and ω-gliadin, no effect on HMW-GS |
| Gliadin | No Significant effect | Not evaluated |
| α-Zein | No results | No significant effect |
| Small intestinal digestion[3,4] | | |
| Wheat gluten | Moderate effect | Significant effect except for B-LMW-GS |
| Gliadin | Significant effect | Not evaluated |
| α-Zein | Significant effect | Significant negative effect |

[1] *In vitro* pepsin digestion for 30 min, [2]*in vivo* stomach chyme of rats after feeding, [3] *In vitro* pepsin (30 min) and pancreatin (120 min) digestion for 150 min, [4]*in vivo* ileal digesta of rats after feeding**;** HMW-GS, high molecular weight glutenin subunits; LMW-GS, low molecular weight glutenin subunits; B-LMW-GS, low molecular weight B glutenin subunit; Source: *Kaur et al., 2010a, 2010b; **Rutherfurd et al., 2011.

These results suggest that the impact of dietary actinidin on the small intestinal degradability of cereal proteins is much less than that observed for gastric protein degradability. This could be due to the broad enzyme specificity of actinidin that may help to hydrolyse a wide range of peptide bonds unavailable to pepsin. This enables opening up protein structures and exposing new sites to pepsin to act on, increasing gastric hydrolysis.

Actinidin was found to be stable against acidic pH and pepsin proteolysis. For instance, it was observed *in vitro* that actinidin was stable at pH 1.2 using SDS-PAGE (Dearman et al., 2014). In an *in vivo* study, actinidin was deemed to be still active after 1 h of gastric digestion when fresh kiwifruit pulp was fed to human volunteers and their gastric content was evaluated using SDS-PAGE (Polovic et al., 2007). Actinidin was also found to be stable against pepsin proteolysis after 1 h of gastric digestion as observed *in vitro* when physiologically relevant protein to pepsin ratios was used and evaluated using SDS-PAGE (Bublin et al., 2008). These observations suggest that, unlike some other possible glutenase enzymes that are unstable in low gastric pH, having their optimum pH at alkaline pH range (pH > 7) and highly susceptible to pepsin-catalysed proteolysis (Kabashima et al., 1998; Moreno Amador et al., 2019; Shan et al., 2004; Szwajcer-Dey et al., 1992), actinidin is active and stable against both the gastric pH and pepsin proteolysis under physiologically relevant conditions.

Based on the above information, it appears that actinidin can enhance the digestion of cereal proteins, particularly wheat gluten and gliadin. Since actinidin appears to be activated under gastric pH conditions as described above (Montoya, Rutherfurd, et al., 2014), it could be hypothesised that actinidin is able to digest gluten proteins reducing or eliminating the production of immunogenic peptides.

During the early and late 1980s, several case studies reported kiwifruit allergic incidents (Falliers, 1983; Fine, 1981; Garcia et al., 1989). Later, in 1996-1998, Pastorello et al. (1996) identified actinidin as the major kiwifruit allergen. Since then, different research groups have documented actinidin allergy in different populations (Kerzl et al., 2007; Novembre et al., 1995; Palacin et al., 2008; Shimizu & Morikawa, 1995; Veraldi & Schianchi-Veraldi, 1990). The clinical symptoms of kiwifruit allergy range from localized symptoms confined to the oral mucosa to severe anaphylactic reactions (Gawrońska-Ukleja et al., 2013; Mancuso & Berdondini, 2001; Mempel et al., 2003). The allergenicity of actinidin is caused by its resistance to both gastric and intestinal digestion (Grozdanovic et al., 2014). The reported studies suggest that actinidin has the capacity to disrupt the intestinal epithelium tight junctions and cross over the intestinal mucosa to induce sensitization in allergenic reactions (Grozdanovic et al., 2016). It has also been suggested that actinidin allergy can be expected in patients with birch pollen allergy exhibiting high levels of IgE (Gall et al., 1994). However, actinidin allergy has also been found in patients who did not have birch pollen allergy (Alemán et al., 2004). Limited information is available in the literature for the prevalence of kiwifruit allergy in the general population. Based on the above information, it seems that kiwifruit allergy is not a common allergic condition among the general population and the symptoms of kiwifruit allergy are not severe among adults, but in children (Lucas et al., 2004).

In this context, if the hypothesis of dietary actinidin is able to reduce the amount of immunogenic gluten peptides can be proven, it may also suggest that eating green kiwifruit may reduce the risk of the unintentional (or intentional) consumption of small amounts of selected gluten-containing products. In addition, kiwifruit consumption may also help to mitigate nutritional deficiencies such as dietary fibre and

micronutrients (Table 2.21) as subjects on a gluten-free diet tend to reduce the intake of dietary fibre and several micronutrients (e.g., vitamin B) **(**Shepherd & Gibson, 2013).

### 2.13 Concluding remarks

Cereal proteins are an important part of human nutrition. However, some cereal proteins, specifically wheat gliadins and glutenins, and prolamins of rye, barley oats, and maize produce immunogenic peptides during GIT digestion due to their high proline/glutamine contents. These immunogenic cereal proteins are collectively known as gluten proteins. Gluten proteins induce gluten-related health disorders i.e., CD, WA, and GS. The incidence of CD, WA, and GS has become a serious public health issue in the world. In contrast to WA and GS, information on CD is available since it is the most complicated gluten health disorder to date, creating chronic and lifelong complications in patients. Maintaining a strict GFD is the sole treatment for CD, WA, and GS. However, maintaining a life-long GFD is not easy because GFDs are expensive, less palatable and their availability in local settings is also low. Therefore, multidisciplinary research efforts are currently being carried out in several directions to find new strategical treatments to reduce or eliminate gluten immunogenicity. However, the proposed strategies to date are still under investigation for human use, and the long-term use of some of these therapies is questionable. Therefore, it is a timely requirement to recognize natural, readily available, and effective therapy for safe long-term usage. Actinidin, a cysteine protease found in kiwifruit has been identified as an effective enzyme, to be utilized as a digestive aid for gluten proteins. Actinidin may help to reduce the risk of unintentional consumption of small amounts of gluten or gluten contaminated GFDs. No studies have been conducted to evaluate the effect of actinidin on the immunogenicity of cereal proteins. Considering the literature to date, it can be

hypothesised that actinidin is able to enhance the digestion of gluten proteins. However, the present information is not sufficient to know if actinidin can reduce or eliminate immunogenic peptides during digestion. Therefore, more studies are required to confirm the effect of actinidin on reducing or eliminating the immunogenicity of gluten proteins during gastrointestinal digestion.

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# Chapter Three

## 3. Hydrolysis of gluten proteins by actinidin during *in vitro* static gastrointestinal digestion

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Jayawardana, I. A., Boland, M. J., Higgs, K., Zou, M., Loo, T., McNabb, W. C., & Montoya, C. A. (2021). The kiwifruit enzyme actinidin enhances the hydrolysis of gluten proteins during simulated gastrointestinal digestion. *Food Chemistry*, *341*, 128239.

**Highlights**

- Actinidin is active under physiological gastric conditions.
- Actinidin can cleave multiple peptide bonds including peptide bonds at the N- and C-terminal of proline residues.
- During the hydrolysis, actinidin at a concentration of > 2.7 U/mL and pH > 2 is considered ideal for gluten hydrolysis.
- Actinidin is more effective in hydrolysing gliadin than two other cysteine proteases, papain, and bromelain.

### 3.1 Abstract

This study investigated the effect of actinidin, a cysteine protease in kiwifruit, on the hydrolysis of gluten proteins and digestion-resistant gluten peptides (synthetic 33-mer peptide and pentapeptide epitopes) under static simulated gastrointestinal conditions. Actinidin efficacy in hydrolysing gliadin was compared with that of other gluten-degrading enzymes. Actinidin hydrolysed usually resistant peptide bonds adjacent to proline residues in the 33-mer peptide. The gastric degree of hydrolysis of gluten proteins was influenced by an interaction between pH and actinidin concentration ($P < 0.05$), whereas the pentapeptide epitopes hydrolysis was influenced only by the actinidin concentration ($P < 0.05$). The rate of gastric degree of hydrolysis of gliadin was greater ($P < 0.05$) by actinidin (0.8%/min) when compared to papain, bromelain, and one commercial enzyme (on average 0.4%/min), while all exogenous enzymes were able to hydrolyse the pentapeptide epitopes effectively. Actinidin is able to hydrolyse gluten proteins under simulated gastric conditions.

**Keywords:** Actinidin; Gastrointestinal tract; Gliadin; Gluten; Hydrolysis

## 3.2 Introduction

Gluten, an important protein group in major cereals, is composed of gliadins and glutelins. These proteins contain proline- and glutamine-rich peptide sequences. The high content of proline in gluten is one of the major contributors to its resistance to gastrointestinal tract (GIT) enzymes. Proline has a unique structure where its N-terminal side is connected to the protein backbone twice forming a five-membered nitrogen-containing ring, which limits rotation around the peptide bond. The ring structure of proline limits the flexibility of the peptide chain by fixing the amino dihedral angle at about -65°, preventing proteolysis by most GIT enzymes (Jayawardana, Montoya, McNabb, & Boland, 2019). The major GIT proteases (pepsin and pancreatic proteases) are not able to hydrolyse proline-containing peptide bonds (Hausch, Shan, Santiago, Gray, & Khosla, 2002). Therefore, gluten is not well digested in the stomach and the upper small intestine. The resistant gluten peptides that are released into the small intestine can trigger immunogenic responses in some individuals. Thus, it is of interest to investigate exogenous proteolytic enzymes that could help degrade proline-rich gluten peptides into small fragments in the stomach.

Actinidin (synonym actinidain, EC 3.4.22.14), is a cysteine protease of the papain superfamily found in kiwifruit *(Actinidia deliciosa*). It has been identified as a potential enzyme that may help to hydrolyse different proteins, including gluten, during *in vitro* simulated gastrointestinal digestion (Kaur, Rutherfurd, Moughan, Drummond, & Boland, 2010) and *in vivo* (Montoya & Hindmarsh, et al., 2014) as highlighted recently (Jayawardana et al., 2019). Actinidin added to a wheat gluten-containing diet enhanced 3.2-fold the gastric protein hydrolysis in rats compared to the control (Montoya & Hindmarsh, et al., 2014). It is of interest, and potential practical value, to better

understand the chemistry of the action of actinidin on gluten proteins, particularly gliadins, during digestion.

The present study aimed at understanding the chemistry of the action of actinidin on hydrolysing gluten proteins and the factors (pH and actinidin concentration) influencing these effects during simulated gastrointestinal digestion. Static *in vitro* digestion studies were conducted to determine the effect of actinidin on the hydrolysis of gluten and the gliadins. Gliadins are highly polymorphic, with multiple gene copies, and the common wheat strains are polyploid, typically containing six or eight sets of chromosomes. For example, a recent study identified 42 expressed genes for different gliadins in a single wheat strain (Wang et al., 2017). This means that simple protein chemistry and peptidomics studies on the hydrolysis of the gliadins, which would otherwise be an obvious investigation, are not feasible because of the complexity of the results. Three alternative analytical approaches were used. The first was a detailed mass spectral analysis of the hydrolysis of a synthetic peptide: the so-called 33-mer peptide, which has been widely reported as one of the most digestion-resistant and immunogenic peptides from α-2 gliadin (Shan et al., 2002). A second approach was a simple measurement of free amino groups, which quantifies the hydrolysis of peptide bonds (excepting those of the amino-terminal of proline). The third approach used the R5 monoclonal antibody-based competitive ELISA, which has been recommended by the Codex Alimentarius for detection of hydrolysed gluten traces in gluten-free food (Codex Alimentarius Commission, 2006). The R5 antibody specifically detects the core epitope sequence QQQ/PFP present in the gluten epitope QQPFP and related sequences, such as QQQFP, LQPFP, and QLPFP (Kahlenberg et al., 2006), which are commonly found in gluten proteins (Sollid, Qiao, Anderson, Gianfrani, & Koning, 2012). Actinidin efficacy in hydrolysing gliadin was compared with that of two other

cysteine proteases (papain and bromelain) and two commercial gluten-degrading enzymes.

## 3.3 Materials and methods

### 3.3.1 Materials

Purified wheat gluten (G5004), purified wheat gliadin (G3375), pepsin from porcine gastric mucosa (P7000; activity $\geq$ 250 U/mg), pancreatin from porcine pancreas (P1750; activity x 4 USP), trypsin (T0303), bromelain (B4882), Tris (2-carboxyethyl)-phosphine (C4706), and N-lauroylsarcosine (61745) were purchased from Sigma Aldrich (St. Louis, Missouri, USA). Purified actinidin was purchased from New Zealand Pharmaceuticals Ltd. (Palmerston North, New Zealand). Papain (from papaya juice; 3.4.22.2) was purchased from BDH chemicals Ltd. (England). Digest Gluten® (Proteases from *Aspergillus oryzae*, activities of 500 dipeptidyl peptidase IV units and 30,000 HUT and *Aspergillus melleus*, activity 8.5 Aminopeptidase Activity Units) was purchased from Pharmacare New Zealand Ltd. (Auckland, New Zealand). GlutnGo® (Protease from *Aspergillus Niger* Prolyl Endoprotease commercially known as Tolerase®G, activity 580 PPI (protein picomol international) per mg) was purchased from Bricker Labs (Arizona, USA). The 33-mer peptide was synthesised by GenScript Biotech (New Jersey, USA), and ELISA kits (RIDASCREEN® Gliadin competitive) were purchased from R-Biopharm AG (Darmstadt, Germany).

### 3.3.2 Reaction conditions for the *in vitro* digestions

The pH and actinidin concentration used in the *in vitro* digestions were selected based on the stomach chyme pH (3.8) and actinidin concentration (2.7 U/mL chyme) at 3 h

postprandial reported in growing pigs fed a diet with green kiwifruit fresh pulp (Montoya, Rutherfurd, et al., 2014).

### 3.3.3 Determining the cleavage sites of 33-mer peptide by actinidin

A 250 µL of 33-mer peptide solution (50 pmol/mL of 50 mM ammonium acetate, pH 4) was mixed with 250 µL of purified actinidin (2.7 U/mL ultra-pure water). The mixture was incubated at 37 °C for 120 min. An aliquot of 100 µL was collected at 0, 30 and 120 min. Actinidin was inactivated by boiling the samples for 1 min. The samples were then centrifuged at 13,000 rpm for 30 min. The supernatant was analysed for peptide sequences using liquid chromatography-tandem mass spectrometry (LC-MS/MS). This experiment was conducted in triplicate.

### 3.3.4 Gluten and gliadin hydrolysis

#### 3.3.4.1 Effect of actinidin on gastrointestinal tract hydrolysis

Ten millilitres of gluten or gliadin solution (5 mg/mL in HCl, pH 4) was mixed with pepsin alone (1:67, w/w, pepsin:protein) and pepsin with purified actinidin (2.7 U/mL hydrolysis medium). The mixtures were incubated, at 37 °C in a water bath for 120 min. Aliquots (1 mL) were collected at 0, 60, and 120 min of hydrolysis. Pancreatin (1:30, w/w, pancreatin:protein) dissolved in phosphate buffer solution (0.02 M, pH 7) was added (1:1, v/v) to the remaining hydrolysis medium, and the mixtures were incubated at 37 °C for another 120 min. Blank tests with digestive enzymes and digestive enzymes with actinidin were run simultaneously. Aliquots (1 mL) were collected at 60 and 120 min of pancreatic hydrolysis (i.e., 180 and 240 min of total hydrolysis time). The aliquots collected during the *in vitro* gastric and small intestinal hydrolysis were immediately boiled for 1 min to inactivate the enzymes. All the

hydrolysates were then stored at -20 °C until analysis. The degree of hydrolysis (DH) was measured as described in detail below. This experiment was conducted in triplicate.

#### 3.3.4.2 Effect of actinidin on small intestinal hydrolysis

To evaluate the effect of actinidin on small intestinal hydrolysis, the above experiment was extended using the cysteine protease inhibitor, E64 (Varughese, Su, Cromwell, Hasnain, & Xuong, 1992). A 0.4 mL aliquot of gluten (or gliadin) solution (5 mg/mL of HCl pH 4) was digested with pepsin and actinidin for 120 min as described above. Phosphate buffer solution (0.02 M, pH 7) containing pancreatin (1:30, w/w, pancreatin:protein) alone or pancreatin and E64 (0.2 mL of 1 mM,) was added to the incubation medium (1:1, v/v) and incubated for another 60 min. Blank enzymes alone (i.e., digestive enzymes and digestive enzymes and actinidin) and blank enzymes and E64 were run simultaneously. Aliquots (0.2 mL) were collected after 120 min of gastric hydrolysis and 60 min of small intestinal hydrolysis. The effect of actinidin in small intestinal hydrolysis was determined by comparing the DH between the samples with and without E64. This experiment was run in triplicate.

#### 3.3.4.3 Effect of pH and actinidin concentration on gastric hydrolysis of gluten, gliadin and their pentapeptide (R5) epitopes

Based on the results of the first gastrointestinal hydrolysis experiment for gluten and gliadin, an experiment was conducted to determine the effect of pH and actinidin concentration on the hydrolysis of gluten and gliadin and their resistant peptides at 60 min of gastric hydrolysis using a surface response analysis (central composite design). The centroid values (pH 4 and 2.7 U actinidin/mL hydrolysis medium) of the response surface analysis were based on an *in vivo* study (Montoya, Rutherfurd, et al., 2014).

The remaining values of pH were selected to simulate the chyme pH at different postprandial times in humans (Omari & Davidson, 2003; Simonian, Vo, Doma, Fisher, & Parkman, 2005). Falcon tubes (15 mL) containing either gluten or gliadin (50 mg) were mixed with 10 mL of Milli-Q water having the required pH (1.2, 2, 4, 6, and 6.8). Mixtures were incubated at 37 °C in a water bath. Immediately, 1 mL of a solution containing either pepsin alone (1:67, w/w, pepsin:protein) or with actinidin at different concentrations (0.3, 1.0, 2.7, 4.4, and 5.1 U/mL hydrolysis medium) was added into the incubation medium. In total, 25 different treatments were simultaneously digested for each protein source. Blank tests with digestive enzymes and digestive enzymes with actinidin were run simultaneously for each actinidin concentration. After 60 min of hydrolysis, two aliquots (1 mL) were collected and boiled as described above. All the hydrolysates were stored at -20 °C until analysis. The DH of whole gluten and gliadin was measured in each sample. In addition, the hydrolysis of R5 epitopes within gluten and gliadin was measured using an ELISA kit as described in detail below. This experiment was run in triplicate.

### 3.3.5 Comparison of the effect of actinidin on gastric hydrolysis of gliadin and its R5 epitopes with other exogenous enzymes

This experiment compared the effect of actinidin with other cysteine proteases (papain and bromelain) and two gluten-degrading commercial enzymes (Digest Gluten® and GlutnGo®), on the gastric hydrolysis of gliadin and its resistant peptides. Based on the similar results observed from the previous experiments for gliadin and gluten, only gliadin was used in this experiment. Briefly, 1.6 mL of gliadin solution (0.5 mg/mL of HCl, pH 4) was mixed and incubated at 37 °C in a water bath for 120 min, with 0.4 mL of pepsin alone (1:67, pepsin:protein) or pepsin and one of the other enzymes (enzyme

activity 5.1 U/mL hydrolysis medium). This enzyme activity was selected based on the DH results obtained from the response surface analysis. Control solutions containing pepsin alone and pepsin with one of the other enzymes, at the same concentrations, were run simultaneously as blanks. Two aliquots (0.2 mL) were collected (15, 30, 60, and 120 min of gastric hydrolysis), inactivated, stored, and analysed as described above. This experiment was run in triplicate.

### 3.3.6 Analysis

#### 3.3.6.1 LC-MS/MS

The hydrolysed samples were separated by reversed-phase chromatography using a Dionex UltiMate™ 3000 RSLCnano System (ThermoFisher Scientific, Waltham, MA USA) with a reversed-phase peptide trap (Acclaim™ PepMap™100 C18, 3 μm particle size, 75 μm inner diameter, 2 cm length and a reversed-phase capillary analytical column Acclaim™ PepMap™ C18, 2 μm particle size, 75 μm inner diameter, 50 cm length (ThermoFisher Scientific, USA)). The liquid chromatography system was coupled on-line to a QExactive™ Plus mass spectrometer equipped with a higher-energy collision-induced dissociation (HCD) collision cell, an Orbitrap mass analyser and a Nanospray Flex™ ion source (ThermoFisher Scientific, USA). Details for the chromatographic parameters were as follows: Flow rates for trap and analytical columns were 15 μL/min and 300 nL/min respectively; column oven temperature of 50 °C; and a gradient of 3-30% acetonitrile in 0.1% formic acid/water over 60 min. The mass spectrometric source and detection settings were as follows: Capillary temperature of 250 °C; S-Lens radio frequency level 50%; source voltage 1.5 kV. Data-dependent tandem MS acquisition method was used, and all runs were performed in duplicates. In all experiments, maximum injection times were 150 ms (full MS), MS*n*

110 ms (HCD) and AGC target values were 3e6 (full MS), MSn (HCD) 1e5. Survey scans were acquired over a mass range of 375-1,600 *m/z* with detection in the Orbitrap mass analyser at a resolution setting of 70,000. Fragment ion spectra produced via HCD were acquired at a resolution setting of 17,500. The top ten most intense ions were selected for fragmentation in each scan cycle. Exclusion conditions were optimized according to observed chromatographic peak width (typically 12 seconds). Peak lists were searched with search engine Proteome Discoverer v 2.4 using the following parameters: Database was from NCBI\α-gliadin with custom synthetic peptide sequences downloaded on 20 June 2019 from NCBI; taxonomy *Triticum aestivum*, protein α-gliadin with custom synthetic peptide sequences; enzyme specificity none; max number of missed cleavages 2; minimum peptide length 4; precursor mass tolerance 10 ppm; fragment mass tolerance 0.02 Da; decoy database search enabled; static modification include carbamidomethylation of cysteine; variable modifications include oxidation of methionine and N-terminal protein acetylation; minimum significant threshold of 0.05; false discovery rate 1%. Only peptides having high confidence values, up to 2,600 Da of molecular weight and having at least 10 peptide spectrum matches were considered when determining cleavage sites of actinidin.

#### 3.3.6.2 Actinidin activity assay

The enzyme activity of the purified actinidin was determined as described previously (Boland & Hardman, 1972). Briefly, purified actinidin powder was dissolved in 0.05 M ice-cold phosphate buffer solution (pH 6) (1 mg/mL). The actinidin substrate, N-α-CBZ-lys-p-nitrophenol hydrochloride was dissolved in deionized distilled water at a concentration of 1.2 g/L. An aliquot of the substrate (100 μL) and 2.85 mL of 0.05 M phosphate buffer solution (pH 6) were added into a spectrophotometer cuvette. After

10−20 s, to determine the rate of spontaneous hydrolysis of the actinidin substrate, 50 µL of a mixture containing the actinidin solution and 0.1 M dithioerythritol (1:1, v/v) were added. The rate of change in absorbance was measured for 300 s at 348 nm using a spectrophotometer. Enzyme activity (U/mL) of the extract was calculated using $\Delta\varepsilon$ = 5,400.

The actinidin concentration was calculated using the following formula:

$$\text{Actinidin concentration (U/mg)} = \frac{(\Delta A348 \text{ test} - \Delta \text{ A } 348 \text{ blank}) \; x \; 1{,}000 \; x \; 3}{(5{,}400 \; x \; \text{X})}$$

$\Delta A_{348}$ is the slope of the initial linear portion of the curve (unit absorbance/minute) for the test (with enzyme) and blank, 5,400 is the molar extinction coefficient (L/ (mol *x* cm) of Z-lys-pNp at 348 nm, 3 is the volume of reaction mix (mL), and X is the quantity (mg) of actinidin in the final reaction mixture (i.e., in the cuvette).

### 3.3.6.3 Determination of the apparent degree of hydrolysis

The DH of the hydrolysed samples was determined using the o-phthaldialdehyde (OPA) method described previously (Church, Swaisgood, Porter, & Catignani, 1983). This method is based on the number of free amino groups generated when peptide bonds are broken. The N-terminal α-amino groups on peptides react quantitatively with OPA to form fluorescent derivatives that can be determined using a spectrofluorometer. Peptide bonds broken in the amino-terminal of proline, if any, cannot be determined with OPA, which could lead to underestimation of the DH. Hence, the DH is reported as apparent DH.

A volume of 25 µL of 20% sodium dodecyl sulphate, 2 µL of β-mercaptoethanol and 20 µL of methanol-OPA (40 mg of OPA in 1 mL of methanol) were mixed with 953

µL of 0.1 M sodium tetraborate decahydrate solution (pH = 9.3). This OPA solution was prepared just before the analysis. The OPA assay was carried out by the addition of 50 µL of the sample or the standard to 1 mL of OPA solution. The mixture was mixed and allowed to stand for 2 min before measuring the absorbance at 340 nm. A standard curve was prepared using glycine (0–20 µg/mL). The total amino groups in hydrolysed gluten and gliadin were determined after acid hydrolysis with 6 M HCl for 24 h at 110 °C. Then, HCl was removed using a centrifugal concentrator and replaced by 0.1 M sodium tetraborate buffer to measure the amino groups as described above.

The apparent DH values were calculated using the following formula:

$$\mathrm{DH\%} = \frac{(\mathrm{NH_{2ti}} - (\mathrm{NH_{2t0}} + \mathrm{NH_{2blank}}))\, x\, 100}{(\mathrm{NH_{2Total}} - \mathrm{NH_{2blank}})}$$

$NH_{2ti}$ and the $NH_{2t0}$ are the free amino groups at time i and at time 0, while $NH_{2blank}$ is the free amino groups in the blank treatments (i.e., amino groups in the digestive enzymes alone or with one of the exogenous enzymes such as actinidin).

### 3.3.6.4 Extraction (solubilisation) of gluten/gliadin for ELISA

The gluten/gliadin peptides in the hydrolysed samples were extracted using a procedure described by Mena, Lombardía, Hernando, Méndez, & Albar (2012). This procedure was required to extract the undigested/resistant peptides from the hydrolysed gluten and gliadin prior to analysing their presence using the ELISA kit. The extraction procedure was based on reducing Tris (2-carboxyethyl)-phosphine (TCEP) and the anionic surfactant N-lauroylsarcosine in phosphate buffer solution. The solution is named as universal prolamin and glutelin extractant (UPEX) solution. A 0.25 mL aliquot of the *in vitro* hydrolysed sample was transferred to a 10 mL Falcon tube. A 2.5 mL aliquot

of the UPEX solution (5 mM TCEP, 2% N-lauroylsarcosine in PBS, pH 7), which was prepared immediately before use, was added to the tube containing the sample. The contents of the tubes were mixed thoroughly and then incubated at 50 °C for 40 min. After cooling for 10 min, 7.5 mL of 80% ethanol were added, mixed, and incubated for 1 h at room temperature in a rotary shaker. The tubes were then centrifuged for 10 min at 2,500 *g* at room temperature. The samples were used in the ELISA analysis.

#### 3.3.6.5 Competitive ELISA

The UPEX extracted supernatants were analysed in duplicate to determine the hydrolysis of gluten and gliadin peptides containing R5 epitopes in the hydrolysed samples by using a commercially available competitive ELISA based on the R5 antibody. Because the molecular weights, sequences, and number of R5 epitopes are not known, the measure is reported as the amount of intact R5 epitope recognised by the monoclonal antibody after hydrolysis. Based on the standard curve of the ELISA kit, it was assumed that the amount of R5 epitopes in a g of gliadin and gluten was 540 and 1080 ng, respectively. All the procedures were carried out according to the manufacturer's instructions. The absorbance was measured at 450 nm using a microplate reader.

#### 3.3.6.6 Statistical analysis

Different statistical analyses were performed using SAS (SAS/STAT version 9.4) or Minitab (Minitab 16.1; Minitab, Inc.). For all the statistical analyses, only significant ($P < 0.05$) terms were kept in the final model, which were, in general, selected by comparing full models with reduced models (i.e., removing predictors that did not affect the response variable) by using the log-likelihood ratio test. A polynomial model was

used to test the effect of adding actinidin to the gastrointestinal hydrolysis over time (120 min pepsin plus 60 min pancreatin). This analysis was also used when different exogenous enzymes were compared. A one-way ANOVA model was used to determine the effect of exogenous enzymes on the gastric hydrolysis of resistant gliadin peptides at 15 and 60 min. A two-way ANOVA model was used to determine the effect of actinidin, the E64 inhibitor and their interaction. A response surface analysis (central composite design) was conducted to determine simultaneously the effect of gastric pH and actinidin concentration during *in vitro* gastric hydrolysis. A polynomial regression analysis was first conducted on each factor (i.e.,, pH and actinidin concentration) and their interaction. The model diagnostics for each response variable were tested using the ODS Graphics procedure and the repeated statement of SAS. When the F-value of the model was significant ($P < 0.05$) for a categorical variable, the means were compared using the adjusted Tukey's test.

## 3.4 Results and discussion

### 3.4.1 Cleavage sites of 33-mer with actinidin

There were 159 possible intermediate fragments that could have been generated from the 33-mer peptide after 120 min hydrolysis with actinidin alone (Figure 3.1). Chromatograms of hydrolysis of the 33-mer peptide by actinidin for 30 (A) and 120 (B) min and actinidin alone for 120 min (C) are shown in Figure 3.2. To determine the cleavage sites of actinidin, only 50 peptides were considered based on their molecular weight and peptide spectrum matches (Table 3.1). Some of the peptides generated could have come from two or more places in the 33-mer sequence. Thus, some of the cleaved bonds are identified tentatively, as it may have been only one of two or more that is cleaved. The tentatively cleaved bonds are indicated by dotted arrows in Figure 3.3.

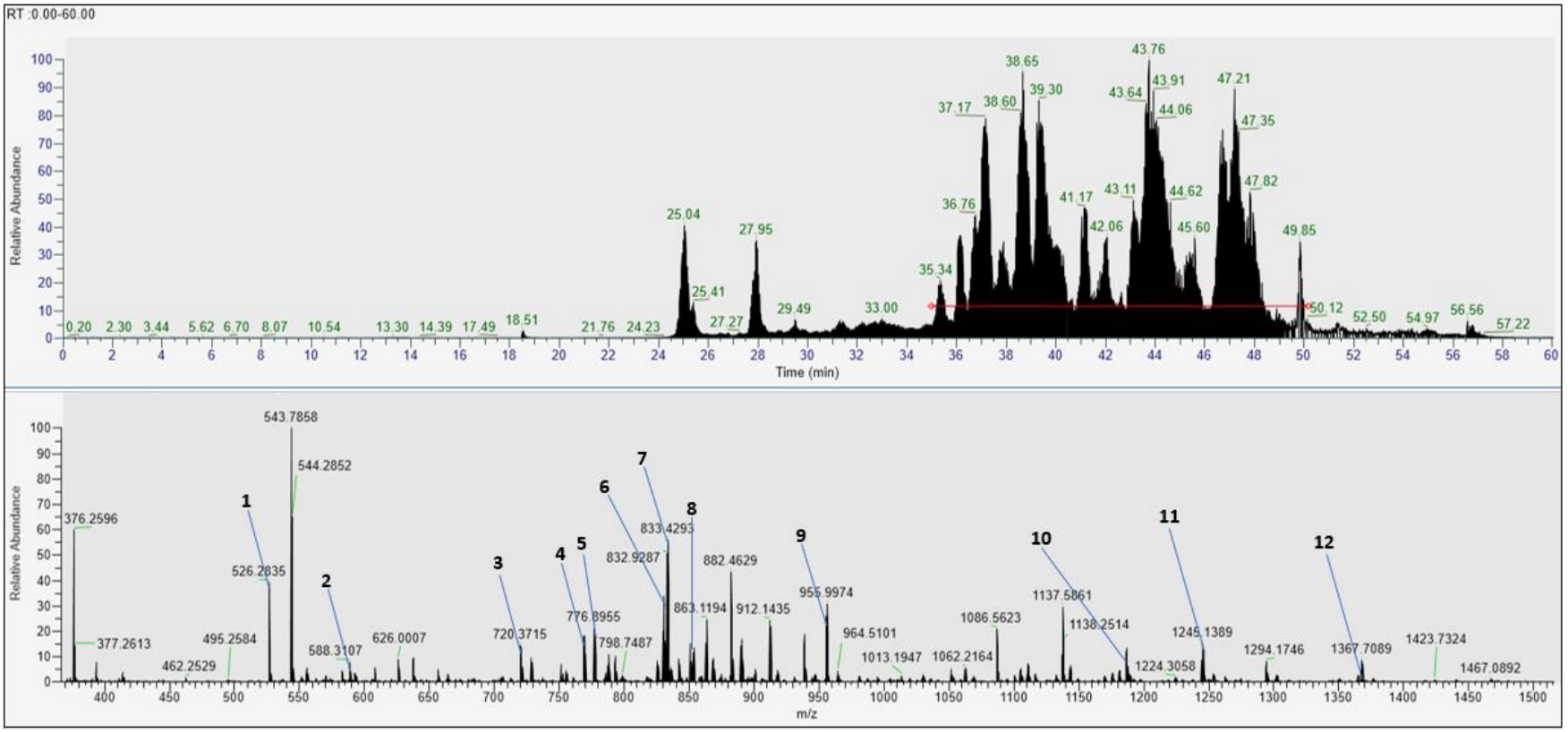



**Figure 3.1** Mass spectrum of possible peptides generated after in vitro hydrolysis of 33-mer peptide with actinidin for 120 min. Relative abundance is based on total ion current.

Some of the main peptides identified are numbered 1-12 and are as follows: 1- QPFPQPQLP; 2- 2 LPYPQPQLPYPQPQP; 3- QPFPQPQLPYPQ; 4- QPFPQPQLPYPQP; 5- YPQPQLPYPQPQP; 6- QPFPQPQLPYPQPQ; 7- FPQPQLPYPQPQLPYPQPQLPYPQPQPF; 8- YPQPQLPYPQPQPF; 9- LPYPQPQLPYPQPQPF; 10- QPFPQPQLPYPQPQLPYPQPQLPYPQPQPF; 11- PQLPYPQPQLPYPQPQLPYPQ or LPYPQPQLPYPQPQLPYPQPQ; 12- LPYPQPQLPYPQPQLPYPQPQPF.

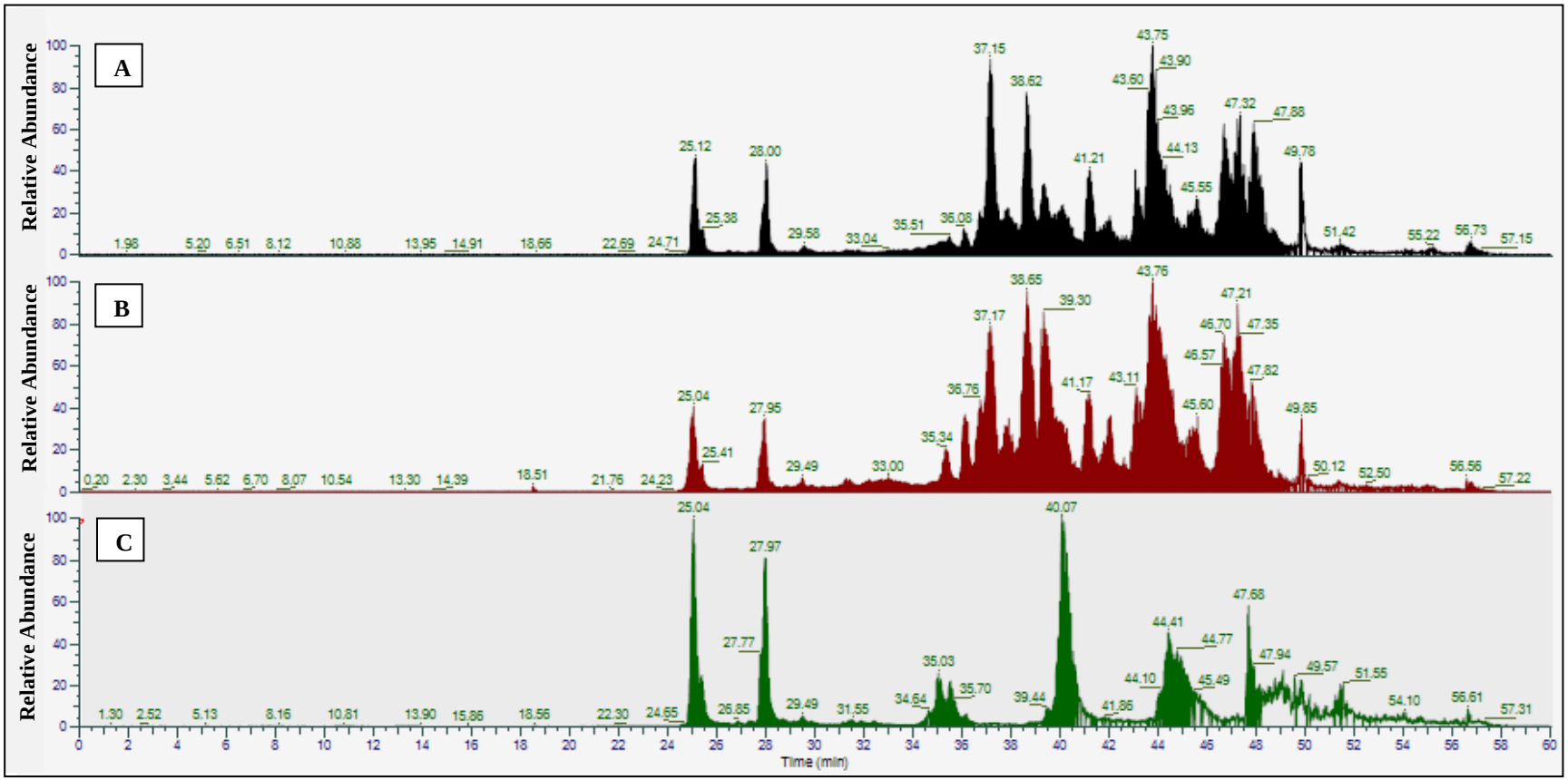



**Figure 3.2** Chromatograms of peptides from *in vitro* hydrolysis of 33-mer peptide with actinidin for 30 (A) min and 120 (B) min, and actinidin alone for 120 min (C).

Relative abundance is based on the total ion current.

**Table 3.1** List of peptides used to determine the cleavage sites of actinidin*.

| Annotated Sequence | Retention time (min) | # peptide spectrum matches | Positions in 33-mer peptide | Theo. MH+ (Da) |
|---|---|---|---|---|
| [L].QPFPQPQLPYP.[Q] | 39.70-40.11 | 16 | [4-14] | 1311.6732 |
| [P].YPQPQLPYPQP.[Q] | 36.09-36.55 | 13 | [13-23] | 1327.66811 |
| [L].QPFPQPQLPYPQ.[P] | 35.97-36.46 | 19 | [4-15] | 1439.73178 |
| [P].YPQPQLPYPQPQ.[PL] | 35.11-35.52 | 23 | [13-24] | 1455.72669 |
| [L].QPFPQPQLPYPQP.[QH] | 38.83-40.03 | 44 | [4-16] | 1536.78454 |
| [P].YPQPQLPYPQPQP.[F] | 35.93-36.42 | 28 | [20-32] | 1552.77945 |
| [L].PYPQPQLPYPQPQP.[F] | 36.38-36.75 | 17 | [19-32] | 1649.83222 |
| [L].QPFPQPQLPYPQPQ.[LP] | 38.31-38.88 | 52 | [4-17] | 1664.84312 |
| [P].YPQPQLPYPQPQPF.[-] | 41.06-41.42 | 31 | [20-33] | 1699.84787 |
| [Q].LPYPQPQLPYPQPQP.[F] | 39.09-39.54 | 17 | [18-32] | 1762.91628 |
| [Q].PQLPYPQPQLPYPQP.[Q] | 39.09-39.54 | 24 | [9-23] | 1762.91628 |
| [L].QPFPQPQLPYPQPQL.[P] | 40.97-41.38 | 14 | [4-18] | 1777.92718 |
| [Q].LQPFPQPQLPYPQPQ.[LP] | 40.85-41.38 | 14 | [3-17] | 1777.92718 |
| [P].QLPYPQPQLPYPQPQ.[PL] | 39.21-39.87 | 12 | [10-24] | 1793.9221 |
| [L].PYPQPQLPYPQPQPF.[-] | 41.67-41.92 | 24 | [19-33] | 1796.90063 |
| [L].QPFPQPQLPYPQPQLP.[Y] | 44.38-44.75 | 16 | [4-19] | 1874.97995 |
| [P].QLPYPQPQLPYPQPQP.[F] | 39.49 - 39.78 | 23 | [17-32] | 1890.97486 |
| [P].QPQLPYPQPQLPYPQP.[Q] | 39.00-39.54 | 20 | [8-23] | 1890.97486 |
| [L].QLQPFPQPQLPYPQPQ.[LP] | 41.11-41.84 | 14 | [2-17] | 1905.98576 |
| [Q].LPYPQPQLPYPQPQPF.[-] | 43.72-43.93 | 45 | [18-33] | 1909.9847 |
| [P].YPQPQLPYPQPQLPYP.[Q] | 43.36-43.77 | 22 | [13-28] | 1925.97961 |
| [Q].PQLPYPQPQLPYPQPQP.[F] | 41.07-41.39 | 20 | [16-32] | 1988.02762 |
| [Q].LQPFPQPQLPYPQPQLP.[Y] | 44.99-45.24 | 14 | [3-19] | 1988.06401 |
| [P].QPQLPYPQPQLPYPQPQ.[PL] | 39.95-40.11 | 15 | [8-24] | 2019.03344 |
| [-].LQLQPFPQPQLPYPQPQ.[LP] | 43.11-43.36 | 10 | [1-17] | 2019.06982 |
| [P].QLPYPQPQLPYPQPQPF.[-] | 43.80-44.17 | 20 | [17-33] | 2038.04327 |

| | | | | |
|---|---|---|---|---|
| [P].YPQPQLPYPQPQLPYPQ.[P] | 41.42-41.88 | 18 | [13-29] | 2054.03819 |
| [Q].PQLPYPQPQLPYPQPQLP.[Y] | 43.42-43.85 | 11 | [9-26] | 2101.11169 |
| [P].QPQLPYPQPQLPYPQPQP.[F] | 40.27-40.52 | 20 | [15-32] | 2116.0862 |
| [L].QPFPQPQLPYPQPQLPYP.[Q] | 44.70-45.12 | 11 | [4-21] | 2135.09604 |
| [P].FPQPQLPYPQPQLPYPQP.[Q] | 44.65-44.98 | 10 | [6-23] | 2135.09604 |
| [P].YPQPQLPYPQPQLPYPQP.[Q] | 42.29-42.50 | 27 | [13-30] | 2151.09095 |
| [FY].PQPQLPYPQPQLPYPQPQP.[F] | 40.37-40.98 | 16 | [14-32] | 2213.13897 |
| [Q].PFPQPQLPYPQPQLPYPQP.[Q] | 38.76-39.13 | 15 | [5-23] | 2232.1488 |
| [L].PYPQPQLPYPQPQLPYPQP.[Q] | 43.89-44.17 | 15 | [12-30] | 2248.14372 |
| [L].QPFPQPQLPYPQPQLPYPQ.[P] | 43.56-43.93 | 26 | [4-22] | 2263.15462 |
| [P].FPQPQLPYPQPQLPYPQPQ.[PL] | 42.66-43.19 | 21 | [6-24] | 2263.15462 |
| [P].YPQPQLPYPQPQLPYPQPQ.[P] | 41.39-41.84 | 22 | [13-31] | 2279.14953 |
| [F].PQPQLPYPQPQLPYPQPQLP.[Y] | 42.13-42.87 | 12 | [7-26] | 2326.22303 |
| [L].QPFPQPQLPYPQPQLPYPQP.[Q] | 44.05-44.42 | 40 | [4-23] | 2360.20738 |
| [P].YPQPQLPYPQPQLPYPQPQP.[F] | 41.84-42.09 | 43 | [13-32] | 2376.20229 |
| [Q].LQPFPQPQLPYPQPQLPYPQ.[P] | 45.20-45.65 | 11 | [3-22] | 2376.23868 |
| [L].PYPQPQLPYPQPQLPYPQPQP.[F] | 47.99-48.60 | 20 | [12-32] | 2473.25506 |
| [P].FPQPQLPYPQPQLPYPQPQLP.[Y] | 45.65-45.85 | 28 | [6-26] | 2473.29144 |
| [L].QPFPQPQLPYPQPQLPYPQPQ.[PL] | 43.44-43.85 | 77 | [4-24] | 2488.26596 |
| [Q].PQLPYPQPQLPYPQPQLPYPQ.[P] | 43.47-43.80 | 18 | [9-29] | 2489.28636 |
| [P].YPQPQLPYPQPQLPYPQPQPF.[-] | 45.40-45.73 | 33 | [13-33] | 2523.27071 |
| [Q].LPYPQPQLPYPQPQLPYPQPQP.[F] | 43.93-44.13 | 67 | [11-32] | 2586.33912 |
| [Q].PQLPYPQPQLPYPQPQLPYPQP.[Q] | 44.01-44.30 | 21 | [9-30] | 2586.33912 |

* Possible peptides generated after digestion of 33-mer with actinidin for 120 min were filtered based on peptide spectrum matches ($\geq$ 10) and molecular weight (< 2,600 Da).

This analysis revealed that actinidin can cleave the 33-mer peptide at a wide range of sites in a broad specific manner, including Q-X (X=L, P), L-Q, F-P, P-X (X=Q, Y, F), and only one Y-P bond.

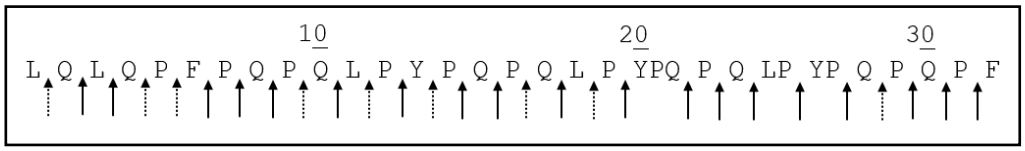



**Figure 3.3** Cleavage sites identified by LC-MS/MS. Arrows represent the locations where actinidin can cleave the 33-mer peptide after digestion for 120 min.
The dotted arrows represent potential cleavages.

To hydrolyse highly resistant gluten peptides, it is essential to cleave proline-containing peptide bonds, as gluten peptides, as exemplified in the 33-mer, are characterised by a high proportion of proline-rich peptide sequences. According to previous studies, the 33-mer peptide remained intact, even after prolonged exposure to the human GIT enzymes both *in vitro* and *in vivo* and it has been identified as one of the most immunogenic peptides arising from the digestion of gliadin (Shan et al., 2002). The current LC-MS/MS results proved that actinidin can break multiple peptide bonds including most of the N- and C-terminal bonds of the prolines in the 33-mer peptide. The ability of actinidin to hydrolyse the peptide bonds Q-P, F-P, P-Q, P-Y, P-F, and P-X-P, and the abundance of these peptide motifs in gluten peptides (Vader et al., 2002) highlights the potential ability of actinidin to hydrolyse most gluten peptides, including immunogenic peptides such as the 33-mer.

### 3.4.2 Effect of actinidin on the apparent DH of gluten and gliadin

There was a significant interaction ($P = 0.01$) between treatments (hydrolysis with or without actinidin) and the time of hydrolysis for the apparent DH of both gluten (Figure

3.4A) and gliadin (Figure 3.4B). For gluten and gliadin, the apparent DH increased over time at different rates due to the presence/absence of actinidin.

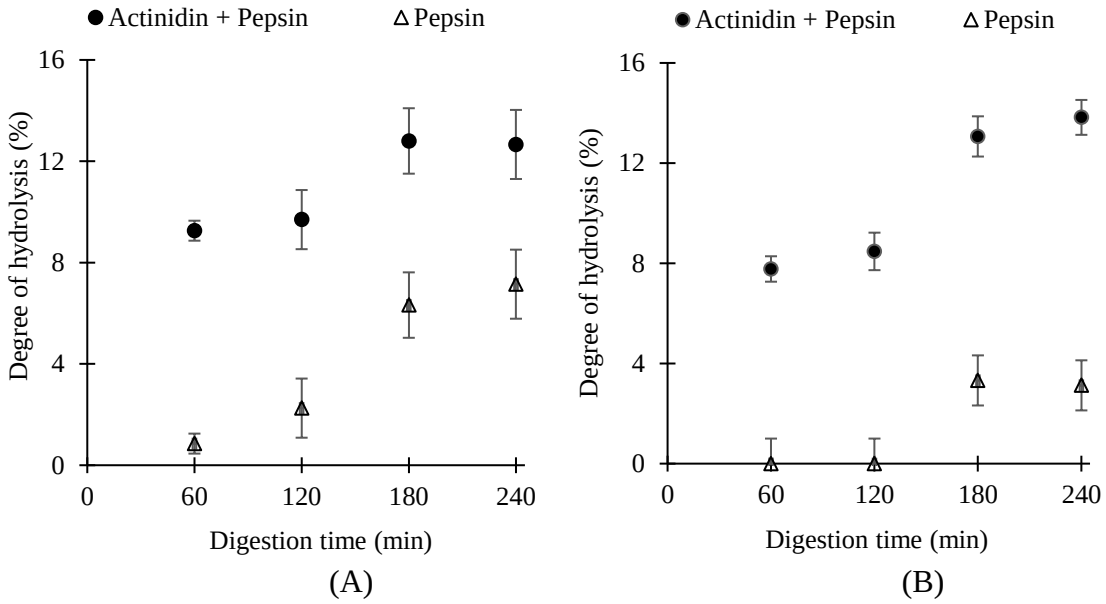



**Figure 3.4** The apparent degree of hydrolysis of gluten (A) and gliadin (B) after *in vitro* gastric (0-120 min) and small intestinal (120-240 min) digestions with and without actinidin, as measured by free amino groups.

For gluten, the P-values for the enzyme, time and their interaction effects were 0.002, 0.007C[1] and 0.01 respectively while for gliadin they were < 0.0010, 0.003C[1] and ≤ 0.001 respectively. [1]C, cubic effect for the time factor. Values are means ± SEMs, n = 3.

For instance, when actinidin was included in the GIT hydrolysis, the apparent DH of gliadin increased ~0.07%/min during the first 180 min of hydrolysis (120 min gastric + 60 small intestinal), but it then reached a plateau. In contrast, there was no hydrolysis of gliadin during the first 120 min of hydrolysis with pepsin alone, but it increased (~0.05%/min) during only the first 60 min pancreatic hydrolysis before reaching a plateau. This revealed that the presence of actinidin increased the gastric hydrolysis of both gluten and gliadin.

This is likely to be due to the broad amino acid specificity of actinidin, as discussed above, that enables it to hydrolyse a wide range of peptide bonds unavailable to pepsin. Similar results were observed in an *in vivo* study, where the gastric hydrolysis (OPA method) of wheat gluten in rats was 3.2-fold greater when actinidin was present in the diet (Montoya & Hindmarsh, et al., 2014).

There was no interaction ($P > 0.05$; Table 3.2) between actinidin and the E64 cysteine protease inhibitor for the small intestinal hydrolysis of gluten and gliadin. The addition of the inhibitor into the small intestinal hydrolysis medium did not change the DH of either gluten or gliadin ($P > 0.05$). Based on these results, actinidin had, if any, a negligible effect on the small intestinal hydrolysis of gluten proteins. However, the present study used a static hydrolysis model where small intestinal hydrolysis started two hours after gastric hydrolysis and this may have resulted in all available bonds being hydrolysed prior to being *in vitro* digested with pancreatic enzymes.

In a real GIT digestion, gastric chyme starts to be released into the small intestine earlier (Montoya, Cabrera, Zou, Boland, & Moughan, 2018), which means that less hydrolysed proteins enter the small intestine. In this situation, actinidin could have an effect on the hydrolysis of gluten proteins and peptides in the small intestine, as actinidin is able to hydrolyse proteins at pH ranging from 3-10 (Arcus, 1959; Sun, Zhang, Yan, & Jiang, 2016).

**Table 3.2** Effect of actinidin and inhibitor on small intestinal apparent degree of hydrolysis of gluten and gliadin.

| | Inhibitor | | | Actinidin | | | P-value[a] | |
|---|---|---|---|---|---|---|---|---|
| | DH% (+) Actinidin | DH% (-) Actinidin | SEM | DH% (+) Inhibitor | DH% (-) Inhibitor | SEM | Actinidin | Inhibitor |
| Gluten | 43.71 | 4.73 | 4.13 | 23.45 | 24.98 | 4.13 | < 0.001 | 0.780 |
| Gliadin | 38.23 | 2.58 | 4.19 | 19.76 | 21.04 | 4.19 | < 0.001 | 0.834 |

[a]The actinidin and inhibitor interaction was not significant. Therefore, it was removed from the final model.

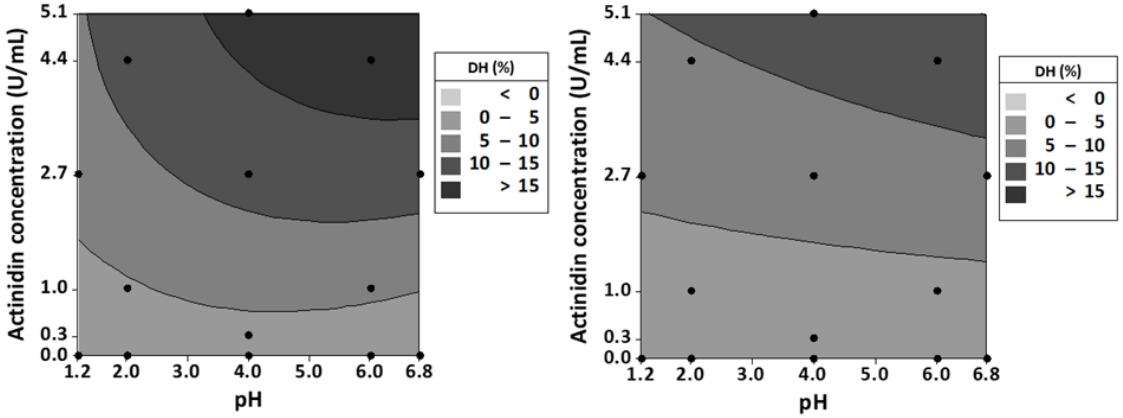
**Table 3.3** Estimated regression coefficients for the apparent degree of hydrolysis (%) of gluten and gliadin after 60 min of *in vitro* gastric hydrolysis with different actinidin concentrations (0.3 to 5.1 U/mL hydrolysis medium) and pH (1.2 to 6.8) levels.

| Terms | Coefficients | |
|---|---|---|
| | Gluten | Gliadin |
| intercept | 1.48 | 1.43 |
| pH | 0.04 | -0.06 |
| actinidin concentration | 1.66** | 1.44** |
| pH x actinidin concentration | 0.33** | 0.19* |
| $r^2$ | 0.87 | 0.86 |

**P < 0.01; *P < 0.05.

### 3.4.3 Effect of pH and actinidin concentration on the gastric hydrolysis of gluten, gliadin and their R5 epitopes

Based on the response surface analysis, the DH after 60 min of gastric hydrolysis of gluten and gliadin was affected by an interaction between pH and actinidin concentration ($P < 0.05$; Table 3.3). In general, the DH of gluten and gliadin increased when the actinidin concentration and pH increased (Figure 3.5A and B).




(A) (B)

**Figure 3.5** Effect of pH and actinidin concentration on the apparent degree of hydrolysis (DH) of gluten (A) and gliadin (B) after 60 min of *in vitro* gastric digestion as measured by free amino groups.

Values are means of three replicates.

However, at pH equal to or lower than 2, the level of actinidin concentration had a reduced effect on the DH of both gluten and gliadin. For instance, at actinidin concentration of 2.7 U/mL, the DH of gluten was 10.1% and 2.3% for pH 6.8 and 2.0 respectively. The selected models explain 0.87 and 0.86 of the variability on the gastric DH of gluten and gliadin respectively. Actinidin is reported to be active over a wide range of pH. The stomach pH (~2 - 5.1) after ~1 h of consuming bread meal in healthy individuals (Hoebler et al., 2002) falls in this active pH range of actinidin. Therefore, it

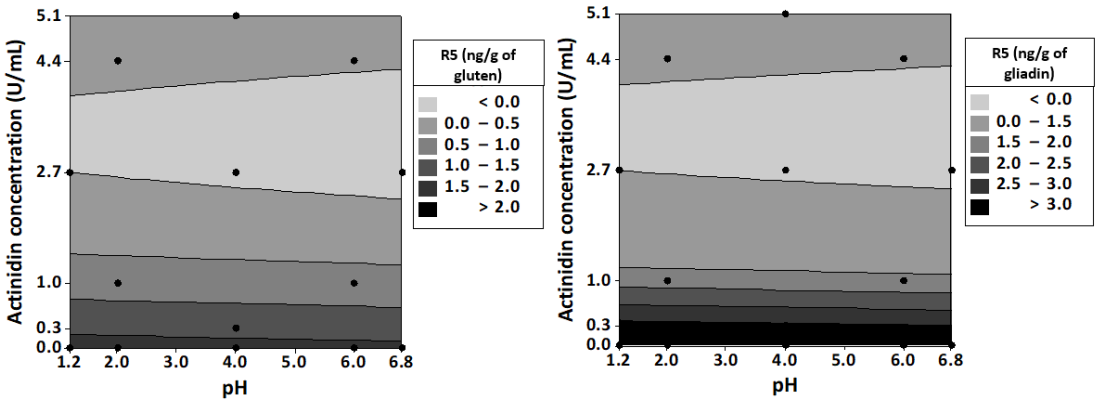
can be assumed that after consuming a gluten-containing meal, the pH in the stomach supports the hydrolysis of gluten proteins by actinidin.

**Table 3.4** Estimated regression coefficients for R5 epitope concentration after 60 min of *in vitro* gastric hydrolysis with different actinidin concentrations (0.3 to 5.1 U/mL hydrolysis medium) and pH (1.2 to 6.8) levels.

| Terms | Coefficients | |
|---|---|---|
| | **Gluten** | **Gliadin** |
| intercept | 3.9 | 1.7 |
| pH | -0.03 | -0.02 |
| actinidin concentration (AC) | -2.41* | -1.08* |
| AC x AC | 0.36* | 0.16* |
| $r^2$ | 0.83 | 0.86 |

*P < 0.01.

After 60 min of gastric hydrolysis, there was no interaction between pH and actinidin concentration on the hydrolysis of resistant gluten and gliadin peptides (P > 0.05; Table 3.4). However, the quantity of R5 epitopes decreased as the actinidin concentration increased in the hydrolysis medium (P < 0.01; Figure 3.6A and B). For both gluten and gliadin, the amount of R5 epitope decreased when the actinidin concentration was 2.7 U/mL and thereafter it was constant (P > 0.05). The pH of the medium did not affect (P > 0.05) the hydrolysis of R5 epitopes.




(A) (B)

**Figure 3.6** Effect of pH and actinidin concentration on the remaining amount of R5 epitope in gluten (A) and gliadin (B) after 60 min *in vitro* digestion.

Values are means of three replicates.

### 3.4.4 Effect of actinidin and other exogenous enzymes on the gastric hydrolysis of gliadin and its R5 epitopes

There was a significant interaction ($P < 0.01$) between the enzyme (pepsin alone or pepsin with one of the exogenous enzymes) and the hydrolysis time on the apparent gastric DH of gliadin (Figure 3.7). The apparent DH of gliadin hydrolysed with pepsin alone did not change over time ($P > 0.05$), while the apparent DH increased ($P < 0.05$) over time at different rates when gliadin was hydrolysed with pepsin supplemented with exogenous enzymes. For instance, the rate of apparent DH during the first 15 min of gliadin hydrolysed with bromelain, papain, and GlutnGo® was on average 0.4%/min, while it was 0.8 and 2%/min for actinidin and Digest Gluten® respectively ($P < 0.05$). At the remaining hydrolysis times, actinidin gave greater apparent DH ($P < 0.05$) than bromelain, papain, and GlutnGo®, but lesser apparent DH ($P < 0.05$) than Digest Gluten®.

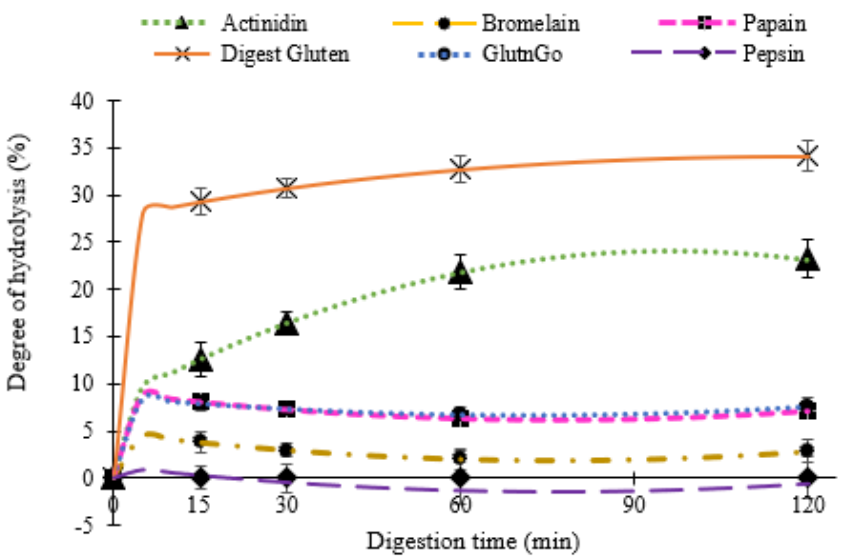



**Figure 3.7** Effect of pepsin alone or supplemented with exogenous enzymes on the apparent degree of hydrolysis of gliadin.

P-values for the enzyme, time and their interaction effects were < 0.001, 0.007, and < 0.001 respectively. Values are means ± SEMs, n = 3.

At 15 min of gastric hydrolysis, all exogenous enzymes significantly hydrolysed (P = 0.009) the R5 epitopic peptides when compared to pepsin alone (Figure 3.8), without a difference (P > 0.05) between them. Moreover, the amounts of remaining resistant R5 epitopes for all exogenous enzymes were not significantly different from the blank (P < 0.05), which did not have any gliadin peptides in it. There was no difference (P > 0.05) in the amount of gliadin epitopes between 15 and 60 min of hydrolysis (data not shown).

The effectiveness of papain and bromelain in hydrolysing gluten has been shown in previous studies. For instance, when papain was incorporated into wheat flour, it reduced the quantity of gliadin epitopes by ~6-fold compared to pepsin as measured using a sandwich ELISA test (enzyme activity 254 U/g flour, roughly about 5.6 U/mg gliadin; antibody not specified) (Li, Yu, Goktepe, & Ahmedna, 2016). In the present study, a higher reduction of antigenic peptides (~35-fold) was observed with papain

compared to pepsin, probably due to the higher papain concentration used in the present study (10.2 U/mg gliadin). It has been observed *in vitro* that bromelain is less effective in detoxifying wheat gluten compared to papain (Pahlavan, Sharma, Pereira, & Williams, 2016).

In contrast, in the present study, all three cysteine proteases were able to reduce the quantity of resistant gliadin peptides to the same extent. This could be attributed to the use of purified gliadin instead of wheat flour, which is less complex to digest and the use of a high enzyme to substrate ratio.

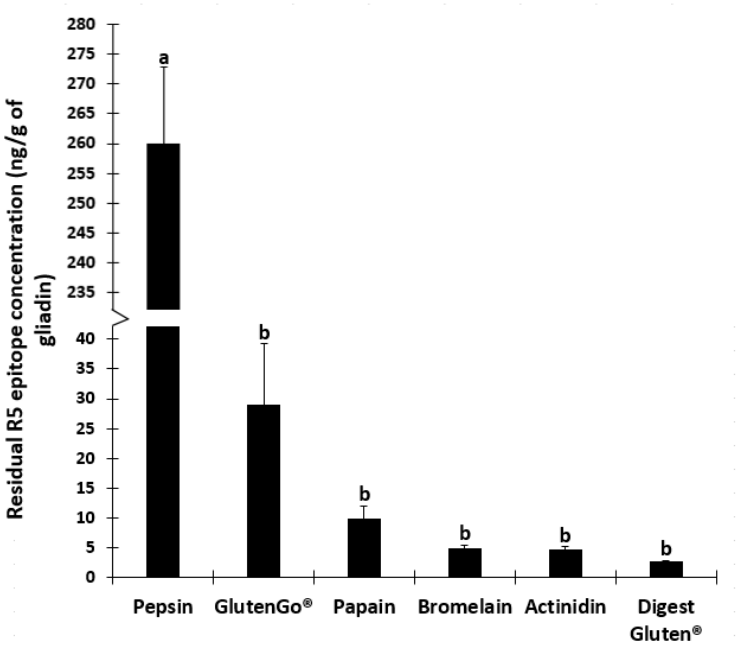



**Figure 3.8** Residual R5 epitope concentration after 15 min of *in vitro* gastric digestion with either pepsin alone or with exogenous enzymes. Columns with different letters differ significantly ($P < 0.05$).

Values from all the exogenous enzymes are not significantly different from the blanks (i.e., 0 gliadin concentration). Values are means ± SEMs, n = 3.

*In vivo* studies have shown that both papain and bromelain reduced the immunogenic effects of gluten. For instance, in a study using mice, the production of total serum IgE was ~2-fold lower in mice sensitized with papain treated gliadins compared to the

control (Xue et al., 2019). Bromelain was able to reduce the toxic effect of gluten by 30% when incorporated into wheat flour in the ratio of 1:100 (enzyme (bromelain activity ~230 U/mg): flour) to make a hypoallergenic bread, without affecting the quality of the bread (Tanabe, Arai, & Watanabe, 1996). Based on the ELISA and LC/MS results of the current study, it can be hypothesised that actinidin could also be able to reduce the immunogenicity of gluten. Further studies, including *in vivo* studies, are warranted to test this hypothesis.

### 3.5 Conclusions

A natural plant enzyme, actinidin, has been investigated for its ability to hydrolyse purified gluten and gliadin under simulated gastrointestinal conditions, using a static hydrolysis model. The results showed that actinidin is active under physiological gastric conditions and it degraded gluten proteins as well as the highly resistant 33-mer gliadin peptide, and peptides containing QQQ/PFP present in gluten epitopes by cleaving multiple peptide bonds including peptide bonds at the N- and C-terminal of proline residues. Actinidin at a concentration of $\geq$ 2.7 U/mL and pH > 2 during the hydrolysis is considered ideal for gluten hydrolysis. Actinidin was more effective in hydrolysing gliadin than two other cysteine proteases, papain, and bromelain. Actinidin is a promising enzyme to hydrolyse gluten and its highly resistant peptides under simulated gastric conditions.

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# Chapter Four

# 4. Effect of actinidin on hydrolysis of gluten and gluten-derived immunogenic peptides in an *in vitro* semi-dynamic gastrointestinal digestion model

This chapter has been submitted to Food Research International:

Jayawardana, I. A., Boland, M., K., Loo, T., McNabb, W. C., & Montoya, C. A. Actinidin reduces gluten-derived immunogenic peptides reaching the small intestine in an *in vitro* semi-dynamic gastrointestinal tract digestion model. Submitted to *Food Research International.*

**Highlights**

- *In vitro* gastric and small intestinal digestion of gluten was accelerated by actinidin.
- Actinidin rapidly hydrolysed immunogenic peptides during *in vitro* gastric digestion.
- Rapid gastric proteolysis of immunogenic peptides reduced the amount of immunogenic peptides reaching the small intestine.
- Fewer immunogenic peptides in the small intestine may reduce the risk of gluten-related health disorders.

**4.1 Abstract**

Actinidin, a cysteine protease in green kiwifruit (*Actinidia deliciosa*), has been identified as a potential enzyme to hydrolyse gluten within the lumen of the gastrointestinal tract (GIT). The present study aimed to further evaluate the effect of purified actinidin sourced from green kiwifruit on the digestion of gluten and the release of immunogenic peptides during GIT digestion using an *in vitro* semi-dynamic GIT digestion model. Purified gluten was digested for 180 min with or without actinidin and subsequently analysed for free amino groups (*o*-phthaldialdehyde) to determine the degree of hydrolysis (DH), gluten R5 epitopes (ELISA), and peptide profiles (mass spectrometry). Strong interactions were observed between treatment (GIT digestion with or without actinidin) and digestion time for the DH of gluten ($P < 0.01$), amount of free amino groups released into the small intestine ($P < 0.01$), and amount of gluten epitopes present in the small intestine ($P < 0.001$). The rate of increase of DH of gluten and the amount of R5 epitopes present in the small intestine during the first 30 min of GIT digestion with actinidin was 0.3%/min and 4.8 ng/g of gluten respectively, whereas it was 0.01%/min and 60.9 ng/g of gluten respectively without actinidin. These results were corroborated by untargeted peptidomics, with a 1.5-fold lower number of known immunogenic epitopes reaching the small intestine at 30 min of GIT digestion when actinidin was present compared to the control. Present results demonstrate that actinidin enhanced the rate of proteolysis of gluten and reduced the number of immunogenic gluten epitopes reaching the small intestine during simulated semi-dynamic GIT digestion.

**Keywords:** Actinidin; Gluten; Immunogenic peptides; Gastrointestinal tract digestion; *In vitro* semi-dynamic gastrointestinal model

## 4.2 Introduction

Gluten-related health diseases (coeliac disease, wheat allergy, and gluten sensitivity) have genetic, environmental, and immunological components involved in their development. The environmental factors, of which gluten intake is the main one, can be controlled to ameliorate these diseases. Gluten proteins are characterised by their high proline content. Endogenous gastrointestinal tract (GIT) enzymes are unable to fully hydrolyse proline-containing peptides like those in gluten, and so gluten proteins are incompletely digested in the GIT, producing proline-rich peptides (Jayawardana et al., 2019). Some of these proline-rich peptides can cross the epithelial layer to trigger adaptive, innate, or allergic immune reactions in genetically predisposed individuals (Scherf et al., 2016).

A lifelong gluten-free diet is the main medical treatment for gluten-related diseases (Haines et al., 2008). Other than complete avoidance of gluten-containing foods, the use of alternative dietary therapies to hydrolyse gluten proteins has also been investigated. The use of exogenous proteolytic enzymes either before or after gluten intake to hydrolyse immunogenic gluten peptides into forms that are “safe”, is one of the dietary alternative therapies that has gained attention from the scientific community due to its success in eliminating immunogenic gluten peptides (De Angelis et al., 2010; Jayawardana et al., 2019; König et al., 2017). Essentially, these exogenous enzymes are capable of hydrolysing proline-containing peptides.

In our previous study, actinidin, a cysteine protease in green kiwifruit (*Actinidia deliciosa*), was identified as a potential enzyme capable of cleaving most N- and C-terminal bonds in peptides including those containing proline. Actinidin was also able to cleave the 33-mer peptide (LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF), and other immunogenic peptides that arise from gluten digestion (Jayawardana et al.,

2021). Our previous study was done using a static *in vitro* GIT digestion model to provide some basic mechanistic understanding of the effect of actinidin on the digestion of gluten proteins and their resultant peptides. However, the static digestion model could not provide an understanding of dynamic variables such as the amount of immunogenic peptides released from the stomach into the small intestine over time. In gluten-related health disorders, the amount of immunogenic peptides and the time of exposure to these peptides in the small intestine are factors that can cause immunogenic reactions (Piper et al., 2004). It is therefore important to determine the efficacy of actinidin in digesting gluten proteins and immunogenic peptides over time.

To be able to understand the chemistry of the action of actinidin on gluten proteins, we needed to start with a simple *in vitro* digestion model and with purified gluten before advancing to a more complex system such as *in vivo* model and a gluten-containing food (e.g., bread). Using purified gluten rather than bread reduces the complexity of removing other proteins present in cereals (e.g., albumins, globulins) and proteins from yeast. This paper describes our second step in a series of experiments increasing in complexity to determine the effect of actinidin on the digestion of gluten proteins and epitopes. The present study, therefore, aimed to evaluate the effect of actinidin on the GIT digestion of gluten and particularly the release of gluten immunogenic peptides into the small intestine over time using an *in vitro* semi-dynamic digestion model. For the simulated semi-dynamic digestion model, a simple *in vitro* model, which considers the change in pH and gastric emptying, was set up to determine important parameters such as the rate of appearance of immunogenic peptides in the small intestine. This simple *in vitro* model could easily be replicated in any research laboratory. Different analytical tools (e.g., competitive enzyme-linked immunosorbent assay (ELISA) and liquid chromatography-tandem mass spectrometry (LC-MS/MS)) were used to

determine the efficacy of actinidin in the reduction of immunogenic peptides that have been associated with gluten-related health disorders.

## 4.3 Materials and Methods

### 4.3.1 Materials

Purified wheat gluten (CAS 8002-80-0), pepsin from porcine gastric mucosa (lyophilized powder, CAS 9001-75-6; activity 3,200-4,500 U/mg protein), pancreatin from porcine pancreas (CAS 8049-47-6; activity x 8 USP), tris (2-carboxyethyl)-phosphine (CAS 51805-45-9), N-lauroylsarcosine (CAS 51805-45-9), and phosphate-buffered saline were purchased from Sigma Aldrich (St Louis, Missouri, USA). Purified actinidin was purchased from New Zealand Pharmaceuticals (Palmerston North, New Zealand). The purified wheat gluten was analysed in duplicate for standard amino acids (by AOAC method 994.12, using HCl acid hydrolysis, followed by Rapid Separation Liquid Chromatography), sulphur-containing amino acids (By AOAC method 43.A08-43.A13, using performic acid oxidation followed by HCl acid hydrolysis) and tryptophan (Allred & Macdonald, 1988, alkali hydrolysis).

### 4.3.2 *In vitro* semi-dynamic gastrointestinal digestion

The INFOGEST standardised *in vitro* gastrointestinal digestion protocol (Minekus et al., 2014) was followed to mimic human gastrointestinal digestion.

Gastric digestion:

Purified gluten (50 mg) was combined with ultrapure water (0.5 mg/mL) and digested with pepsin alone (2,000 U/mL of digestion medium) or pepsin with purified actinidin (3 U/mL) for 180 min in a flask maintained at 37 °C. This actinidin activity was selected based on the actinidin activity in the stomach chyme of pigs fed a diet containing 20%

of green kiwifruit (on a dry matter basis), which is equivalent to the consumption of two kiwifruit with an average-sized meal (Montoya & Rutherfurd et al., 2014). Actinidin activity was measured according to the method described by Boland, & Hardman (Boland & Hardman, 1972). The initial pH of the mixture was pH 5.0 and every 30 min, the pH was reduced with 1 M HCl to represent the gastric pH drop that can occur after consumption of a meal. The changes in pH values were selected based on the postprandial chyme pH of pigs fed diets containing green (with actinidin) and yellow (without actinidin) kiwifruit (Table 4.1). An aliquot of 5 mL was taken from the gastric digestion medium into a separate 15 mL Falcon tube every 30 min to represent chyme from gastric emptying at that time and for subsequent small intestinal digestion.

**Table 4.1** pH of gastric chyme in pigs fed a gluten-containing diet with and without actinidin.

| Time | Actinidin | No actinidin |
|---|---|---|
| 30 | 4.34 | 4.21 |
| 60 | 4.23 | 4.18 |
| 180 | 3.85 | 3.69 |
| 300 | 3.31 | 3.20 |
| 420 | 2.34 | 2.87 |

The description of the animal study is reported in Montoya & Rutherfurd et al., 2014, but the pH values in the Table have not been published.

Small intestinal digestion:

To continue the simulated small intestinal digestion, the gastric emptied samples were mixed 1:1 (V/V) with pancreatin solution (100 U/mL trypsin activity (Minekus et al., 2014)) and the pH was adjusted to pH 7.0 at 37 °C for up to 180 min. For analysis, 2 mL aliquots were taken into Eppendorf tubes representing gastric and gastrointestinal digestion at different time points as presented in Figure 4.1. The tubes were immersed in boiling water for 1 min to inactivate enzymes and frozen at –20 °C until analysis.

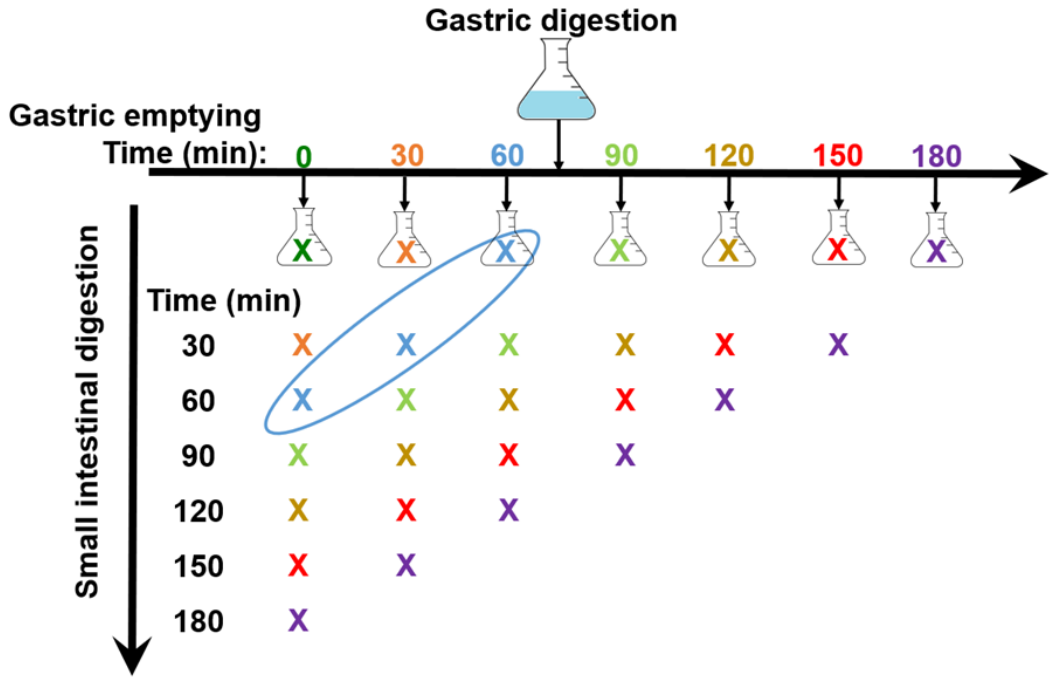



**Figure 4.1** Collection of gastric and small intestinal samples (denoted as X) during continued *in vitro* gastrointestinal digestion; single colour X represents the samples that belong to a particular gastrointestinal digestion time.

The 60 min gastrointestinal digested sample (in blue colour) = 60 min gastric digested sample + (30 min gastric digested sample + 30 min small intestinal digested sample) + (0 min gastric digested sample + 60 min small intestinal digested sample). The small intestinal digestion time reflects not only duodenal digestion, but digestion occurring throughout the small intestine over time, as it is expected that the digested material entering the small intestine at different times moves throughout the small intestine.

To evaluate the effect of actinidin on small intestinal digestion, a sample from the gastric medium was taken at 0 min and digested with pancreatin (100 U/mL trypsin activity, pH 7) at 37 °C for 180 min in the presence or absence of actinidin. Gastric emptying can occur directly after a meal intake (Malagelada et al., 1976), so the small intestinal digestion alone also represents gastric chyme released during the first postprandial minutes, which is assumed in this study to not be digested by pepsin. Aliquots were taken to Eppendorf tubes every 30 min for analysis as described above.

The whole experiment was conducted in triplicate. The samples were analysed for free amino groups by the OPA method, the residual R5 gluten epitope content by a commercially available competitive ELISA kit, and the immunogenic gluten peptides generated during gastric and small intestine digestion by untargeted LC/MS-MS as detailed below.

### 4.3.3 Determination of free amino groups by OPA

The free amino groups in each sample were measured using the OPA method (Church, et al., 1983). Briefly, an OPA solution was prepared just before the analysis using 25 µL of 20% sodium dodecyl sulphate, 2 µL of β-mercaptoethanol and 20 µL of methanol-OPA (40 mg of OPA in 1 mL of methanol) and 953 µL of 0.1 M sodium tetraborate decahydrate solution (pH 9.3). A volume of 50 µL of the sample or the standard was added to 1 mL of OPA solution, and the mixture was mixed and allowed to stand for 2 min before measuring the absorbance at 340 nm using a spectrofluorometer. Glycine was used to prepare the standard curve. To quantify the total amino groups in gluten, the sample was hydrolysed with 6 M HCl for 24 h at 110 °C and the amino groups were measured as described above after removing HCl using a centrifugal concentrator and replacing it with 0.1 M sodium tetraborate buffer. The amount of free amino groups determined using OPA was used to estimate the DH of gluten in the digested samples as described in the calculations.

### 4.3.4 Quantification of residual R5 gluten epitopes by ELISA

A commercially available competitive ELISA based on an antibody to the R5 (QQPFP, QQQFP, LQPFP, and QLPFP) epitope (RIDASCREEN® Gliadin competitive, R-Biopharm AG, Darmstadt, Germany) was used to quantify the remaining R5 epitopes

in all the digested samples. The gluten peptides in the digested samples were first extracted using the procedure of Mena et al. (2012). The extracted samples were used in the ELISA analysis. The extracted samples were analysed in duplicate according to the manufacturer's instructions.

### 4.3.5 Identification of immunogenic gluten peptides by untargeted LC-MS/MS

An untargeted LC-MS/MS analysis was conducted to determine the release pattern of immunogenic gluten peptides after semi-dynamic GIT digestion with and without actinidin. Gastrointestinal digested samples at 30 (0 min gastric + 30 min small intestinal), 60 ([30 min gastric + 30 min small intestinal] + [0 min gastric + 60 min small intestinal]), 90 ([30 min gastric + 60 min small intestinal] + [60 min gastric + 30 min small intestinal] + [0 min gastric + 90 min small intestinal]), and 120 ([30 min gastric + 90 min small intestinal] + [60 min gastric + 60 min small intestinal] + [90 min gastric + 30 min small intestinal] + [0 min gastric + 120 min small intestinal]) min were used to calculate the number of immunogenic peptides at each gastrointestinal digestion time present in the small intestine. The digested samples were defrosted, vortexed and 200 µL of each sample was taken into separate tubes. A trypsin solution (10 µL of 0.1 mg/mL in 0.5 M ammonium bicarbonate, pH 7) was added to each sample and incubated at 37 °C overnight. This step was included to further hydrolyse the complex gluten proteins (and/or larger peptides), especially for the gastric samples, to release cryptic immunogenic peptides trapped in the protein matrix, which otherwise might not be identified in the analysis and therefore would underestimate the potential amount of immunogenic peptides calculated to be present in the GIT. These cryptic immunogenic peptides are expected to be released during digestion. Most known immunogenic peptides are resistant to tryptic digestion (Shan et al., 2002), therefore it was assumed

that the tryptic step does not affect the identification of immunogenic peptides. Then, 800 µL of 100% methanol and an isotopically-labelled internal standard, P1H (LQLQPF*PQPQLPY isotopic l-Phenylalanine-$^{13}C_9$,$^{15}N$ at residue six) was added at 3 ppm into each tube and vortexed for 1 min. The samples were incubated at –20 °C for 30 min and vortexed for another 1 min prior to being centrifuged for 10 min at 4,000 *g*. The supernatants were removed into separate tubes and concentrated using a vacuum evaporator until the contents reduced to ~25 µL. The contents were then reconstituted to 50 µL with 0.1% formic acid and 5% acetonitrile, vortexed for 5 min, and centrifuged for 15 min at 4,000 *g*. The supernatants were used for LC-MS/MS analysis.

Liquid-phase chromatography was done using a Dionex UltiMate™ 3000 RSLCnano System (ThermoFisher Scientific, Waltham, MA USA) with a reversed-phase peptide trap (Acclaim™ PepMap™100 C18, 3 µm particle size, 75 µm inner diameter, 2 cm length) and a reversed-phase capillary analytical column (Acclaim™ PepMap™ C18, 2 µm particle size, 75 µm inner diameter, 50 cm length), both from ThermoFisher Scientific, Waltham, MA USA. The liquid chromatography system was coupled on-line to a QExactive™ Plus mass spectrometer equipped with a higher-energy collision-induced dissociation (HCD) collision cell, an Orbitrap mass analyser and a Nanospray Flex™ ion source (ThermoFisher Scientific, Waltham, MA USA).

Details for the chromatographic parameters were as follows: Flow rates for trap and analytical columns were 15 µL/min and 300 nL/min respectively; column oven temperature was 50 °C; and a gradient of 3-30% acetonitrile in 0.1% formic acid/water over 60 min and 30-90% acetonitrile over 15 min for regeneration was used. The mass spectrometric source and detection settings were as follows: Capillary temperature of 250 °C; source voltage 1.6 kV, S-Lens RF 50%. Data-dependent tandem MS acquisition (Top 10) was used, and one run per replicate was performed. In all

experiments, survey scans were acquired over a mass range of 375 - 1600 *m/z* with detection in the Orbitrap mass analyser at a resolution setting of 70,000. Fragment ion spectra produced via HCD were acquired at a resolution setting of 17,500. The top ten most intense ions were selected for fragmentation in each scan cycle. Exclusion conditions were optimized according to observed chromatographic peak width (typically 12 seconds). Peak lists were searched with Proteome Discoverer v2.4 using the following parameters: The database was from NCBI (1 July 2020), taxonomy *Triticum aestivum*, proteins α-gliadin and glutenin with custom synthetic peptide sequences; enzyme specificity none; max number of missed cleavages 2; minimum peptide length 6; precursor mass tolerance 10 ppm; fragment mass tolerance 0.02 Da; variable modifications included oxidation of methionine, N-terminal protein acetylation and deamidation and Gln -> pyro-Glu; target FDR for peptide spectrum matches and peptides ≤ 0.01.

Immunogenic gluten peptide profiles for each time point were identified across the three replicates and they were combined to represent particular GIT digestion time (Figure 4.1). The immunogenic gluten peptides were recognized by the presence of at least one epitope within their primary peptide sequence using the online database ProPepper (Juhász et al., 2015).

### 4.3.6 Calculations

The amount of free amino groups present in the samples measured with OPA was used to calculate the degree of hydrolysis (DH) of samples at different times as described previously (Church et al., 1983). The DH at each time point was calculated using the following formula:

$$\text{DH } (\%) = \frac{(\text{NH}_{2\text{ti}} - (\text{NH}_{2\text{t0}} + \text{NH}_{2\text{blank}})) \times 100}{(\text{NH}_{2\text{Total}} - \text{NH}_{2\text{blank}})}$$

$NH_{2ti}$ and the $NH_{2t0}$ are the amounts of free amino groups at the time i and at time 0 of digestion, while $NH_{2blank}$ is the amount of free amino groups in the blank treatments (i.e., amino groups in digestive enzymes alone or with actinidin prior to digestion). $NH_{2total}$, is the total amino groups present in the digestion medium, which was determined in this study as 150 µg/mg gluten.

Gastric emptying values obtained in rats gavaged with gluten diets with and without actinidin (Montoya & Hindmarsh et al., 2014) (Table 4.2) were combined with the present *in vitro* data (i.e., DH value of each digested sample calculated using the above equation) to calculate the DH of gluten at each GIT digestion time.

**Table 4.2** Gastric emptying (%) of rats gavaged a gluten-containing diet with and without actinidin.

| Time | Actinidin | No actinidin |
|---|---|---|
| 30 | 8.55 | 9.61 |
| 60 | 19.16 | 14.77 |
| 90 | 35.69 | 30.91 |
| 120 | 41.34 | 37.12 |
| 150 | 49.69 | 46.31 |
| 180 | 60.64 | 54.62 |

Adapted from Montoya & Hindmarsh et al., 2014.

All the calculations were done assuming an intake of 1 g of gluten. An example of the calculations of the DH at 60 min of GIT digestion with actinidin is shown in Appendix 1. The same procedure using gastric emptying data was followed to calculate the residual R5 epitopes at each GIT digestion time and the amount of R5 epitopes released into the small intestine at different times.

The immunogenic gluten peptides identified through untargeted peptidomics were combined with the gastric emptying data to calculate the number of immunogenic peptides present in the small intestine at each time point (Appendix 2). This is a semi-quantitative calculation as it only considered the number of identified peptides during GIT digestion, but not the total amount of each peptide identified in the digested samples.

### 4.3.7 Statistical analysis

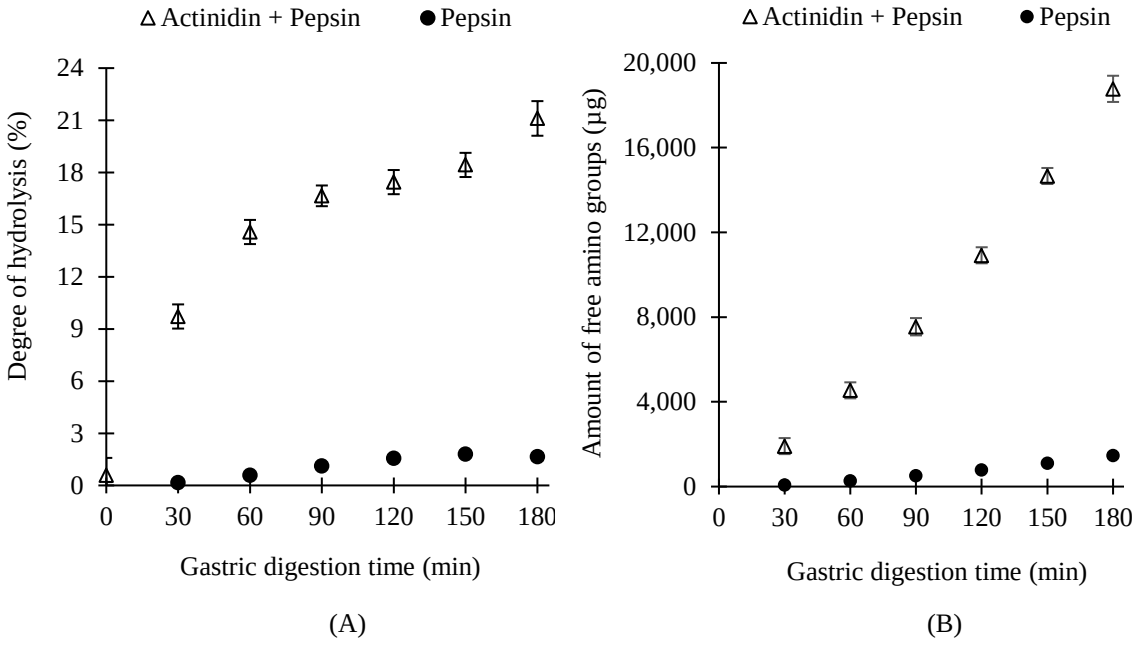
Statistical analyses were performed using SAS (SAS/STAT version 9.4). A polynomial regression analysis (up to quintic order) was first conducted on each main factor (i.e., treatment and time) and their interaction. Only significant ($P < 0.05$) terms were kept in the final polynomial model, which was selected by comparing full models with reduced models (i.e., removing predictors that did not affect the response variable) by using the log-likelihood ratio test. The selected polynomial model was then compared with a selected model with time as a categorical variable using the log-likelihood ratio test. For all the analysed responses, except the calculated number of residual R5 gluten epitopes present in the small intestine, the final best model had time as a numerical covariate.

The model diagnostics (e.g., normal distribution) for each response variable were tested using the ODS Graphics procedure and the repeated statement of SAS. A correlation analysis was conducted between the gastric digestion alone and small intestinal digestion alone of gluten with actinidin using the average values obtained at each digestion time. Similarly, a correlation analysis was conducted between gastric digestion and the respective small intestinal digestion time.

## 4.4 Results

### 4.4.1 Digestion of gluten

When only the gastric phase was considered, there was a significant interaction ($P < 0.001$) between treatment (digestion with or without actinidin) and the gastric digestion time for DH of gluten (Figure 4.2A). The DH of gluten increased in the presence of actinidin at a rate of 0.25%/min ($P < 0.05$) during the first 60 min of gastric digestion, whereas without actinidin, digestion was negligible ($P > 0.05$). After 60 min, the rate of increase in DH of gluten with actinidin was reduced to 0.05%/min on average until 180 min. Throughout the whole gastric digestion (180 min), the rate of DH of gluten without actinidin was 0.01%/min. There was a significant interaction ($P < 0.01$) between treatment and gastric digestion time for the amount of free amino groups released into the small intestine (Figure 4.2B). The rate of free amino groups released into the small intestine at 60 min gastric digestion was calculated to be ~265 µg/min with actinidin and it was only ~14 µg/min without actinidin.

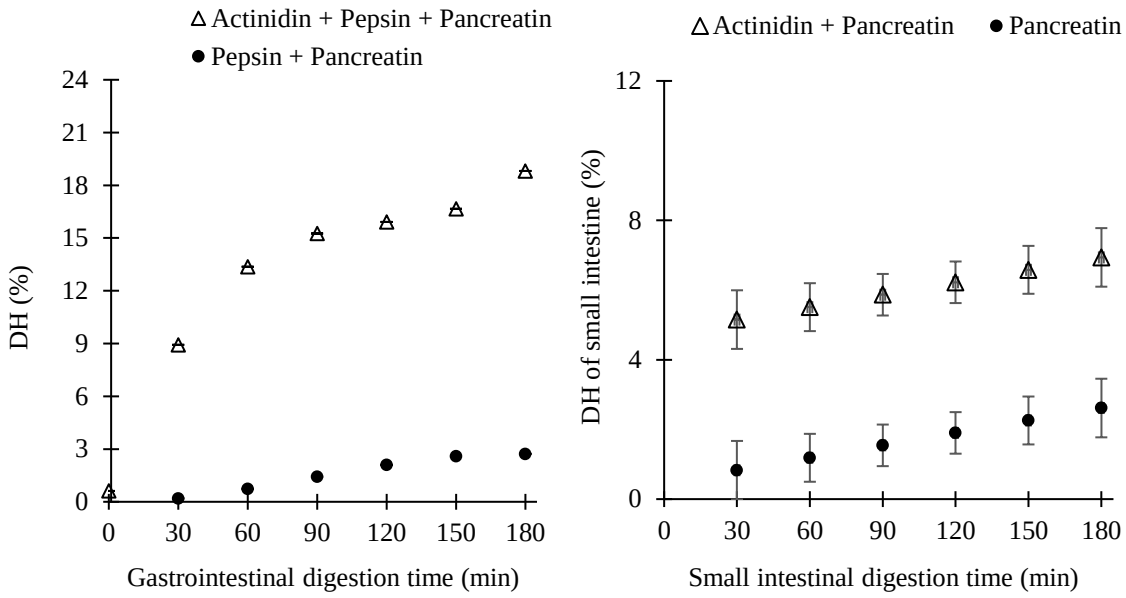



(A) (B)

**Figure 4.2** Degree of hydrolysis of gluten as measured by free amino groups (A) and calculated amount of free amino groups released (cumulative) into the small intestine (B) after *in vitro* gastric digestion with and without actinidin.

For the degree of hydrolysis, the P-values for the enzyme, time, and their interaction effects were 0.555, 0.093C, and 0.001C respectively, while for the number of free amino groups released into the small intestine the P-values were 0.67, 0.01Q, and 0.04Q respectively. C or Q, cubic or quadratic effect for the time factor. Values are means ± SEMs, n = 3.

A similar pattern to the gastric phase was observed when the whole semi-dynamic GIT digestion of gluten is considered. For instance, the rate of DH of gluten during the first 60 min of GIT with actinidin was 0.22%/min, whereas it was only 0.01%/min without actinidin (Figure 4.3A). The effect of actinidin on the small intestinal digestion of gluten was also evaluated using the samples that underwent only small intestinal digestion with and without actinidin. The DH was 5.6-fold higher after 60 min of small intestinal digestion with actinidin compared to the control. The rate of DH was 0.15%/min and 0.02%/min during the first 30 min of digestion and thereafter the rate increased only 0.01%/min on average up to 180 min for both with and without actinidin respectively (Figure 4.3B).

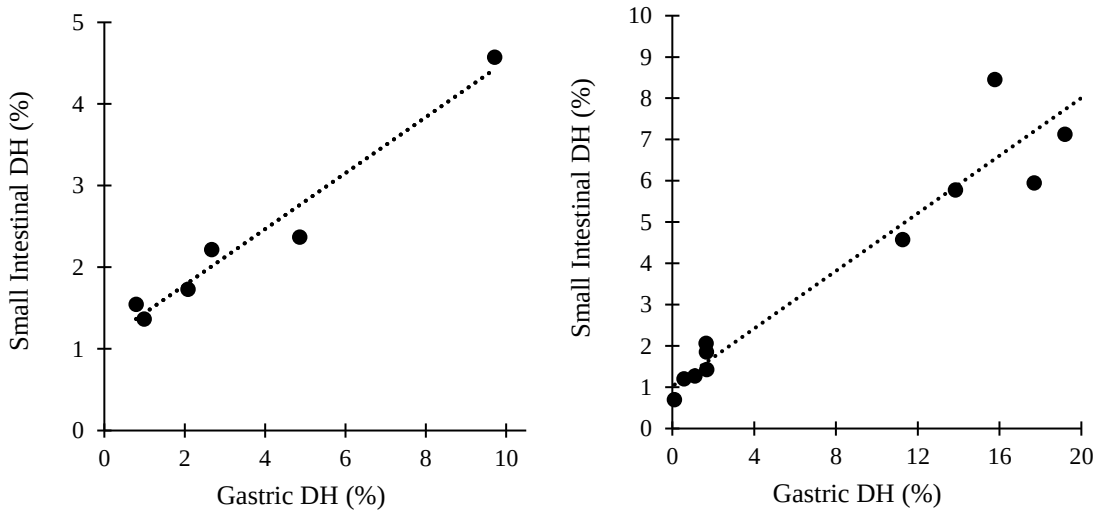



(A) (B)

**Figure 4.3** Degree of hydrolysis (DH) of gluten during *in vitro* gastrointestinal digestion (i.e., whole gastric and small intestinal DH together at each gastrointestinal digestion time) (A) and DH of gluten during *in vitro* small intestinal digestion of gluten (i.e., sample with no gastric digestion, but only small intestinal digestion) (B) as measured by free amino groups with and without actinidin.

For the DH during gastrointestinal digestion, the P-values for the enzyme, time, and their interaction effects were 0.523, 0.330C, and 0.001C respectively, while for the small intestinal digestion the P-value for the enzyme, time and their interaction effects were < 0.001, < 0.0001L, and < 0.0001L respectively. L or C, linear or cubic effect for the time factor. Values are means ± SEMs, n = 3.

There was a significant positive correlation between the DH of gastric digestion alone (pepsin + actinidin) and small intestinal digestion alone (pancreatin + actinidin) of gluten with actinidin r = 0.981 (P < 0.001, n = 6 values are the average values at the different time points) (Figure 4.4A). A positive significant correlation (0.935; P < 0.001, n=12) was also observed between gastric digestion and the respective subsequent small intestinal digestion time of gluten with and without actinidin (Figure 4.4B).




(A) (B)

**Figure 4.4** Correlation between gastric alone (pepsin + actinidin) and small intestinal alone (pancreatin + actinidin) degree of hydrolysis (DH) of gluten (A) and correlation between the gastric and subsequent small intestinal DH (pepsin + pancreatin alone and ± actinidin) of gluten.

Correlation and P-value for the gastric and small intestinal DH alone of gluten with actinidin were r = 0.962 and < 0.001 respectively (values (n=6) are the average values at the different time points). Correlation and P-value for the DH of gluten between gastric digestion and the respective subsequent small intestinal digestion time were r = 0.935 and P < 0.001 respectively (values (n=12) are the average values at the different time points).

### 4.4.2 Amount of residual R5 gluten epitopes in the GIT

There was a treatment and digestion time interaction effect (P < 0.001) on the amount of gluten R5 epitopes during both gastric (Figure 4.5A) and GIT digestion.

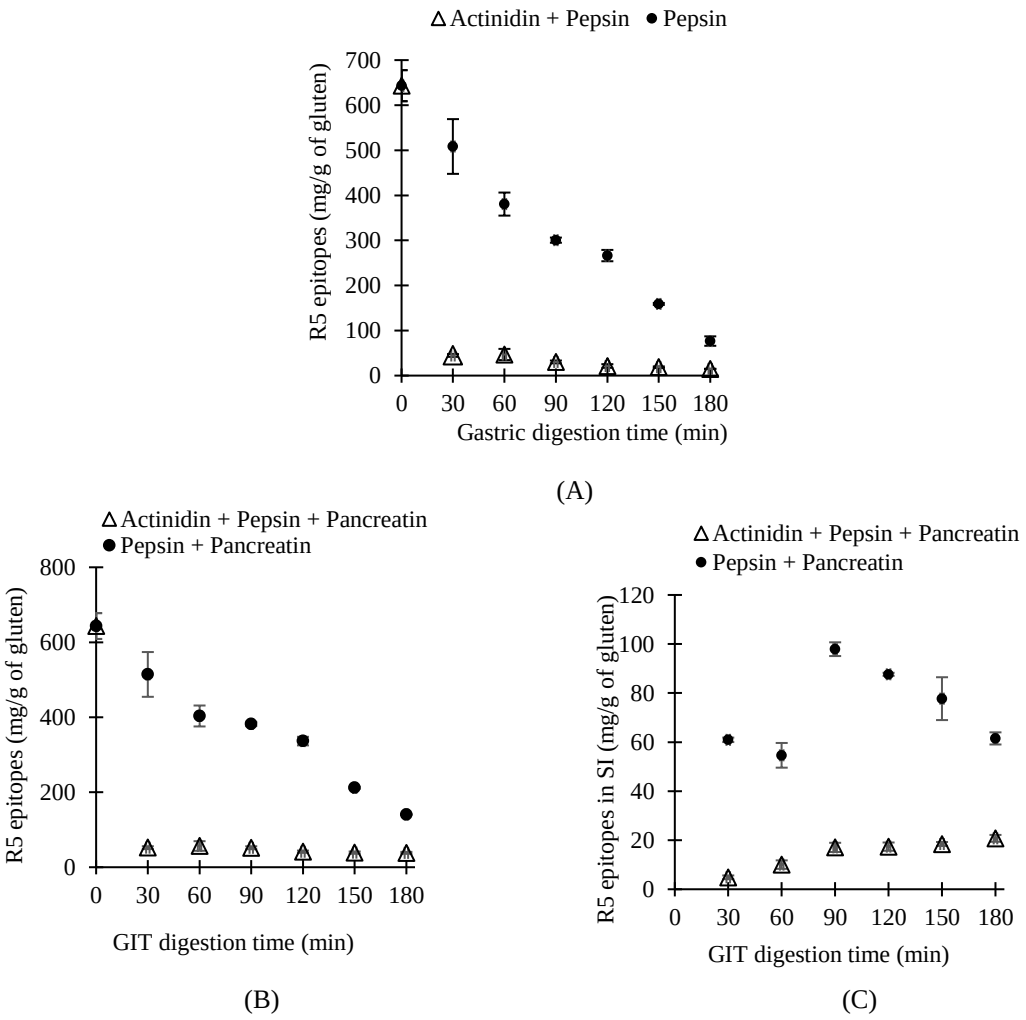



**Figure 4.5** Amount of residual R5 gluten epitopes present in the stomach during *in vitro* gastric digestion (A) and amount of residual R5 gluten epitopes present in the gastrointestinal tract (GIT) (i.e., whole gastric and small intestinal R5 epitopes together at each gastrointestinal digestion time) (B) and calculated R5 epitopes present in the small intestine (SI) (i.e., R5 epitopes entering the small intestine and R5 epitopes in the small intestine at each GIT digestion time) (C) during *in vitro* GIT digestion with and without actinidin as measured with competitive enzyme-linked immunosorbent assay.

For the amount of residual R5 gluten epitopes present in the gastric digestion, the P-values for the enzyme, time and their interaction effects were 0. 0.55, 0.09Q and < 0.001C while for GIT, they were 0.991, 0.0241Q1, and < 0.001Q1, and for the calculated residual R5 epitopes in the SI, they were < 0.001, < 0.001L, and < 0.001L respectively. C, Q, Q1 or L, cubic, quadratic, quintic, or linear effect for the time factor. Values are means ± SEMs, n = 3.

For example, the amount of epitopes in the GIT reduced 10-fold ($P < 0.05$) during the first 30 min of digestion with actinidin and then remained constant ($P > 0.05$) throughout the GIT digestion (Figure 4.5B). In the control, the amount of R5 epitopes was reduced by 2.8 mg/min throughout the GIT digestion, and at 180 min it remained 3.6-fold higher than the actinidin treatment.

The calculated amount of R5 epitopes in the small intestine (sum of R5 epitopes both released from the stomach and already present in the small intestine at a particular time point) at different time points was lower ($P < 0.001$) when actinidin was included in the digestion (Figure 4.5C). For instance, the calculated amount of R5 epitopes in the small intestine at 30 min was 12-fold lower with actinidin compared to the control. A rapid increase in the calculated amount of R5 epitopes was observed during 60 to 90 min of digestion in the control, whereas with actinidin, there was a slight increase during this time. After 90 min, the calculated amount of R5 epitopes in the small intestine remained constant when actinidin was present.

### 4.4.3 Immunogenic gluten peptides in the small intestine

The number of different immunogenic gluten peptides identified in the small intestine with actinidin was lower compared to the control throughout the GIT digestion (Table 4.3). For instance, the number of identified immunogenic gluten peptides present in the small intestine at 30 min was 1.5-fold lower when actinidin was present compared to the control. More than 50% of the immunogenic peptides were α-gliadin-derived epitopes. The full immunogenic peptide profiles in the stomach and small intestine during GIT digestion are presented in the google drive folder: https://drive.google.com/drive/folders/1JPm_499OsfVS1LNsPYUxb_0yKTHRedSd?usp=sharing.

**Table 4.3.** Number of immunogenic gluten peptides present in the small intestine* calculated from untargeted LC-MS/MS after gastrointestinal digestion with and without actinidin[1].

| | Without actinidin | With actinidin |
|---|---|---|
| 30 min | 63 | 40 |
| 60 min | 64 | 54 |
| 90 min | 112 | 64 |
| 120 min | 81 | 49 |

[1]Peptides were identified across the three replicates; * R5 epitopes entering the small intestine through gastric emptying and identified in the small intestine at each GIT digestion time during *in vitro* gastrointestinal digestion

### 4.5 Discussion

The results of this study revealed that the gastric digestion of gluten proteins was strongly accelerated by actinidin. Actinidin reduced the amounts of immunogenic peptides in the stomach and thereby reduced the amount of immunogenic peptides reaching the small intestine.

In this study, the OPA method was used to determine the free amino groups in the digested samples. The OPA forms fluorescent moieties when combined with free α-amino groups in the presence of reduced sulfhydryl groups. However, OPA cannot determine whether the N-terminal side of proline has been cleaved since proline does not have a free α-amino group to react with OPA. Actinidin can cleave proline at the N-terminal side, but this was not observed with pepsin and trypsin (Jayawardana et al., 2021). Therefore, the DH of the samples treated with actinidin could be underestimated. Based on the whole amino acid composition of wheat (full amino acid profile is presented in Appendix 3), proline represented 11.7% of the total amino acids. Thus, the DH can be underestimated by up to 11.7% and therefore the DH reported in this study was an apparent DH.

### 4.5.1 Gastric digestion

Our previous *in vitro* study has also shown that actinidin can cleave immunogenic gluten peptides during gastric digestion (Jayawardana et al., 2021). However, that study was done using a static *in vitro* digestion model, which does not consider parameters such as gradual acidification in the stomach and the continual release of chyme to the small intestine (i.e., gastric emptying). In the present study, an *in vitro* semi-dynamic GIT model was used that considers those parameters. This model allowed us to calculate important information such as the amount and number of different types of immunogenic peptides released into the small intestinal lumen during GIT digestion. Gastric emptying data obtained in rats gavaged gluten diets with and without actinidin (Montoya & Hindmarsh et al., 2014) were used in setting up the model since this study gives information on how gastric emptying occurs with gluten in the presence and absence of actinidin. Our semi-dynamic model did not include any gastric contractions and secretions as some dynamic *in vitro* models do (Kong & Singh, 2010). This simple model mimicked important aspects of GIT digestion and it is a simpler alternative for research laboratories than fully dynamic models. The material entering the small intestine at different post-gastric digestion times has a different rate of digestion throughout the small intestine (Montoya & Cabrera et al., 2018). This model integrates gastric and small intestinal data to understand what is happening in the small intestine at each GIT digestion time, which is physiologically important. Similar semi-dynamic models have been used by other research groups (Mulet-Cabero et al., 2020; Iqbal et al., 2021), though the gastric secretions were not adapted in the present study.

The effect of actinidin on the gastric digestion of gluten was significant compared to pepsin alone throughout the *in vitro* gastric digestion. This effect was substantial during the first 30 min of gastric digestion and this could be due to the pH of the gastric

medium supporting the action of actinidin (Jayawardana et al., 2021) and the broad substrate specificity of actinidin (Storer & Ménard, 1996). This initial reaction rate could also be explained by Michaelis-Menten kinetics. Thus, the lower rate of increase of DH after 90 min of gastric digestion can be ascribed to a reduction in the number of peptide bonds that actinidin can cleave (i.e., a decrease in the concentration of substrate available). The lower rate of DH after 90 min of digestion could also be attributed to the inactivation of actinidin. It has been observed *in vitro* that actinidin lost its catalytic activity after 90 min of gastric digestion when bread was digested with actinidin (Jayawardana et al., unpublished data). After 150 min of gastric digestion, a slight increase in the DH of gluten was observed, and this increase could be due to the action of pepsin on gluten under the low pH (~3.5) of the medium.

Corresponding to a higher rate of gastric DH during the first 30 min, actinidin cleaved R5 epitopes (core epitope sequence is QQQ/PFP and present in gluten epitope QQPFP and related sequences such as QQQFP, LQPFP, and QLPFP (Kahlenberg et al., 2006)) during the first 30 min of gastric digestion. Actinidin is able to hydrolyse the peptide bonds Q-P, F-P, P-Q, P-Y, P-F, and P-X-P (Jayawardana et al., 2021), which highlights the hydrolysis of R5 epitopes. After 30 min, the R5 epitopes remained constant throughout the gastric digestion which means the effect of actinidin on R5 epitopes was substantial only during the first 30 min of digestion. Similarly, the number of immunogenic peptides present in the stomach was substantially lower with actinidin during the first 30 min of gastric digestion and remained lower throughout the gastric digestion (Figure 4.5A).

### 4.5.2 Small intestinal digestion

For actinidin, when only small intestinal digestion of gluten was conducted, the DH was lower compared to the DH observed under gastric conditions. This could be due to the lower activity of actinidin since the upper limit of the actinidin activity falls around pH 7. Unlike pepsin, pancreatic enzymes can partially hydrolyse actinidin at pH ~7.0 (Bublin et al., 2008), which could also explain the lower DH determined during small intestinal digestion. However, it was reported in an *in vitro* study that a considerable amount of actinidin retained its primary structure and proteolytic activity after 2 h of simulated gastric digestion, followed by 2 h of intestinal digestion, as assessed qualitatively by gel-electrophoresis, zymography and mass spectroscopy (Grozdanovic et al., 2014). The significant positive correlation between gastric digestion alone (pepsin + actinidin) and small intestinal digestion alone (pancreatin + actinidin) suggests that the digestion of gluten under small intestinal conditions can also be explained by factors such as those described above for gastric digestion (e.g., a reduction in the number of peptide bonds that actinidin can cleave).

A significant positive correlation between the gastric and subsequent small intestinal digestion (pepsin + pancreatin alone and ± actinidin) of gluten was observed in this study as has been reported by others after *in vitro* digestion (Torres et al., 2016). It is expected that pepsin cleaves specific peptide bonds increasing the surface of exposure of the protein to pancreatic enzymes. Surprisingly, this correlation still holds when only the result of the actinidin treatment is considered ($r=0.55$; $P < 0.05$; $n=6$) despite that actinidin cleaved a greater number of peptide bonds during gastric digestion when compared with pepsin alone. This is mainly explained by the broad substrate specificity of actinidin, allowing it to cleave peptide bonds during gastric digestion that cannot be hydrolysed by pancreatic proteases (e.g., proline-containing peptide bonds).

### 4.5.3 Physiological relevance of rapid gluten digestion

Both the amount of immunogenic peptides released into the small intestine and the time of exposure of these immunogenic peptides to the small intestinal epithelium are important factors responsible for triggering an immune response in those subjects with gluten-related disorders (Hausch et al., 2002). In the present study, it was observed that actinidin was able to substantially reduce the concentration of R5 epitopes that would be released into the small intestine during the first 30 min of GIT digestion, and this concentration remained low during subsequent digestion. This reduction was mainly explained by the higher DH of material calculated to enter the small intestine in the presence of actinidin. To be specific, during gastric digestion, actinidin was able to hydrolyse R5 epitopes rapidly reducing the amount of R5 epitopes calculated to enter the small intestine. Untargeted mass spectrometry data also suggested that actinidin reduced the number of immunogenic peptides present in the small intestine at 30 min of digestion and this was observed throughout the digestion period. During small intestinal digestion alone, actinidin hydrolysed R5 epitopes (data not shown), but at a lower rate than in gastric digestion.

The small intestinal digestion of R5 epitopes by actinidin is physiologically important for the gluten that could be released into the small intestine during the first postprandial minutes since gastric emptying can occur soon after consumption of a meal (Malagelada et al., 1976). Consequently, actinidin could reduce both the number of immunogenic peptides present in the small intestinal lumen and the time of exposure of the small intestinal epithelium to those immunogenic gluten peptides. These results rely on an *in vitro* digestion model, which is unable to replicate the complex processes within the human GIT and was conducted using purified gluten. The effect of actinidin on the digestion of purified gluten is expected to be affected when the gluten is present

within a food matrix, as the food matrix would be expected to reduce the contact between actinidin and peptide bonds. Thus, in this promising scenario, more work is warranted with gluten-containing model foods (e.g., bread or pasta) using *in vivo* digestion models to assess the effect of actinidin on hydrolysing immunogenic gluten peptides and gluten immunogenicity, prior to conducting a clinical study.

### 4.6 Conclusion

The findings of the present study have shown that actinidin is able to rapidly hydrolyse gluten and its immunogenic peptides during simulated *in vitro* gastric digestion, reducing the number and amount of immunogenic peptides calculated to enter the small intestine. Some of the immunogenic peptides entering the small intestine were also rapidly hydrolysed by actinidin. Further work to investigate the effect of actinidin in gluten-containing foods *in vivo* is warranted.

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# Chapter Five

# 5. Hydrolysis of wheat proteins and gluten immunogenic peptides in bread by actinidin in an *in vitro* semi-dynamic gastrointestinal digestion model

This chapter has been submitted to Food and Function:

Jayawardana, I. A., Boland, M. J., Loo, T., McNabb, W. C., & Montoya, C. A. (Submitted to Food & Function). Rapid proteolysis of gluten-derived immunogenic peptides in bread by actinidin in an *in vitro* semi-dynamic gastrointestinal digestion model.

**Highlights**

- Actinidin was stable against both the gastric pH and pepsin proteolysis under physiologically relevant conditions up to 90 min.
- Action of actinidin was rapid to hydrolyse wheat proteins present in a food matrix during simulated gastric conditions.
- Actinidin decreased the occurrence of immunogenic peptides in the small intestine in a normal gluten-containing diet.

## 5.1 Abstract

This study aimed to determine the ability of actinidin, a cysteine protease in green kiwifruit (*Actinidia deliciosa*), to hydrolyse wheat proteins and gluten-derived immunogenic peptides from a commonly consumed food matrix (bread) using an *in vitro* semi-dynamic gastrointestinal tract (GIT) model. A chewed and spat composite bolus of bread was digested with or without purified actinidin using a human gastric simulator. Gastric digestion was conducted for 150 min with gastric emptying occurring at different time points. Emptied samples were immediately digested under small intestinal conditions. Gastric and small intestinal aliquots were collected to quantify peptide profiles and nine marker immunogenic peptides (untargeted and targeted mass spectrometry, respectively), R5 epitopes (by monoclonal antibody-based competition assay), and free amino groups released by digestion (by o-phthaldialdehyde method). There was a significant effect ($P < 0.05$) of actinidin and digestion time on the hydrolysis of wheat proteins and the amount of gluten R5 epitopes of that material emptying the human gastric simulator. Actinidin accelerated 1.2-fold the gastric hydrolysis of wheat proteins during the first 20 min of digestion, which was reflected in a faster (5.5 µg/min) reduction in the evolution of R5 epitopes. Actinidin accelerated ($P < 0.05$) the rate of disappearance of most of the immunogenic marker peptides. For example, in the first 20 min of small intestinal digestion, the 33-mer peptide decreased 2-fold faster (0.25 vs 0.12 µg/g of bread per min) in the presence of actinidin than in the control. Untargeted peptidomics showed actinidin markedly decreased the amounts of known immunogenic peptides in the small intestine. These findings demonstrated that actinidin accelerates the hydrolysis of wheat proteins and known gluten immunogenic peptides in a commonly consumed food matrix (bread) in an *in vitro* semi-dynamic GIT model.

**Keywords:** Actinidin, Bread, Human gastric simulator, Gastric and small intestinal digestion, Gluten immunogenic peptides

## 5.2 Introduction

Gluten-containing cereals (wheat, barley, rye, and oats) are globally consumed in different food formats (e.g., bread, pasta, roti, beer, etc.). Bread is the most commonly consumed gluten-containing food. For instance, the average daily intake of bread in Europe in 2013 was 59.4 g per person (Eglite & Kunkulberga, 2017). Consumption of gluten is sometimes associated with immune responses such as coeliac disease, gluten sensitivity, and wheat allergy in genetically predisposed or otherwise vulnerable individuals. Gluten proteins are highly resistant to gastrointestinal proteases due to their proline- and glutamine-rich peptide sequences, which result in the accumulation of indigestible peptides, many of which are immunogenic, in the gastrointestinal tract (GIT) (Jayawardana et al., 2019). These immunogenic peptides can trigger immune reactions in the small intestine of predisposed individuals (Hausch et al., 2002; Piper et al., 2004). The poor gastric digestion of gluten proteins and their short residence time in the stomach (Montoya & Hindmarsh et al., 2014) may be responsible for the accumulation of the indigestible peptides in the small intestine. This could be alleviated by using exogenous proteases to rapidly hydrolyse gluten proteins in the stomach (M'hir et al., 2012). It is expected that greater gastric digestion of gluten by exogenous enzymes (like actinidin from kiwifruit) will improve the digestion of gluten peptides directly in the small intestine or by rendering them more accessible to pancreatic and brush-border enzymes. Some brush-border enzymes (dipeptidylcarboxypeptidase and dipeptidylaminopeptidase) can hydrolyse proline-containing peptide bonds (Gray,

2010; Hausch et al., 2002), which in turn decreases the accumulation of these peptides in the small intestine.

Actinidin, a cysteine protease in green kiwifruit (*Actinidia deliciosa*), has been identified previously as a potential exogenous enzyme that can rapidly hydrolyse gluten and its peptides in the stomach and small intestine due to its broad substrate specificity (Jayawardana et al., 2021; Jayawardana et al. unpublished data). For instance, the degree of hydrolysis of gluten increased during the first 30 min of *in vitro* GIT digestion at a rate of 0.3%/min with actinidin, whereas it was only 0.01%/min without actinidin. The former led to a 9.6-fold decrease in gluten R5 epitopes (QQPFP, QQQFP, LQPFP, and QLPFP) during the first 30 min of GIT digestion with actinidin. These results were obtained when purified gluten was digested with actinidin. However, the ability of actinidin to digest gluten is expected to be affected when gluten is within a food matrix. Nutrients such as carbohydrates, fats, other proteins, and their interactions in gluten-containing foods could create a specific matrix that can limit the access of actinidin to gluten proteins (Mat et al., 2016). We hypothesise that actinidin can effectively hydrolyse gluten-derived peptides, including those that are immunogenic, present in food matrices such as bread.

The aim of the present study was to evaluate the efficacy of actinidin to digest wheat gluten proteins and gluten peptides in a commonly consumed food model (whole bread) using a semi-dynamic *in vitro* GIT digestion model. In the present study, oral digestion of bread was conducted using a human panel. To simulate gastric digestion, a human gastric simulator (HGS) (Ferrua & Singh, 2015) was used, which mimicked *in vitro* dynamic parameters of gastric digestion such as reduction of pH, temperature, gastric secretions, peristaltic mixing, and gastric emptying, while a static digestion model was used to simulate small intestinal digestion.

## 5.3 Materials and methods

### 5.3.1 Materials

Whole-wheat flour (*Triticum aestivum* cv. "VICEROY") was purchased from Plant and Food Research Institute, Christchurch, New Zealand. Pepsin (from porcine gastric mucosa, P6887, lyophilized powder, 3,200-4,500 units/mg protein), pancreatin from porcine pancreas (P7545; activity x 8 USP), Tris (2-carboxyethyl)-phosphine [TCEP] (CAS 51805-45-9), N-lauroylsarcosine (CAS 51805-45-9), N-α-CBZ-lys-p-nitrophenol hydrochloride (CAS 2179-15-9), phosphate-buffered saline (PBS), DL-dithioerythritol (DTT) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Synthetic peptides including internal standard peptides were purchased from New England Peptide Inc. (Gardner, Massachusetts, USA). Purified actinidin was purchased from New Zealand Pharmaceuticals (Palmerston North, New Zealand). The ELISA kits (RIDASCREEN® Gliadin competitive) were purchased from R-Biopharm AG (Darmstadt, Germany).

### 5.3.2 Preparation of soda bread

Soda bread was used in the study to ensure all proteins in the bread were from wheat only. To make soda bread, whole-wheat flour (*Triticum aestivum* cv. "VICEROY") was used. Soda bread was prepared using a standard recipe: 400 mL water, 5.0 g table salt, 30 g baking soda, 40.0 g vegetable oil, and 600 g whole-wheat flour. The bread was prepared using the yeast-free option in the bread maker (Model BB380, Breville, Melbourne, Australia). After baking, the bread was immediately removed and allowed to cool for 1 h at room temperature (23 °C). The bread was sliced and placed in sealed plastic bags to minimize moisture loss. The bread was used in the experiments within 24 h of preparation.

### 5.3.3 *In vivo* oral digestion of bread

Ethical approval was obtained from the Human Ethics Committee, Massey University, Palmerston North, New Zealand (No:4000019772). A group of volunteers (n=6, age 25 – 35 years), who do not have a history of gluten-related health disorders were selected. Each individual was asked to chew two slices of bread normally and at the stage of swallowing, to expectorate the bolus onto a container. A composite sample was prepared by combining the boluses obtained from each subject. This procedure was repeated three times and each composite was used as a replicate for the *in vitro* digestion. Before each replicate, the composite bread was divided into two parts. The first part was used directly in the first digestion, while the second part of the composite was kept at 4 °C before being used in the second digestion. For each *in vitro* digestion, 160 g of the chewed composite bread representing 4 slices of bread was used either alone (control) or with actinidin, as described below. The order of the digestion was randomly allocated to the control or actinidin treatment on the same day.

### 5.3.4 *In vitro* gastric digestion of bread

The *in vitro* gastric digestion was conducted in an HGS. All the solutions required for the digestion were prepared according to the INFOGEST protocol (Minekus et al., 2014). The composite bread (160 g) alone (control) or with purified actinidin were added to the HGS, which contained 25 mL of simulated gastric fluid at pH 1.7 with a pepsin concentration of 27 U/mL, to mimic the conditions of a fasted stomach (Ong, Palahniuk, & Cumming, 1978). For the actinidin treatment, purified actinidin powder (3 U/mg actinidin activity) was thoroughly mixed into the composite bread to get an actinidin activity equivalent to the enzyme activity in two green kiwifruit (Montoya & Rutherfurd et al., 2014).

*Gastric pH and pepsin:* The gastric pH profile used in this study was based on pH data measured in the fundus of eight subjects fed a bread meal (Hoebler et al., 2002). The pH of the composite bread bolus after oral digestion was taken as the initial pH of the masticated bread added into the HGS. A solution of 0.2 M HCl (1.1 pH) was continuously added (inflow 1) into the HGS at different flow rates that changed every 30 min to maintain the pH profile throughout the gastric digestion. Pepsin-containing simulated gastric fluid (pH 6.9) was continuously added (inflow 2) into the HGS at different flow rates that changed each 30 min to simulate the gastric secretions and to maintain a pepsin concentration of 2,000 U/mL of the gastric medium throughout the digestion (Minekus et al., 2014). A preliminary test was conducted with the composite bread to determine the flow rates of HCl and pepsin containing simulated gastric fluid to maintain the required pH and to maintain 2,000 U/mL pepsin concentration respectively. The two inflows (Figure 5.1) mimicked the gastric secretions and dilution of food in the human stomach fed a solid meal (Malagelada et al., 1976).

*Gastric emptying:* Gastric emptying data reported elsewhere obtained in humans fed a solid bread-based diet (Malagelada, 1977; Malagelada et al., 1976) was used to determine the material removed from the HGS at 10, 20, 30, 60, 90, 120, and 150 min. To simulate the sieving effect of the pylorus, a thin polyester net bag (pore size 1.5 mm) was placed inside the bottom of the HGS to let small particles (< 1 - 2 mm) pass through the mesh for emptying. Aliquots (20 mL) of samples exiting the HGS were simultaneously and immediately allocated for simulated small intestinal digestion. Other emptying aliquots (2 and 4 mL) were taken to determine actinidin activity or boiled for 3 min for later determination of free amino groups, R5 epitopes and immunogenic peptides. Aliquots were stored at -80 °C (actinidin activity) or -20 °C (free amino groups, epitopes, and peptides) until analysis.

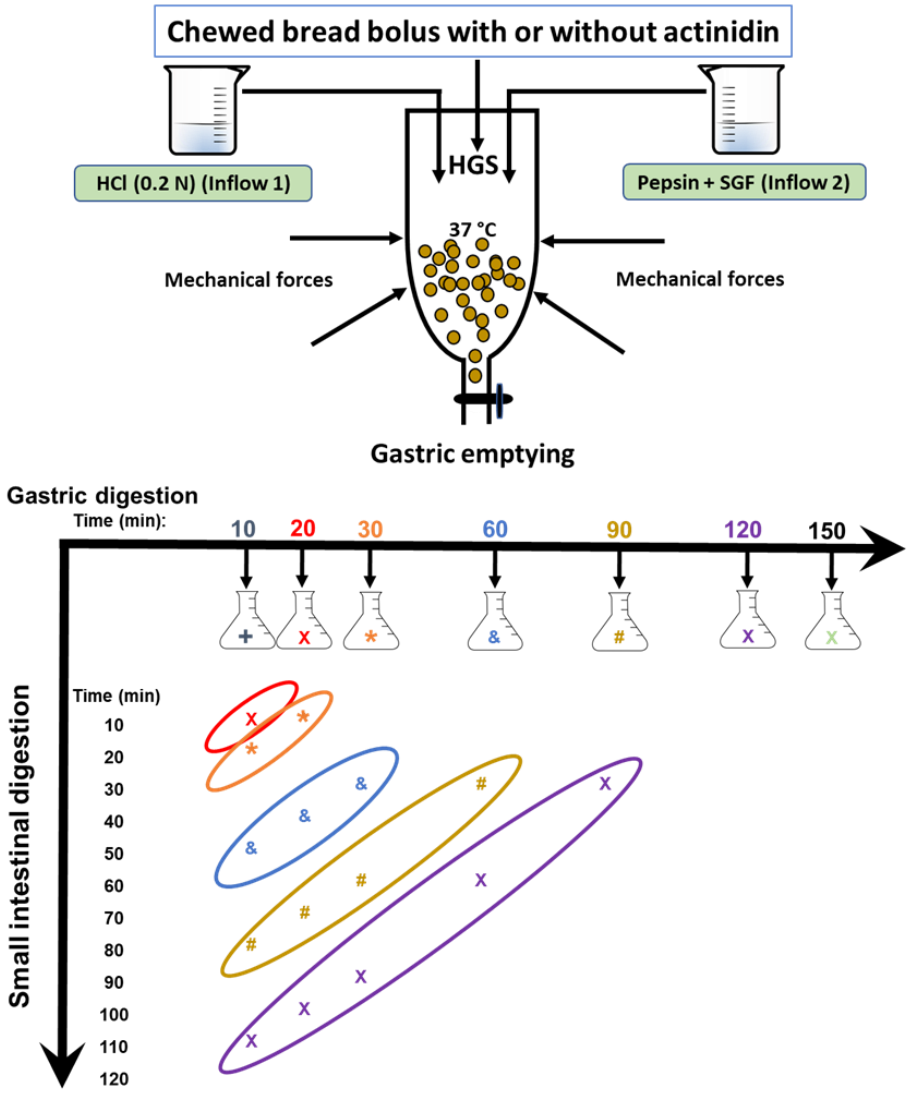



**Figure 5.1** Graphical illustration of dynamic gastrointestinal digestion and gastric emptying using the human gastric simulator (HGS). Collection of gastric and small intestinal samples (denoted by symbols) during continued dynamic *in vitro* gastrointestinal digestion.

A single colour symbol represents the samples that belong to a particular gastrointestinal digestion time. For instance, the 60 min sample in the small intestine (in blue colour) = (10 min gastric + 50 min small intestinal digested sample) + (20 min gastric + 40 min small intestinal digested sample) + (30 min gastric + 30 min small intestinal digested sample). SGF; Simulated gastric fluid.

### 5.3.5 *In vitro* small intestinal digestion of bread

A static model was used to simulate the small intestinal phase. Gastric emptying samples up to 120 min (20 mL) were mixed in a 50 mL Falcon tube with a simulated intestinal fluid (pH 7.0) (1:1 v/v) containing pancreatin (100 U/mL of trypsin activity in the final mixture) (Minekus et al., 2014). The samples were kept in a shaking incubator (20 turns/min) at 37 °C up to 120 min. Aliquots (4 mL) were collected at different time points to represent the gastrointestinal digestion at 0, 10, 20, 30, 60, 90, and 120 min (Figure 5.1). The aliquots were boiled and stored at -20 °C until analysis.

### 5.3.6 Determination of dry matter and crude protein

Chewed bread samples, material entering the small intestine and small intestinal digested samples were and analysed for dry matter in duplicate (oven-dry at 105 (AOAC 930.15)) and soda bread was analysed for crude protein in triplicate (nitrogen x 6.25; Kjeldahl method; AOAC 991.20).

### 5.3.7 Extraction of gluten proteins and peptides from digested samples

Gluten proteins and peptides in the digested sample were extracted using a solution (universal prolamin and glutelin extractant (UPEX) solution) containing Tris (2-carboxyethyl)-phosphine and N-lauroylsarcosine reagents in PBS (Mena et al., 2012). These extracted samples were used for the o-phthaldialdehyde (OPA), and competitive enzyme-linked immunosorbent assay (ELISA) described below. It is expected that other wheat proteins (albumins and globulins) were also extracted as they are soluble.

### 5.3.8 Determination of free amino groups

The UPEX extracted samples were used to determine the free amino groups present in the gastric and small intestinal digested samples using the OPA method described previously (Church et al., 1983; Torres et al., 2016). Briefly, a 0.025 mL of 20% sodium dodecyl sulphate, 2 µL of β-mercaptoethanol and 0.02 mL of methanol-OPA (40 mg of OPA in 1 mL of methanol) were mixed with 953 µL of 0.1 M sodium tetraborate decahydrate solution (pH = 9.3) just before the analysis. The OPA assay was carried out by the addition of 50 µL of the sample or the standard to 1 mL of OPA solution. The mixture was mixed and allowed to stand for 2 min before measuring the absorbance at 340 nm with a UV-VIS spectrophotometer (GENESYS™, Thermo Fisher Scientific, Waltham, MA, USA). A standard curve was prepared using glycine (0 - 500 µg/mL).

### 5.3.9 Determination of the quantity of residual R5 gluten epitopes

A commercial competitive ELISA was used to quantify the presence of R5 gluten epitopes (QQPFP, QQQFP, LQPFP, and QLPFP), which are common gluten motifs derived from the digestion of gluten proteins present in soda bread. The UPEX extracted samples were analysed in duplicate following the manufacturer's instructions.

### 5.3.10 Untargeted and targeted Liquid Chromatography-Tandem Mass Spectrometry (LC/MS-MS) for peptides and immunogenic peptides, respectively

*Sample Preparation:* Digested samples were defrosted, and 200 mg of each sample was weighed into separate tubes. A 100 µL of trypsin (0.1 mg/mL) in 100 mM ammonium bicarbonate (pH 7.5) was added into each tube, vortexed and incubated overnight at 37 °C. This step was added to release cryptic peptides in the bread matrix. Pure methanol (700 µL) and an isotopically-labelled internal standard, P1H (l-Phenylalanine-$^{13}C_9$,$^{15}N$

at N terminal residue six) at 3 ppm were added into each tube. Samples were vortexed and incubated at -20 °C for 30 min, then centrifuged at 13,300 rpm for 15 min. The supernatants (900 µL) were purified using polymeric reversed-phase solid extraction (SPE) Strata™-XL 100 µm, 30 mg/mL cartridges (Phenomenex® Inc. Torrance, CA, USA) as follows. The cartridges were pre-equilibrated with 100% acetonitrile and water prior to being loaded with the supernatants. Once loaded, the cartridges were washed with 2 x 0.2 mL 40% methanol, followed by drying under full vacuum for 10 min. The target peptides were then eluted using 200 µL of 70% acetonitrile/2% formic acid. Samples were concentrated to 25 µL and reconstituted the contents to 50 µL with 5% acetonitrile/0.1% formic acid. Samples were vortexed and centrifuged at 13,300 rpm for 15 min and transferred to glass inserts for MS analysis.

*Untargeted LC-MS Analysis:* A few of the gastric digested bread samples exiting the HGS (10, 20 and 60 min digestion) and gastrointestinal digested samples representing 20 and 60 min of GIT digestion (Figure 5.1) with and without actinidin were pooled across replicates and analysed for their peptide profiles using discovery proteomics. The SPE-extracted samples described above were separated by online reversed-phase chromatography using a Dionex UltiMate™ RSLCnano system (ThermoFisher Scientific, Waltham, MA, USA) with reversed-phase C18 peptide trap and capillary analytical column. The liquid chromatography system was coupled online to a Q Exactive™ Plus mass spectrometer equipped with a higher-energy collision-induced dissociation (HCD) collision cell, an Orbitrap mass analyser and a Nanospray Flex™ ion source (ThermoFisher Scientific, Waltham, MA, USA). Data-dependent tandem MS acquisition method (Top10) was employed where the top ten most intense ions in each scan cycle were selected for fragmentation. In all experiments, MS1 scans were acquired over a mass range of 375-1,600 *m/z* with detection in the Orbitrap mass

analyser at a resolution setting of 70,000. The dd-$MS^2$ spectra produced via HCD were acquired with a resolution setting of 17,500. The column oven temperature was 50 °C and a gradient of 3-30% acetonitrile in 0.1% formic acid/water over 60 min and 30-90% acetonitrile over 15 min for regeneration was used. Ion exclusion conditions were optimized according to observed chromatographic peak width (typically 12s). The raw data files were searched using Proteome Discoverer™ version 2.4 search engine (ThermoFisher Scientific, Waltham, MA, USA). The following search parameters used matched the specifications of the Q Exactive instrument, and variables resulting from chemical treatment; The database was from NCBI (1 July 2021), taxonomy *Triticum aestivum*, proteins α-gliadin and glutenin with custom synthetic peptide sequences; enzyme specificity none; max number of missed cleavages 2; minimum peptide length 6; precursor mass tolerance 10 ppm; fragment mass tolerance 0.02 Da; static modification none; variable modifications included oxidation of methionine and N-terminal protein acetylation; target false discovery rate for peptide-spectrum matches and peptides was $\leq 0.01$. The validation node of choice was percolator and target decoy. Only for samples exiting the HGS, peptides having high confidence values and having at least 10 peptide spectrum matches were considered when identifying peptide profiles due to a higher number of peptides generated during *in vitro* gastric digestion. For small intestinal digested samples, all the peptides generated were considered. The immunogenic gluten peptides were recognized by the presence of at least one epitope within their primary peptide sequence using the online database ProPepper (Juhász et al., 2015).

*Targeted LC-MS Analysis:* Targeted MS was undertaken for nine synthetic marker immunogenic gluten peptides (Table 5.1). Seven of these peptides (P1 – P7) were selected based on a previous study (Ogilvie et al., 2020). The other two marker peptides

(P8 and P9) were identified from the untargeted proteomics experiment of the present study based on their common presence in gastric digested samples with and without actinidin.

A Q Exactive™ Focus (ThermoFisher Scientific, MA, USA) coupled to a Dionex UltiMate™ 3000 quaternary RS system (ThermoFisher Scientific, Waltham, MA, USA) was used with a HESI-II (ThermoFisher Scientific, Waltham, MA, USA) ionization source. Reverse-phase chromatography was conducted on an analytical column Phenomenex Aeris™ 2.6 µm PEPTIDE XB-C18 100 Å, 100 x 2.1 mm at 45 °C with a flow rate of 0.35 mL/min. Parallel reaction monitoring tandem MS acquisition method with an inclusion list of ions was used. Fragment ion spectra produced via HCD were acquired with a resolution setting of 35,000. The sample was injected onto the pre-equilibrated column, then eluted by buffer A (0.1% formic acid, 5 mM ammonium formate in water) and buffer B (0.1% formic acid, 5 mM ammonium formate in acetonitrile). Data were collected using spray voltage (4.0 kV), aux gas flow rate (8 L/min), sheath gas flow rate (40 L/min), scan range (3 *m/z),* capillary temperature (350 °C), and normalised collision energy (15). Details for the chromatographic and mass spectrometric settings are listed in Appendix 4, Tables 1-5. The peak areas from the total ion current of target peptides were integrated and analysed by Trace Finder version 4.1 SP5 (ThermoFisher Scientific, Waltham, MA USA). A standard curve for each peptide was established and was used to calculate concentrations of peptides in each unknown sample. Standard peptide concentrations were 0.05, 0.1, 0.5, 1, 5, 10, 50 and 100 ppm/µL and 1 µL was injected per run for 5 repeats. Unknown samples were run in triplicates and 5 µL was injected for each.

**Table 5.1** Parameters of marker peptides for quantitative mass spectrometry.

| Peptide ID | Amino acid sequence | Molecular weight (kDa) | Retention time (min) |
|---|---|---|---|
| P1 | LQLQPFPQPQLPY | 1.568 | 4.37 |
| P2 | LQLQPFPQPQLPYPQPQPF | 2.263 | 4.67 |
| P3 | LQLQPFPQPQLPYPQPHLPYPQPQPF | 3.096 | 4.58 |
| P4 | LQLQPFPQPQLPYPQPQLPYPQPQPF | 3.087 | 4.87 |
| P5 | LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF (33-mer) | 3.912 | 5.00 |
| P6 | RPQQPYPQPQPQY | 1.627 | 0.52 |
| P7 | RPQQPYPQSQPQY | 1.617 | 0.50 |
| P8 | FQPSQQNPQAQGSFQPQQLPQF | 2.529 | 2.48 |
| P9 | VRVPVPQLQPQNPSQQQPQEQVPLVQQQQF | 3.491 | 3.22 |
| P1H | LQLQPF*PQPQLPY | 1.578 | 4.37 |

Amino acid sequence denoted in one-letter code. *Isotopic label, l-Phenylalanine-$^{13}C_9$,$^{15}N$.

### 5.3.11 Actinidin activity assay

The actinidin activity was measured using the method described previously (Boland & Hardman, 1972). Actinidin activity of fresh green kiwifruit was measured to calculate the amount of actinidin powder required (equivalent to two fresh kiwifruit) to use in the experiments. Three randomly selected ready-to-eat green kiwifruit were taken and their pulp was separately extracted to measure the actinidin activity. The kiwifruit pulp from each fruit was manually homogenised at 4 °C. Then the pulp was filtered through a muslin cloth. The filtrate was centrifuged at 13,000 rpm for 30 min at 4 °C and the supernatant was collected. To activate the enzyme, 100 µL of the supernatant and 100 µL of 0.1 M DTT, the enzyme activator, were mixed 3 min before measuring the absorbance. The actinidin substrate, N-α-CBZ-lys-p-nitrophenol hydrochloride (CBZ)

was dissolved in Milli-Q water at a concentration of 1.2 g/L. A 4 mL cuvette was placed in the UV-Vis-spectrophotometer (GENESYS™, ThermoFisher Scientific, Waltham, MA, USA) and 2.85 mL of 0.05 M phosphate buffer (pH 6) and 100 µL CBZ were added. After 20 s, to determine the rate of spontaneous hydrolysis of CBZ, 50 µL of the sample mixture (sample and DTT) was added. The rate of change in absorbance was measured for 100 seconds at 348 nm to calculate the actinidin activity. The average actinidin activity of three kiwifruit was taken as the actinidin activity of fresh green kiwifruit. The same procedure was followed to measure the actinidin activity of purified actinidin powder and samples exiting the gastric digestion with actinidin. Purified actinidin powder was dissolved in 0.05 M phosphate buffer at a concentration of 1 mg/mL before the analysis. The samples exiting the HGS were thawed on ice and centrifuged at 13,000 rpm for 15 min at 4 °C and the supernatants were analysed for actinidin activity.

The actinidin activity was calculated using the following formula:

$$\text{Actinidin activity (U/mg)} = \frac{\Delta A348 \text{ test} - \Delta \text{ A } 348 \text{ blank } x \ 1000 \ x \ 3}{(5{,}400 \ x \ \text{X})}$$

$\Delta A_{348}$ : slope of the initial linear portion of the curve (unit absorbance/minute) for the test (with enzyme) and blank

5,400: molar extinction coefficient (L/ (mol *x* cm)) of CBZ at 348 nm

3: volume of reaction mix (mL)

X: quantity of actinidin in the final reaction mixture (in the cuvette) (mg).

### 5.3.12 Calculations

The amount of free amino groups in samples measured using the OPA method was used to calculate the amount of new free amino groups present in 1 g of bread at a particular time point of *in vitro* GIT digestion using the following formula:

$$\text{Free amino groups (mg/g bread)} = \frac{(\text{NH}_{2\text{ti}} - \text{NH}_{2\text{blank}})}{\text{Amount of bread (g)}}$$

$NH_{2ti}$ : amount of free amino groups at the time i of GIT digestion

$NH_{2blank}$ : free amino groups in the blank treatments (i.e., amino groups in both gluten prior to digestion with the digestive enzymes alone or with actinidin)

*In vitro* data obtained from the present study (free amino groups, R5 epitopes and quantity of immunogenic marker peptides) were combined with the gastric emptying rate at each time point (Appendix 4, Table 6) to calculate free amino groups, R5 epitopes and the nine marker immunogenic gluten peptides entering the small intestine at each GIT digestion time. All the calculations were done for 1 g of digested bread on a dry matter basis. An example showing the calculations for the amount of free amino groups present in the small intestine at 30 min with actinidin is presented in Appendix 5.

### 5.3.13 Statistical analysis

The statistical software SAS (SAS/STAT version 9.4) was used to analyse the data. A polynomial regression analysis (up to sextic order) was first conducted on each factor (i.e., treatment and time) and their interaction. Only significant ($P < 0.05$) terms were kept in the final polynomial model, which was selected by comparing full models with reduced models (i.e., removing predictors that did not affect the response variable) by

using the log-likelihood ratio test. The selected polynomial model was then compared with a selected model with time as a categorical variable using the log-likelihood ratio test. The best model with time as numerical or categorical variable is reported only. The model diagnostics (e.g., normal distribution) for each response variable were tested using the ODS Graphics procedure and the repeated statement of SAS.

### 5.4 Results

The soda bread had a dry matter content of 47.1 ± 1.6% and a crude protein content of 6.3 ± 0.3%. The pH of the chewed soda bread before digestion was 7.8 ± 0.18 and the pH dropped to 1.3 ± 0.20 and 1.5 ± 0.10 over 150 min of gastric digestion with and without actinidin, respectively (Figure 5.2A). The amount of gastric digested bread entering the small intestine was adjusted to the starting amount of bread used in the present study (68.6 g dry matter) (Figure 5.2B) based on the gastric emptying data obtained in humans fed a solid bread-based diet (Malagelada, 1977; Malagelada et al., 1976).

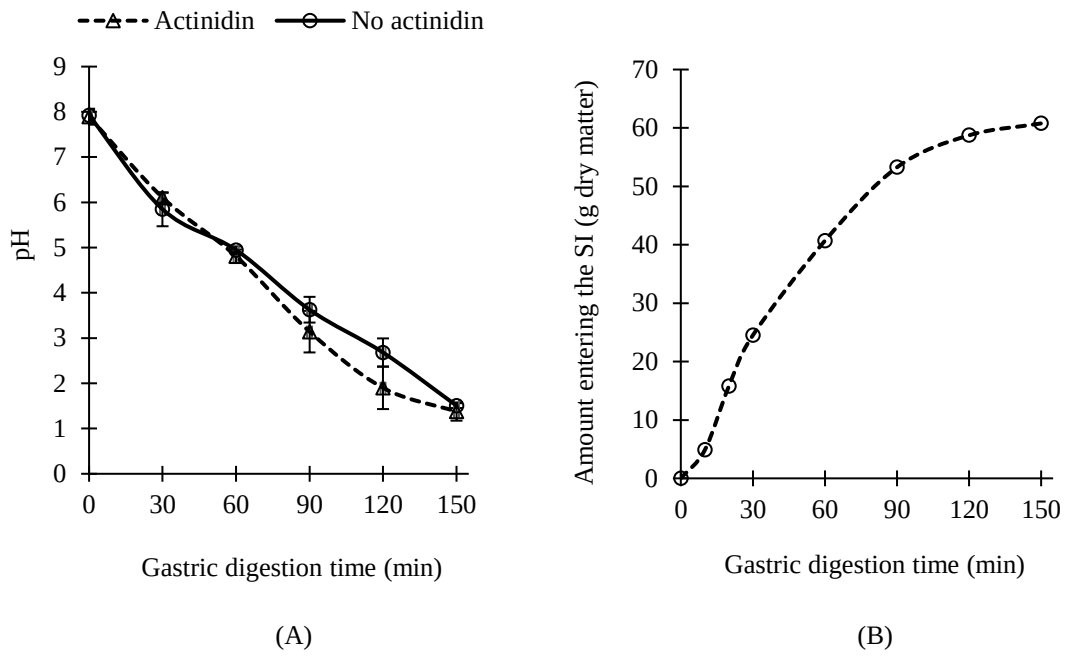



**Figure 5.2.** Mean pH measured in the *in vitro* gastric digested bread sample entering the small intestine with and without actinidin (A) and the cumulative gastric digested bread entering the small intestine (SI) (B) during 150 min of gastric digestion.

The values are means ± SEMs, n = 3.

### 5.4.1 Actinidin activity and pH of the samples exiting the gastric digestion

The actinidin activity of fresh green kiwifruit and purified actinidin powder was 15.0 ± 0.6 U/mL and 3.1 ± 0.34 U/mg, respectively. Actinidin remained active in the samples that left the stomach (i.e., entering the small intestine) after 90 min. The actinidin activity in digesta entering the small intestine after 10 min of gastric digestion was 2.7 ± 0.3 U/mL (Figure 5.3) and the value increased to 3.6 ± 0.1 U/mL at 20 min and then decreased over time. For example, at 60 min, the actinidin activity decreased by 50% (1.4 ± 0.06 U/mL), and there was no actinidin activity after 120 min of gastric digestion.

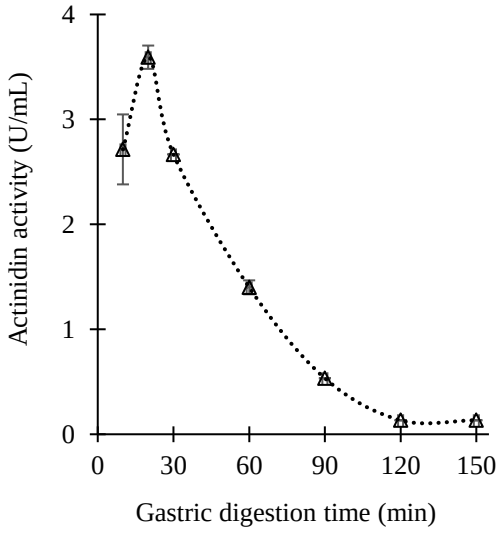
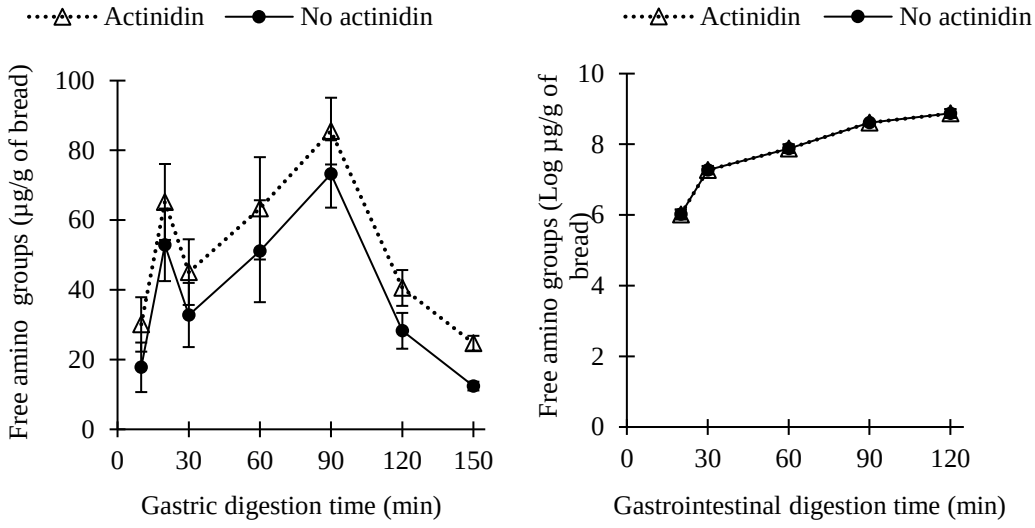



**Figure 5.3.** Actinidin activity (U/mL of digested bread chyme entering the small intestine) at different gastric digestion times.

Values are means ± SEMs, n = 3.

### 5.4.2 Actinidin accelerated the hydrolysis of wheat proteins during gastric digestion

Actinidin and the digestion time had significant effects ($P < 0.01$) on the amount of free amino groups present in bread samples entering the small intestine at different gastric digestion times (Figure 5.4A). A rapid increase in the amount of free amino groups in the samples entering the small intestine was observed at 20 min of gastric digestion and the release of free amino groups decreased after 90 min of gastric digestion for both treatments. For instance, at 20 min, the rate of released free amino groups with actinidin was 3.2 µg/g of bread per min whereas it was only 2.6 µg/g of bread per min in the control.




E, P = 0.003;
T, P = 0.002S
(A)

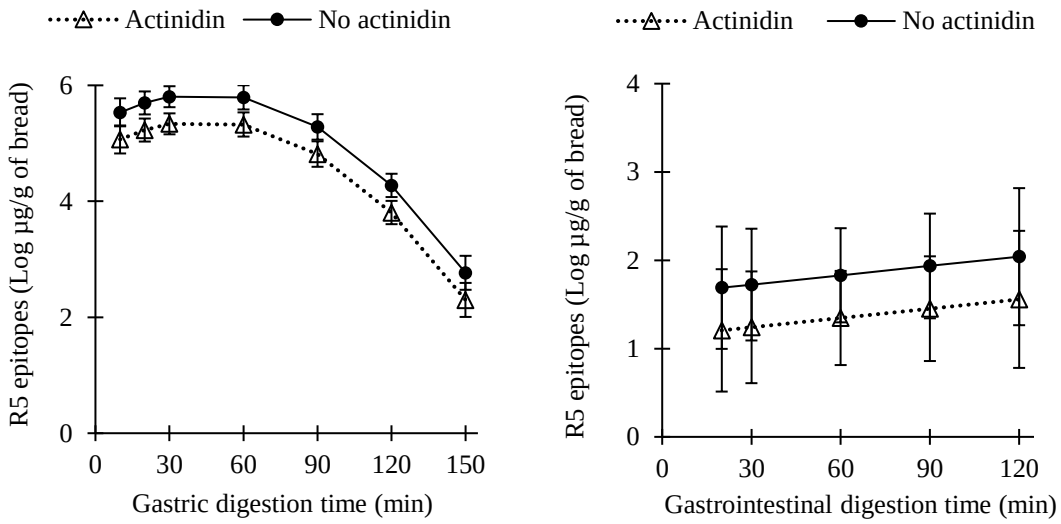
E, P = 0.77;
T, P < 0.001L
(B)

**Figure 5.4.** Amount of free amino groups in bread entering the small intestine over time (A) and amount of free amino groups released during *in vitro* small intestinal digestion of bread over gastrointestinal digestion time (B) with and without actinidin.

The interaction between enzyme and time was removed from the final model as it was not significant. E, and T, P-values for the enzyme and time respectively. S, and L, Sextic and linear effect of the time factor. Values are means ± SEMs, n = 3.

### 5.4.3 Actinidin accelerated the hydrolysis of R5 epitopes present in bread during gastric digestion

The gastric hydrolysis of R5 gluten epitopes was significantly affected by the treatment (digestion with or without actinidin) ($P < 0.05$), and digestion time ($P < 0.01$) (Figure 5.5A). The amount of residual R5 epitope present in the samples entering the small intestine decreased with time, however, based on the back-transformed natural log results, this amount was 37% lower with actinidin at all time points. However, the amount of R5 epitopes present in the small intestine was not significantly affected by either the treatment or digestion time ($P > 0.05$), although a trend was observed (Figure 5.5B).




E, P = 0.043;
T, P < 0.001Q
(A)

E, P = 0.53;
T, P = 0.73L
(B)

**Figure 5.5.** Amount of residual R5 epitopes present in bread entering the small intestine over gastric digestion time (A) and amount of residual R5 epitopes present in the small intestine over gastrointestinal tract digestion time (B) with and without actinidin.

The interaction between enzyme and time was removed from the final model as it was not significant. E, and T, P-values for the enzyme and time respectively. Q, and L, the quadratic and linear effect of the time factor. Values are means ± SEMs, n = 3.

### 5.4.4 Actinidin decreased the number of immunogenic peptides present in the digesta during gastrointestinal digestion of bread

Untargeted peptidomics was conducted to identify peptides and also enabled the further identification of specific peptides. In this study, immunogenic peptides, comprising ≥ 6 amino acid residues were generated during the digestion (google drive folder: https://drive.google.com/drive/folders/1JPm_499OsfVS1LNsPYUxb_0yKTHRedSd?usp=sharing). The number of identified immunogenic peptides present in the samples entering the small intestine at 10, 20, and 60 min of gastric digestion with actinidin was lower compared to the control (Table 5.2). For instance, at 60 min, the number of immunogenic peptides was 2-fold higher in the control composite than those in the actinidin composite. Similar trends were observed during small intestinal digestion.

**Table 5.2** Number of immunogenic gluten peptides identified by untargeted LC-MS/MS in pooled samples exiting the human gastric simulator and samples in the small intestine after *in vitro* gastrointestinal tract digestion times (10, 20, and 60 min) with and without actinidin[1].

| | Without actinidin | With actinidin |
|---|---|---|
| Samples exiting the stomach after gastric digestion | | |
| 10 min | 48 | 32 |
| 20 min | 41 | 37 |
| 60 min | 64 | 30 |
| Samples in the small intestine after GIT digestion | | |
| 20 min | 12 | 09 |
| 60 min | 21 | 10 |

[1]*Peptides were identified across three pooled replicates. The total immunogenic peptides are shown in the google drive folder.* GIT, gastrointestinal tract.

### 5.5.5 Actinidin decreased the quantity of the immunogenic marker peptides present in bread in the small intestine

The peptides detailed in Table 5.1 contain immunogenic epitopes commonly found in gluten proteins some of which have been used by previous mass spectrometry studies. All nine marker peptides (P1 – P9) were released during the GIT digestion of soda bread with and without actinidin. Treatment (presence or absence of actinidin) and the digestion time had significant effects ($P < 0.05$) on the amount of all immunogenic marker peptides present in the samples entering the small intestine (Figure 5.6). Actinidin increased ($P < 0.05$) the amounts of P1 - P7 present in the samples entering the small intestine at all time points compared to the control. For instance, at 30 min gastric digestion, the amount of P5 (33-mer peptide) was 1.3-fold higher with actinidin compared to the control (Figure 5.6E).

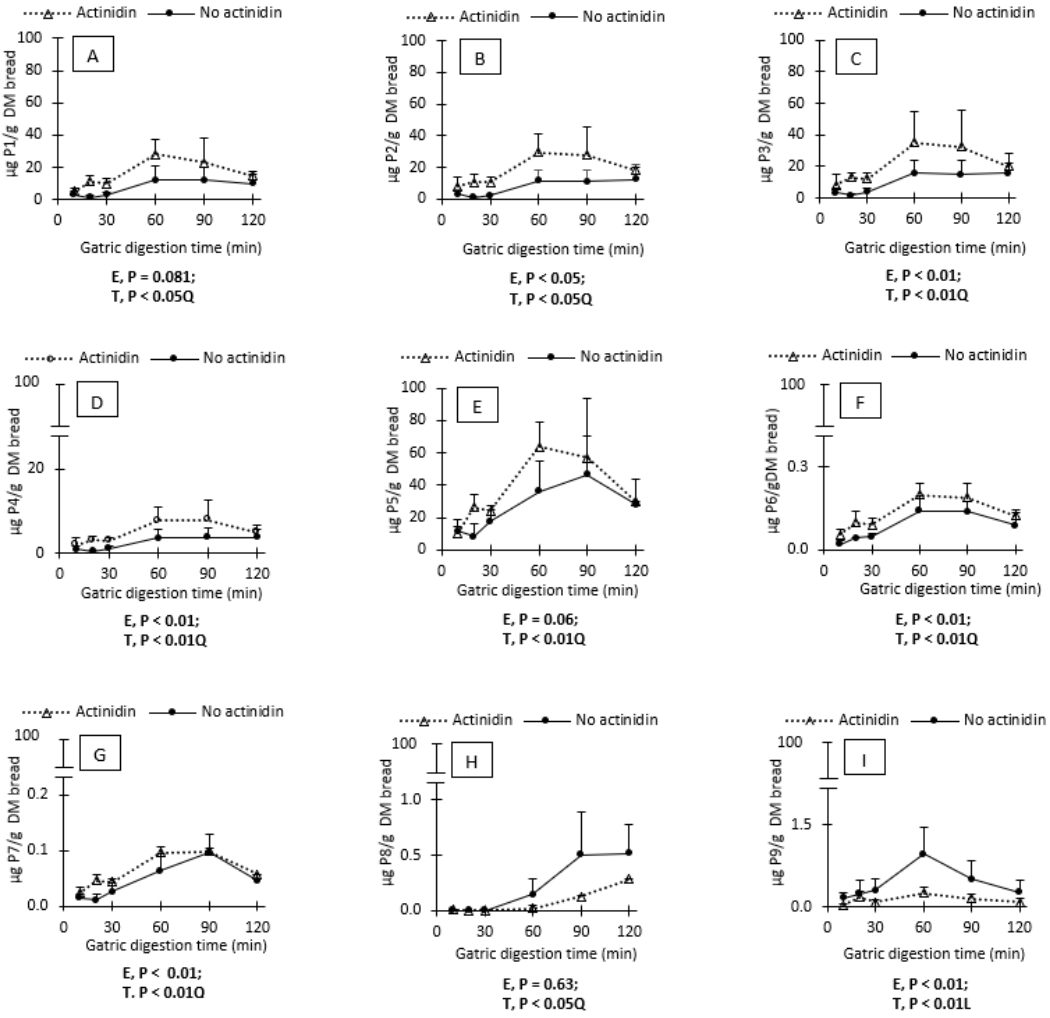



**Figure 5.6** Amount of nine immunogenic marker peptides (P1 – P9) present in bread entering the small intestine over time with and without actinidin.

A – I amount of peptide P1 – P9. Q & L, quadratic, and linear effect of the time factor. DM, dry matter. E, and T, P-values for the enzyme and time respectively. Values are means ± SEMs, n = 3. Amino acid sequence denoted in one-letter code: P1, LQLQPFPQPQLPY; P2, LQLQPFPQPQLPYPQPQPF; P3, LQLQPFPQPQLPYPQPHLPYPQPQPF; P4, LQLQPFPQPQLPYPQPQLPYPQPQPF; P5, LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF (33-mer); P6, RPQQPYPQPQPQY; P7, RPQQPYPQSQPQY; P8, FQPSQQNPQAQGSFQPQQLPQF; P9, VRVPVPQLQPQNPSQQQPQEQVPLVQQQQF.

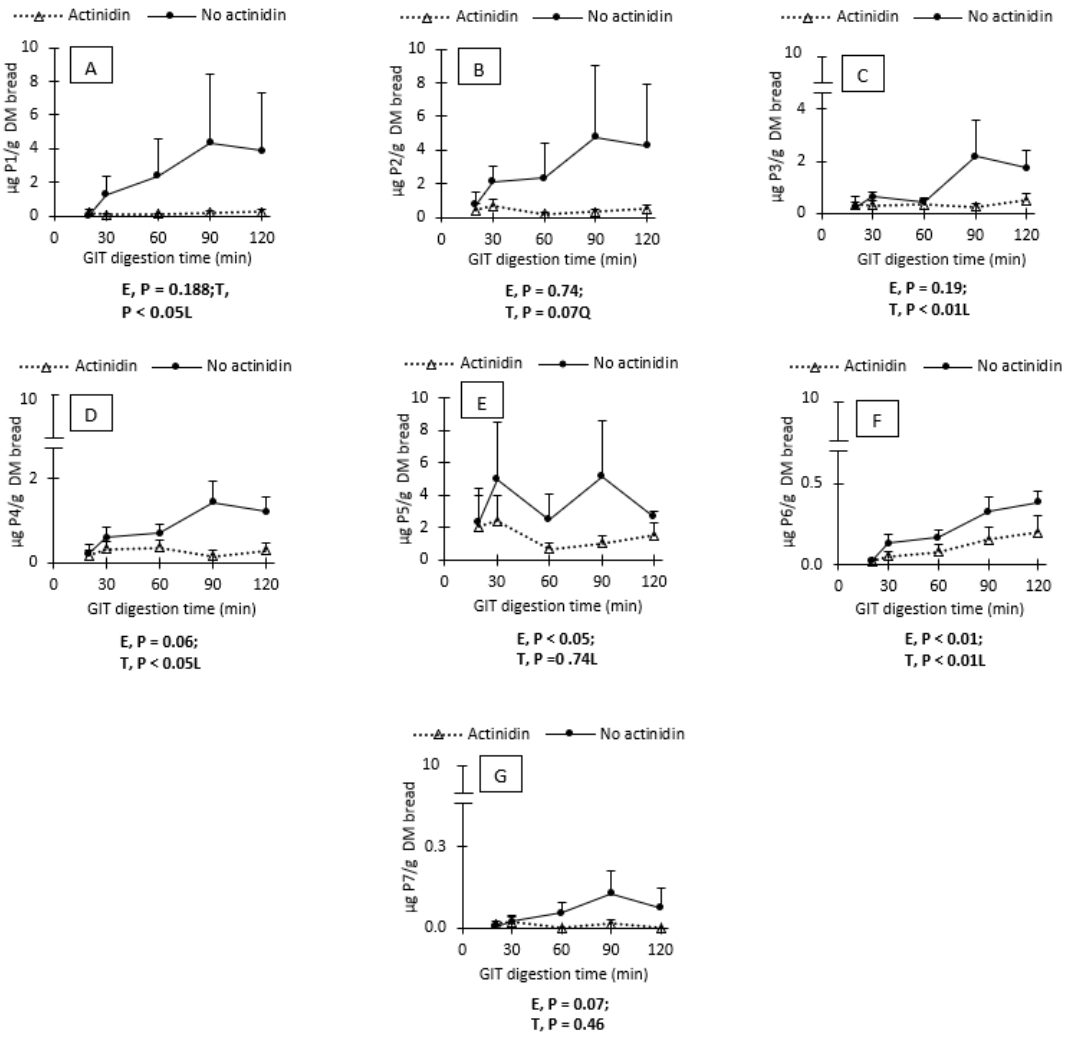



**Figure 5.7** Amount of nine immunogenic marker peptides (P1 – P9) present in bread during *in vitro* small intestinal digestion of bread over gastrointestinal tract digestion time with and without actinidin.

A – I amount of peptide P1 – P9. Q & L, quadratic, and linear effect of the time factor. DM, dry matter. E, and T, P-values for the enzyme and time respectively. P8 and P9 values in the small intestine were negligible. Values are means ± SEMs, n = 3. Amino acid sequence denoted in one-letter code: P1, LQLQPFPQPQLPY; P2, LQLQPFPQPQLPYPQPQPF; P3, LQLQPFPQPQLPYPQPHLPYPQPQPF; P4, LQLQPFPQPQLPYPQPQLPYPQPQPF; P5, LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF (33-mer); P6, RPQQPYPQPQPQY; P7, RPQQPYPQSQPQY.

In the small intestine, the amounts of P4 – P7 were significantly lower (or tended to be) ($P \leq 0.05$) with actinidin compared to the control (Figure 5.7). For instance, the amount of P5 was 2-fold lower with actinidin compared to the control at 30 min of GIT digestion (Figure 5.7E). At 30 min of GIT digestion, actinidin decreased 90% of the P5 which entered the small intestine, while it was only 70% in the control (Table 5.3).

**Table 5.3** Decrease of immunogenic marker peptides P1-P7 from the human gastric simulator to the small intestine at 30 min of gastrointestinal tract digestion with and without actinidin.

| **Peptide** | **% Reduction** | |
|---|---|---|
| | **Without actinidin** | **With actinidin** |
| P1 | 60.4 | 99.3 |
| P2 | 7.0 | 93.8 |
| P3 | 83.4 | 97.4 |
| P4 | 45.4 | 88.9 |
| P5 | 72.0 | 90.2 |
| P6 | 0 | 42.3 |
| P7 | 9.7 | 51.2 |

P1, LQLQPFPQPQLPY; P2, LQLQPFPQPQLPYPQPQPF; P3, LQLQPFPQPQLPYPQPHLPYPQPQPF; P4, LQLQPFPQPQLPYPQPQLPYPQPQPF; P5, LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF (33-mer); P6, RPQQPYPQPQPQY; P7, RPQQPYPQSQPQY

## 5.5 Discussion

In the present study, we hypothesised that actinidin from green kiwifruit would mitigate the immunogenic gluten-derived peptides generated during *in vitro* digestion of gluten-containing bread. To test this hypothesis, a semi-dynamic *in vitro* HGS accompanying a static small intestinal digestion model was used with a gluten-containing model food, whole wheat soda bread.

The hydrolysis of wheat proteins and the immunogenic gluten peptides derived from the *in vitro* gastric digestion of the soda bread was accelerated by the presence of actinidin, and thereby both the number and the amount of immunogenic gluten peptides (R5 epitopes) that reached the small intestine were lower with actinidin. Further, a lower number and lower amounts of immunogenic gluten peptides were observed in the small intestine model over time.

The present study used four slices of bread (160 g of chewed bread) per digestion with actinidin, equivalent to consuming two kiwifruit, which represents a typical meal size. The gluten content of this meal was around 4.5 g considering that 90% of the protein present in bread is gluten. In contrast, most of the other studies testing the effect of exogenous enzymes have tested inadvertent gluten intake (Di Cagno et al., 2004; Montserrat et al., 2015). However, this study aimed to examine how actinidin hydrolyses gluten immunogenic peptides in a meal that contained a standard amount of gluten.

The physical disintegration of food is a crucial factor for the hydrolysis of food materials throughout the GIT. Food disintegration starts in the mouth. The size of the food particles in the bolus after mastication plays an important role in the digestion of the food in the stomach and subsequently in the small intestine. Thus, in the present study, a group of human volunteers was used to masticate the bread to have a more physiological chewed bread prior to the *in vitro* gastric digestion. A similar type of "chew and spit" approach has been used by others to determine protein digestion *in vitro* (Baugreet et al., 2019; Chen et al., 2020). The HGS used in this study to conduct gastric digestion is a validated *in vitro* model which was set up based on *in vivo* data to simulate human gastric digestion (Kong & Singh, 2010). This model mimics gastric pH changes, gradual gastric secretions, fluid dynamics in the stomach, gastric emptying,

sieving and continuous motility and has been frequently used by others for gastric digestion studies (Guo et al., 2015; Ye et al., 2017; Wang et al., 2018).

The actinidin activity of fresh green kiwifruit reported in the present study agreed with a previous study reported by Kazem & Habeeb (2020) where they have reported an actinidin activity of 15.6 U/mL, however, Kaur et al., (2010) reported a slightly higher value of 26.4 U/mL for the same. This could be due to many reasons including the maturity of the fruits used in the study (Lewis & Luh, 1988). The effect of actinidin was significant throughout gastric digestion to accelerate the hydrolysis of wheat proteins in the samples entering the small intestine. Actinidin specificity played a role in this acceleration during gastric and small intestinal digestion. Actinidin has broad specificity and can hydrolyse many peptide bonds, including those that contain proline and glutamine (Jayawardana et al., 2021). The pH of the gastric medium (Figure 5.2A) supported the action of actinidin on wheat proteins since actinidin acts on gluten when the pH of the medium is > 2 (Jayawardana et al., 2021).

It was observed in the present study that actinidin was still active in the material entering the small intestine until 90 min of gastric digestion. This suggests that actinidin is active and stable against the gastric pH, pepsin proteolysis and self-hydrolysis under physiologically relevant conditions. *In vivo* evidence has shown that actinidin activity increased during gastric digestion (Montoya & Rutherfurd et al., 2014). However, in the present study, actinidin activity decreased over time in the samples entering the small intestine. Other *in vitro* and *in vivo* studies have found actinidin remained intact against acidic pH and pepsin proteolysis based on dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). For instance, it was observed *in vitro* that actinidin continued to be intact at pH 1.2 (Dearman et al., 2014) and pepsin proteolysis after 1 h of gastric digestion when physiologically relevant protein to pepsin ratios was used

(Bublin et al., 2008). In an *in vivo* study, actinidin was still intact after 1 h of gastric digestion when fresh kiwifruit pulp was fed to human volunteers and their gastric content was evaluated using SDS-PAGE (Polovic et al., 2007). However, SDS-PAGE data can not specify whether the actinidin is in its active state or not.

The rate of wheat protein hydrolysis was notable during the first 10 min of gastric digestion, and this initial effect remained constant over the whole *in vitro* digestion. The amount of marker immunogenic peptides was higher in the samples entering the small intestine, indicating that actinidin was able to hydrolyse gluten proteins present in the food matrix and release immunogenic fragments which were present within the original proteins. The released marker immunogenic peptides reaching the small intestine were rapidly hydrolysed by both actinidin and some pancreatic proteases, preventing them from being accumulated in the small intestine. For example, the marker peptides P1 – P5 including the highly immunogenic 33-mer peptide (P5) were up to 90% decreased during the first 30 min of small intestinal digestion with actinidin, while the reduction was only 70% in the control. This is supported by the untargeted peptidomics results where the number of immunogenic peptides (having $\geq$ 6 amino acid residues) identified in the small intestine was lower with actinidin. In the control, the greater release of immunogenic peptides during the small intestinal digestion may be due to the presence of other pancreatic enzymes such as amylase and lipase that digest other nutrients of the bread matrix, which could have increased the surface exposure of gluten to the pancreatic proteases. This observation is consistent with other MS analyses of similar gluten immunogenic peptides in bread where the amounts of immunogenic peptides peaked during the small intestinal digestion but not during the peptic digestion (Ogilvie et al., 2020).

The R5 epitopes (QQPFP, QQQFP, LQPFP, and QLPFP) measured with ELISA are common motifs found in gluten immunogenic peptides (Kahlenberg et al., 2006). For instance, five of the nine marker immunogenic peptides (P1 - P5) used in this study contained the R5 epitope LQPFP. The R5 ELISA antibodies detect R5 epitopes present in gluten peptides and proteins (Mitea et al., 2008), thus the R5 epitopes unreleased and present within the parent gluten protein structure may also be detected by these antibodies. Therefore, the ELISA results indicate an overall (either released or unreleased) estimation of R5 peptides present in gluten. The lower amount of R5 epitopes with actinidin in the samples reaching the small intestine indicates that peptides containing these motifs were hydrolysed when actinidin was present during gastric digestion. In other words, actinidin was able to hydrolyse the peptide sequences containing R5 motifs. These ELISA results are consistent with the untargeted peptidomics results showing the number of immunogenic peptides reaching the small intestine was lower with actinidin. These results are consistent with our previous *in vitro* study results when pure wheat gluten was digested with actinidin using both static (Jayawardana et al., 2021) and semi-dynamic (Jayawardana et al. submitted) *in vitro* digestion models.

The level of gluten tolerance varies across individuals. Codex Alimentarius proposed a limit for gluten of less than 20 mg/kg for naturally gluten-free food (Codex Alimentarius Commission, 2003). It has been reported that a daily intake of 50 mg of gluten has a definite effect on the mucosal histology of coeliac patients and even with 10 mg of gluten, observable mucosal changes have been reported (Akobeng & Thomas, 2008; Catassi et al., 2007). Therefore, it has been identified that the safe limit could be a daily intake of less than 10 mg of gluten for subjects with coeliac disease. The minimum daily intake of gluten to initiate wheat allergy has been reported to be higher

than that for coeliac disease, around 1-2 g of wheat proteins (Hischenhuber et al., 2006). In the present study and based on the R5 epitopes, the "intact" total gluten content reaching the small intestine within the first 30 min of gastric digestion was 35 mg with actinidin while it was 43 mg in the control. An important factor in gluten-related health disorders is the amount of immunogenic peptides present in the small intestine, where immune responses occur. These reactions arise within a short postprandial time, around 10 – 60 mins (Silvester et al., 2016). Based on the marker immunogenic peptide results, it has been shown that the rapid action of actinidin on gluten decreased both the amount of and time of exposure to immunogenic peptides in the small intestine after consumption of normal gluten-containing food.

It is important to note that the results of the present study could be underestimated as in an *in vivo* situation, gluten immunogenic peptides could be hydrolysed by brush border and microbial proteases (Shan et al., 2002; Zamakhchari et al., 2011). This (combined with our previous studies (Jayawardana et al., 2021) suggests that actinidin might decrease/eliminate gluten immunogenicity when a small amount of gluten is accidentally ingested by individuals with gluten-related health disorders. Pre-clinical and clinical trials are warranted to determine the efficacy of actinidin to combat gluten immunogenicity *in vivo*.

### 5.6 Conclusion

To conclude, actinidin was stable against both the gastric pH and pepsin proteolysis under physiologically relevant conditions up to 90 min and actinidin was able to rapidly hydrolyse wheat proteins present in a food matrix during simulated gastric conditions. The presence of actinidin markedly decreased the amounts of immunogenic gluten peptides, including the highly immunogenic 33-mer peptide, in the small intestine. The

greater hydrolysis of gluten with actinidin in gastric conditions increased the hydrolysis of gluten peptides in the small intestine and decreased the occurrence of immunogenic peptides in the small intestine in a normal gluten-containing diet.

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# Chapter Six

# 6. Effect of actinidin from kiwifruit (*Actinidia deliciosa* cv. Hayward) on hydrolysis of wheat proteins and gluten immunogenic peptides in the gastrointestinal tract of growing pigs

This chapter is in preparation to be submitted to Gastroenterology:

Jayawardana, I. A., Boland, M. J., Loo, T., McNabb, W. C., & Montoya, C. A. (In preparation to be submitted to Gastroenterology). Mapping of digestion of gluten-derived immunogenic peptides along the gastrointestinal tract of the growing pig model.

**Highlights**

- When a normal bread meal was fed to growing pigs, low gastric hydrolysis of wheat proteins and high immunogenic gluten peptides in the gastrointestinal tract was observed.
- Actinidin rapidly hydrolysed wheat proteins in the stomach of growing pigs.
- Actinidin reduced the amount of immunogenic peptides in the gastrointestinal tract of pigs postprandially when a normal bread meal was fed.
- Actinidin was able to survive peptic proteolysis and gastric pH conditions until 300 min postprandial.

## 6.1 Abstract

### Background and Aims

This study investigated the digestion of wheat proteins and gluten-derived immunogenic peptides in the gastrointestinal tract (GIT) of growing pigs with and without the presence of an exogenous enzyme, actinidin, a protease in green kiwifruit (*Actinidia deliciosa*).

### Methods

Entire male pigs (n=54, starting bodyweight 21.2 ± 2.1 kg) were fed whole wheat soda bread either with green kiwifruit (+ actinidin) or yellow kiwifruit (- actinidin). Pigs were euthanised at 0, 20, 60, 120, and 300 min postprandially (n=6 per time point for each diet). Gastrointestinal contents were collected to determine the hydrolysis of wheat proteins in the stomach and immunogenic peptides in the GIT of pigs.

### Results

The rate of digestion of wheat proteins was significantly higher ($P < 0.01$) in the stomach of the pigs fed the actinidin-containing diet compared to that observed without actinidin (0.07% vs 0.03%/min). This was reflected in higher hydrolysis ($P < 0.01$) of R5 epitopes in the stomach (0.3 mg/min higher), and subsequently in different small intestinal regions ($P < 0.05$) (e.g., 0.04 mg/min higher for the first half of the small intestine). The LC/MS-MS showed that the stomach of the pigs fed the actinidin-containing meal had ($P < 0.05$) on average 56% lower amounts of seven of nine immunogenic marker peptides and at 20 min, two-fold lower number of immunogenic peptides compared to that without actinidin.

### Conclusion

The intake of green kiwifruit with a gluten-containing diet increased the gastric digestion of wheat proteins and reduced the immunogenic peptides along the GIT.

**Keywords:** Actinidin, Digestion, Gluten, Growing pigs, Immunogenic peptides

## 6.2 Introduction

Human gastrointestinal proteases lack the ability to effectively hydrolyse the proline-glutamine-rich peptides in gluten proteins, leading to only partial hydrolysis of gluten in the gastrointestinal tract (GIT) of humans. This leads to the release of proline-glutamine-rich peptides, some of which are immunogenic, producing immune responses in genetically predisposed individuals to create gluten-related health disorders (celiac disease, or sensitivity or allergy to gluten). A considerable proportion of the world population suffers from gluten-related health disorders (Fasano et al., 2003; Tack et al., 2010). The only valid and effective medical treatment for these disorders to date is to stick to a strict gluten-free diet (Discepolo & Guandalini, 2017; Fasano & Catassi, 2001; Norström et al., 2012) which is a difficult prescription to follow, affecting the quality of life of the people effected. Gluten traces are commonly found in gluten-free products due to cross-contamination (Farage et al., 2017) and the dose to trigger an immunogenic response may be as small as 20 ppm of gluten as proposed in the Codex Alimentarius (Codex Alimentarius Commission, 2008). Alternative dietary and non-dietary strategies have been investigated to enable those with gluten-related health disorders to have a normal life (Gass et al., 2007; Leffler et al., 2015; Montserrat et al., 2015; Pinier et al., 2012; Toft-Hansen et al., 2014; Tye-Din et al., 2010). Among dietary strategies, exogenous proteolytic enzymes are being explored for their potential to supplement human GIT proteases. Exogenous enzymes such as prolyl endopeptidases (from *Flavobacterium meningosepticum, Sphingomonas capsulate, Myxococcus xanthus*) and cysteine endoproteases (from germinating barley) appear to reduce or eliminate gluten immunogenicity (Davy et al., 1998; Moreno

Amador et al., 2019; Piper et al., 2004; Stepniak & Koning, 2006; Stepniak et al., 2006). Only a few of the exogenous enzymes are able to survive the gastric environment in their active form (Gass et al., 2007; Stepniak & Koning, 2006).

Actinidin, a cysteine protease from kiwifruit *(Actinidia deliciosa*), has been identified as a potential natural enzyme to assist in hydrolysing gluten proteins. Several *in vitro* and *in vivo* studies have shown that actinidin can enhance the digestion of gluten and gliadin peptides (Jayawardana et al., 2021; Carlos & Hindmarsh, et al., 2014; Rutherfurd et al., 2011). In rats, actinidin increased 1.4-fold, the degradation of ω-gliadins during gastric digestion (Rutherfurd et al., 2011). In our previous *in vitro* studies, actinidin has a broad substrate specificity hydrolysing multiple peptide bonds, including those adjacent to proline (Jayawardana et al., 2021). Actinidin hydrolysed immunogenic gluten peptides in simulated GIT digestion (Jayawardana et al., 2021), including the 33-mer peptide which is known to be one of the most antigenic gluten peptides (Shan et al., 2002). Most importantly, actinidin during *in vitro* digestion of gluten was able to reduce the amount of immunogenic peptides reaching the small intestine, where immunogenic reactions take place in individuals with gluten health-related disorders. However, limited information is available on whether actinidin is able to hydrolyse immunogenic gluten peptides under *in vivo* conditions.

The aim of this study was two-fold: First, to map the digestion of gluten and gluten-derived immunogenic peptides in the GIT of a pig model fed a gluten-containing meal since this information is scarce in the literature. Second, to evaluate the effect of actinidin on the digestion of gluten and the immunogenic peptides resulting from gluten digestion. The pig was used as an animal model of the adult human due to the anatomical and physiological similarities of the GIT (Deglaire & Moughan, 2012). It was hypothesised that the inclusion of actinidin-containing kiwifruit with a gluten-

containing food would lessen or eliminate the immunogenic gluten peptides generated during GIT digestion in growing pigs.

## 6.3 Materials and Methods

### 6.3.1 Animals and housing

Ethics approval (No:20/46) for the animal trial was obtained from the Animal Ethics Committee, Massey University (Palmerston North, New Zealand). Entire male pigs of nine weeks of age (n=54; 21.2 ± 2.1 mean ± standard error kg body weight) were used in this study. Pigs were housed individually in metabolism crates in a room maintained at 22 ± 2 °C with a 12 h:12 h light/dark cycle (Animal Physiology Unit, Massey University, Massey University).

### 6.3.2 Experimental diets

Two experimental diets; green kiwifruit (*Actinidia deliciosa* cv. Hayward) + whole wheat soda bread (treatment) and yellow kiwifruit (*Actinidia chinensis* cv. Hort16A) + whole wheat soda bread (control) were used in the study. Yellow kiwifruit (Hort16A) was used in the control diet, as this kiwifruit variety does not contain any actinidin activity, but it has other compounds present in green kiwifruit that could affect transit time and digestion in the GIT, notably dietary fibre. Kiwifruit pre-ripened to a similar firmness were used in both experimental diets (Henare et al., 2012). Whole wheat soda bread was used to minimise the addition of other proteins (such as yeast proteins). The adaptation diets were formulated to have a similar nutrient composition across diets with the nutrient requirements of the growing pig (Table 6.1). The quantity of fresh kiwifruit included in the diets was calculated to provide an intake equivalent to consuming two kiwifruit with an average-sized meal, thus, kiwifruit represented around

19% of the daily dry matter intake (Montoya & Rutherfurd, et al., 2014). Purified gluten was added to the adaptation diets to reach the daily protein requirements. However, in the last meal, the protein from bread was the only protein source. The last meal contained four slices of whole wheat soda bread and two kiwifruit (either green or yellow) per pig (Table 6.1). When preparing the meal, whole wheat soda bread was cut into small pieces. The fresh kiwifruit were peeled, hand crushed and made into a pulp and were thoroughly mixed with the bread immediately before feeding. Only in the last meal, a known amount of titanium dioxide ($TiO_2$) was added as an indigestible marker to determine the flow of that meal along the GIT.

**Table 6.1** Ingredient and nutrient compositions of the experimental diets**.**

| | Adaptation diet (g/kg dry matter) | Final Meal (g dry matter) | |
|---|---|---|---|
| | | Green kiwifruit | Yellow kiwifruit |
| **Ingredients** | | | |
| Green kiwifruit | 190.0 | 25.6 | - |
| Yellow kiwifruit | 190.0 | - | 27.2 |
| Bread | 641.7 | 178.1 | 178.1 |
| Gluten | 90.0 | - | - |
| Oil | 50.0 | - | - |
| Sugar | - | - | - |
| Vitamin/Mineral* | 5.0 | - | - |
| Cellulose | - | - | - |
| Dicalcium | 19.0 | - | - |
| Phosphate | | | |
| Celite | 4.2 | - | - |
| Starch | - | - | - |
| Water | - | | |
| Titanium dioxide | - | 1.01 | 1.02 |
| **Nutrient composition** | | | |
| Crude protein | 193.0 | 22.8 | 22.2 |
| Fat | - | 11.2 | 11.0 |
| Dietary fibre | 47.0 – 58.0 | 18.1 | 14.5 |
| Starch | - | 103.8 | 105.1 |
| Gross energy (kJ/g) | 168.0 – 171.0 | 18.0 | 17.9 |

*Calculated amounts of commercially purchased vitamin and mineral premixes were added to the adaptation diets to meet the daily nutrient requirements of the pigs for all the nutrients as prescribed by the National Research Council (1998).

### 6.3.4 Experimental design

On the day of arrival, pigs were assigned randomly to one of the two dietary treatments, green kiwifruit diet (n=24, four-time points and six pigs per time point) and yellow kiwifruit diet (n=30, five-time points and six pigs per time point), and they received the same commercial diet provided at the farm *ad libitum*. From the following day, the adaptation diets replaced the commercial diet gradually (33% per day). The quantity of dry matter per day was based on the 90 g dry matter/kg metabolic body weight (metabolic body weight=body weight$^{0.75}$) per day (Montoya & Rutherfurd, et al., 2014). The diet was given at 09.00 and 16.00 hours and the proportion of the portion sizes were 2:1 respectively. The aim of smaller afternoon meals was to attempt to have an emptier stomach after fasting on the final day of the experiment. Water was provided *ad libitum*, and pigs were adapted to their experimental diet for 8 days. On day 8, the afternoon meal was provided at different times to ensure a fasting period of 16 h before feeding the morning (final) meal on day 9. A known amount of $TiO_2$ was added to the dinner of the pigs euthanised at time 0 to determine the location (or transit) of the meal at fasting. On day 9, water was withheld from the pigs 2 h before each animal received an experimental meal with a known amount of TiO2 after a 16 h fasting period. Pigs were anaesthetised and euthanised at times 0, 20, 60, 120, and 300 min post-feeding. The times for euthanasia were selected based on our previous *in vitro* digestion study of bread with and without actinidin (Jayawardana et al., unpublished data). Any feed refusal from the last meal was collected, dried, and weighed to obtain an accurate measure of dry matter feed intake. The anaesthesia mixture contained Zoletil 100 (zolazepam and tiletamine, both 50 mg/mL) reconstituted with 2.5 mL Ketamine and 2.5 mL Xylazine, both 100 mg/mL. The final solution contained 50 mg/mL of each drug. This was administered at a dose rate of 120 μL of the mixed solution/kg body

weight by intramuscular injection. Pigs were then euthanised by an intracardial injection of sodium pentobarbitone (0.3 mL/kg BW of Pentobarb 300; Provet) (Montoya & Rutherfurd, et al., 2014). The whole GIT was dissected out. The stomach, small intestine, caecum, and colon including the rectum were ligated before dividing these sections with minimal handling and the digesta samples were collected as described in Appendix 6 Supplementary methods 1.

### 6.3.5 Sample analysis

The experimental diets were analysed for dry matter (Official methods of analysis of the association of official analytical chemists, 1990), crude protein (N × 6.25, method 968.06 using a LECO elemental analyser; LECO Corporation), gross energy (LECO AC-350 Automatic Calorimeter; LECO Corporation), total dietary fibre (Proskyet al.,1988), starch (α-amylase Megazyme kit, AOAC 996.11), fat (Soxtec, (Grains, feed), AOAC 2003.06) and $TiO_2$ marker (Short et al., 1996).

The gluten in the diet and GIT contents was extracted (Mena et al., 2012) (detailed in Appendix 6 Supplementary methods 2) to increase the recovery from heat-treated and hydrolysed gluten present in the bread. The extracted samples were used to determine free amino groups and residual R5 epitopes using the o-phthaldialdehyde (OPA) method and enzyme-linked immunosorbent assay (ELISA) respectively as described in Appendix 6 Supplementary methods 3 and 4 in detail.

### 6.3.6 Degree of hydrolysis and R5 epitopes

The degree of hydrolysis (DH) of wheat proteins present in the stomach was calculated using the free amino groups determined using the OPA method (Church et al., 1983). All stomach chyme samples and selected small and large intestinal digesta samples

were analysed in duplicate to quantify the residual R5 epitopes (QQPFP, QQQFP, LQPFP, and QLPFP) using a commercially available competitive ELISA (RIDASCREEN® Gliadin competitive, R-Biopharm AG, Darmstadt, Germany) based on the R5 antibody.

### 6.3.7 Marker immunogenic peptides and immunogenic peptide profiles

Freeze-dried stomach chyme or proximal small intestinal digesta (200 mg) were prepared for the mass spectrometry analysis of immunogenic peptides using polymeric reversed-phase solid extraction cartridges to separate gluten peptides (Detailed in Appendix 6 Supplementary methods 5). Quantitative liquid chromatography-tandem mass spectrometry (LC-MS/MS) was conducted to quantify nine immunogenic marker peptides (Table 6.2) in gastric chyme and proximal small intestine samples. The marker immunogenic peptides could not be detected in the proximal small intestinal samples therefore, we did not analyse those in other small intestinal samples. Gastric chyme and proximal small intestinal digesta samples at 60 min and 120 min were pooled per GIT location to determine the immunogenic peptide profiles using discovery proteomics. Details of the methods are given in Appendix 6 Supplementary methods 6 and 7.

### 6.3.8 Actinidin activity

The actinidin activity was measured in fresh green and yellow kiwifruit and fresh stomach chyme and small intestinal samples. Separately extracted pulps of three randomly selected green and yellow kiwifruit and fresh chyme samples were centrifuged at 13,000 rpm for 30 min at 4 °C and the supernatants were used to measure the actinidin activity (Units/g dry matter) using the method described previously (Boland & Hardman, 1972) (see Appendix 6 Supplementary methods 8 for details). As

the activity did not differ across the treatments in the proximal small intestinal samples, actinidin activity was not analysed in the remaining small intestinal samples.

**Table 6.2** Marker peptide parameters for quantitative mass spectrometry.

| Peptide name | Amino acid sequence | Molecular weight (kDa) | Retention time (min) |
|---|---|---|---|
| P1 | LQLQPFPQPQLPY | 1.568 | 4.37 |
| P2 | LQLQPFPQPQLPYPQPQPF | 2.263 | 4.67 |
| P3 | LQLQPFPQPQLPYPQPHLPYPQPQPF | 3.096 | 4.58 |
| P4 | LQLQPFPQPQLPYPQPQLPYPQPQPF | 3.087 | 4.87 |
| P5 | LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQ PF (33-mer) | 3.912 | 5.00 |
| P6 | RPQQPYPQPQPQY | 1.627 | 0.52 |
| P7 | RPQQPYPQSQPQY | 1.617 | 0.50 |
| P8 | FQPSQQNPQAQGSFQPQQLPQF | 2.529 | 2.48 |
| P9 | VRVPVPQLQPQNPSQQQPQEQVPLVQQQQ F | 3.491 | 3.22 |
| P1H | LQLQPF*PQPQLPY | 1.578 | 4.37 |

Amino acid sequence denoted in one-letter code. *Isotopic label, l-Phenylalanine-$^{13}C_9$,$^{15}N$.

### 6.3.9 Calculations

The DH of gluten was calculated as follows:

$$\text{DH}\ (\%) = \frac{(\text{NH}_{2\text{chyme}} - \text{NH}_{2\text{diet}})}{(\text{NH}_{2\text{diet total}} - \text{NH}_{2\text{diet}})} \times 100$$

where $NH_{2diet\ total}$, $NH_{2diet}$ and $NH_{2chyme}$ are the total amino groups in the diets after acid hydrolysis, free amino groups in the diets before digestion, the free amino groups in the chyme at each postprandial time respectively in μg/g dry matter.

The total amount of R5 epitopes present in the stomach was calculated as follows:

$$\text{Total R5 epitopes } (\mu g) = \text{R5}_{\text{chyme}} \times \text{DM}_{\text{Chyme}}$$

where $R5_{chyme}$ and $DM_{Chyme}$ are the R5 epitopes in the chyme at each postprandial time in µg/g dry matter and the chyme dry matter content in the stomach respectively.

The same calculation was used to calculate the R5 epitopes present in each part of the small intestine (proximal small intestine, distal small intestine, and terminal ileum) and the large intestine (caecum and colon). The same procedure of calculation was used to calculate the amount of marker immunogenic peptides present in the stomach.

The amount of total digested protein entering the small intestine was calculated as follows:

$$\text{Total digested protein entering the small intestine}(\mu g) = \text{NH}_{2\text{chyme}} \times \text{DM}_{\text{Chymereleased}}$$

where $NH_{2chyme}$ and $DM_{Chymereleased}$ are the free amino groups in the chyme at each postprandial time in µg/g dry matter and the chyme dry matter content released from the stomach to the small intestine (g) calculated using stomach emptying data respectively.

The same procedure of calculation was used to calculate the amount of R5 epitopes and marker immunogenic peptides released into the small intestine.

### 6.3.10 Statistical analysis

Statistical analyses were performed using SAS (SAS/STAT version 9.4). For the parameters such as degree of hydrolysis of protein, the quantity of residual R5 epitopes

and immunogenic marker peptides, a polynomial regression analysis including treatment (green or yellow kiwifruit), time (as either a categorical or a numerical variable up to cubic order) and their interaction as fixed effects were used. When the P-value was lower or equal to 0.05, the difference was declared significant. A correlation analysis was conducted between the gastric emptying rate of the dry matter and the $TiO_2$ marker.

### 6.4 Results

All pigs were healthy during the experiment. A significant positive correlation ($P < 0.01$, $r^2 = 0.994$, n=54, Figure 6.1) was observed between the gastric emptying rate of dry matter and $TiO_2$.

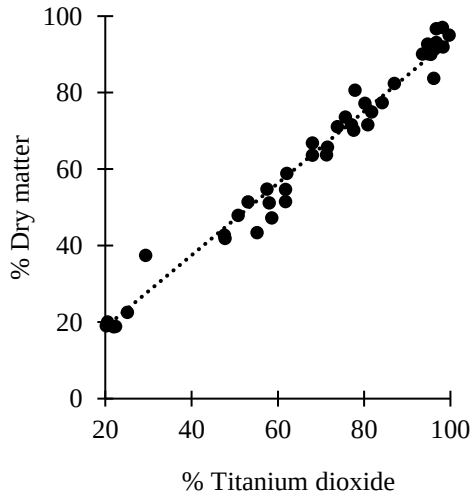


**Figure 6.1** Correlation between gastric emptying of the dry matter (%) and the indigestible marker titanium dioxide (%) in the stomach chyme at different digestion times in growing pigs fed green kiwifruit diet (with actinidin) and yellow kiwifruit diet (no actinidin).

P < 0.01, r2 = 0.994, n=54.

The % dry matter gastric emptying of the green kiwifruit diet was similar (P > 0.05) to that of the yellow kiwifruit diet (Figure 6.2). The amount of chyme observed in the pigs euthanised at time 0 was 2.5 ± 1.1 g (Table 6.3). Based on this negligible amount (empty stomach), no correction was done for the other time points when analysing different parameters such as stomach emptying.

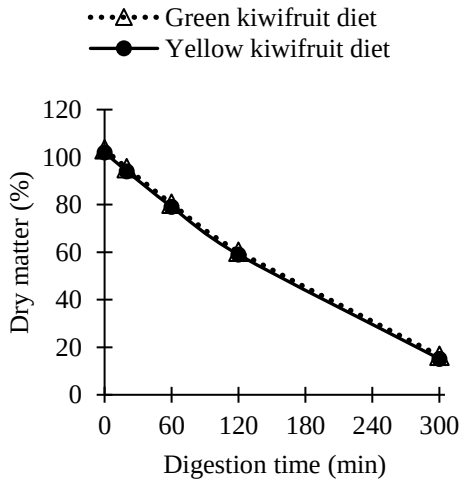


**Figure 6.2** Gastric emptying of the dry matter (%) with time in growing pigs fed the green kiwifruit diet (with actinidin) and the yellow kiwifruit diet (no actinidin).

The P-values for the enzyme and time were 0.55 and < 0.01Q respectively. Q, quadratic effect of time factor. Values are means ± SEMs, n = 6.

### 6.4.1 Actinidin activity in stomach chyme and proximal small intestine

The actinidin activity of fresh yellow kiwifruit pulp was negligible when compared to the fresh green kiwifruit (0.22 ± 0.15 vs 27.08 ± 1.20 Units/g dry matter). Thus, as expected, the actinidin activity over time in the stomach chyme of the pigs fed the green kiwifruit meal was higher (P < 0.01) than those fed the yellow kiwifruit meal (Table 6.4). The actinidin activity of the proximal small intestinal digesta (e.g., ~10 U/mL at 20 min) was similar between both dietary treatments.

**Table 6.3** Dry matter content of each gastrointestinal location at each postprandial time.

| Location | Time (min) | Dry matter content (g) Green kiwifruit meal | Yellow kiwifruit meal |
|---|---|---|---|
| Stomach | 0 | - | 2.5 ± 1.1 |
| | 20 | 202.8 ± 1.8 | 207.0 ± 6.7 |
| | 60 | 168.2 ± 12.1 | 163.2 ± 4.6 |
| | 120 | 113.2 ± 11.4 | 125.7 ± 18.4 |
| | 300 | 49.5 ± 14.9 | 27.2 ± 27.3 |
| Proximal small intestine | 0 | - | 1.1 ± 0.1 |
| | 20 | 3.1 ± 0.4 | 2.2 ± 0.4 |
| | 60 | 6.7 ± 1.1 | 5.8 ± 1.0 |
| | 120 | 6.2 ± 1.0 | 4.0 ± 0.7 |
| | 300 | 3.2 ± 0.3 | 4.5 ± 1.3 |
| Distal small intestine | 0 | | 2.6 ± 0.6 |
| | 20 | 3.3 ± 0.8 | 4.2 ± 1.0 |
| | 60 | 7.6 ± 1.8 | 9.6 ± 0.4 |
| | 120 | 15.5 ± 1.1 | 13.2 ± 1.3 |
| | 300 | 11.0 ± 1.3 | 10.4 ± 1.5 |
| Terminal ileum | 0 | | 0.5 ± 0.1 |
| | 20 | 0.4 ± 0.3 | 0.5 ± 0.2 |
| | 60 | 0.9 ± 0.3 | 0.7 ± 0.2 |
| | 120 | 0.5 ± 0.2 | 0.4 ± 0.2 |
| | 300 | 0.4 ± 0.2 | 1.7 ± 0.5 |

Values are means ± SEMs, n = 6.

**Table 6.4** Actinidin activity of fresh kiwifruit, final diet and stomach chyme of growing pigs fed the diets containing kiwifruit at different postprandial times.

| | **Meal** | | **P** | |
|---|---|---|---|---|
| | **Green kiwifruit** | **Yellow kiwifruit** | **Enzyme** | **Time** |
| | Units/g (DM) | | | |
| Fresh fruit | 27.08 ± 1.20 | 0.22 ± 0.15 | | |
| Final meal | 21.2 ± 0.94 | 0.17 ± 0.01 | | |
| Chyme | | | < 0.01 | < 0.05 |
| 20 min | 5.30 ± 1.44 | 0.89 ± 0.24 | | |
| 60 min | 9.31 ± 1.44 | 0.68 ± 0.24 | | |
| 120 min | 7.22 ± 1.44 | 0.93 ± 0.24 | | |
| 300 min | 7.58 ± 1.44 | 1.79 ± 0.24 | | |

DM, dry matter; Values are means ± SEMs, n =6 per chyme, n = 3 for fresh kiwifruit.

### 6.4.2 Degree of hydrolysis of wheat proteins in the stomach

There was an interaction ($P < 0.01$) between digestion time and treatment (with or without actinidin) on the DH of wheat proteins in the stomach (Figure 6.3A). The rate of increase in gastric DH of wheat proteins at 120 min digestion was 0.14%/min for the pigs fed green kiwifruit meal, while it was only 0.04%/min for the pigs fed the yellow kiwifruit meal. At 300 min, the gastric DH for the pigs fed the green kiwifruit meal was 2-fold higher than those fed the yellow kiwifruit meal.

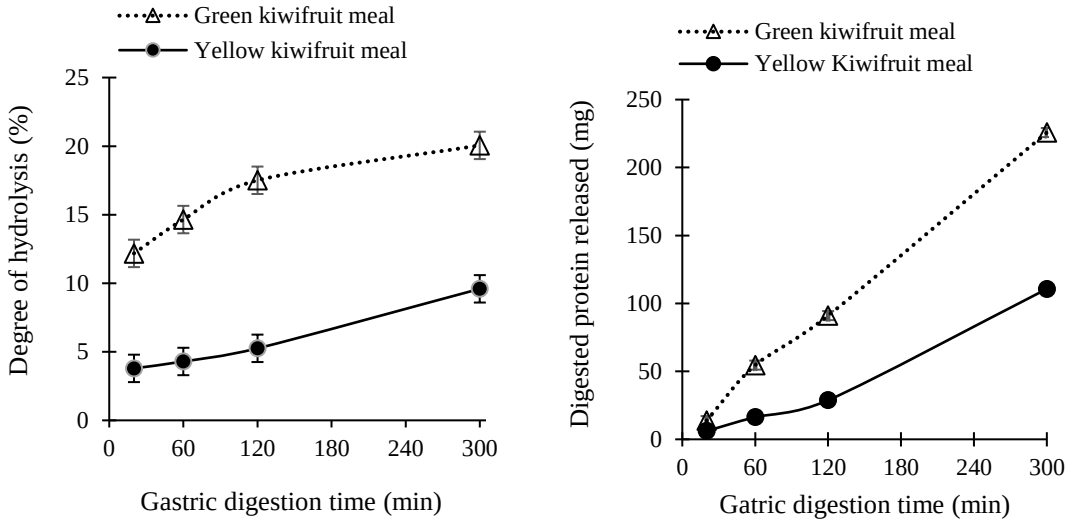


**Figure 6.3** Degree of hydrolysis of wheat proteins in the stomach chyme (A) and the amount of digested protein released into the small intestine (B) at different digestion times in growing pigs fed a bread meal containing either green kiwifruit (with actinidin) or yellow kiwifruit (no actinidin).

The P-values for the enzyme, time, and their interaction effects for degree of hydrolysis were < 0.01, 0.076Q and < 0.05Q respectively and the P-values for the enzyme, time, and their interaction effects for the amount of digested proteins released to the small intestine were < 0.01, < 0.01L and < 0.01L respectively. Q and L, quadratic, and linear effect for the time factor. Values are means ± SEMs, n = 6.

There was an interaction ($P < 0.01$) between digestion time and treatment (with or without actinidin) on the amount of digested protein that entered the small intestine. At

120 min, the pigs fed the green kiwifruit meal had a rate of 0.7 g/min digested protein entering the small intestine whereas it was only 0.2 g/min in the pigs fed the yellow kiwifruit meal (Figure 6.3B).

### 6.4.3 Residual R5 epitopes present in the gastrointestinal tract

The average amount of residual R5 epitopes present in the final meals was 11.6 ± 0.8 mg/dry matter. This amount was reduced over time in the GIT of pigs during digestion with and without actinidin. The interaction ($P < 0.01$) between treatment (with or without actinidin), digestion time and the GIT location (stomach, proximal small intestine, and distal small intestine + terminal ileum) affected the amount of total R5 epitopes present in the GIT of pigs (Table 6.5). Actinidin reduced ($P < 0.01$) the total R5 epitopes present in the stomach, proximal small intestine, and distal small intestine + terminal ileum. The effect of actinidin on R5 epitopes was rapid in the stomach. For instance, at 20 min, the rate of reduction of R5 epitopes in the stomach of the pigs fed the green kiwifruit meal was 4-fold higher than that of pigs fed yellow kiwifruit.

The interaction ($P < 0.05$) between digestion time and treatment (with or without actinidin) had an effect on the amount of residual R5 epitopes that entered the small intestine. The rate of release of R5 epitopes during the first 60 min of digestion was 4-fold lower with the green kiwifruit meal compared to that of the yellow kiwifruit meal (Figure 6.4A). At 120 min, the rate of release of R5 epitopes into the small intestine was 0.3 mg/min in pigs fed the green kiwifruit meal whereas it was 2.2 mg/min in the pigs fed yellow kiwifruit meal.

**Table 6.5** Amount of total residual R5 epitopes along the gastrointestinal tract of growing pigs fed the green kiwifruit diet (with actinidin) and the yellow kiwifruit diet (no actinidin).

| GIT location/ Digestion time (min) | Total residual R5 epitopes* (mg) | | P | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Green kiwifruit meal | Yellow kiwifruit meal | E | T | GIT | E x T | GIT x T | E x GIT | E x T x GIT |
| Stomach | | | < 0.01 | < 0.01 | < 0.01 | > 0.05 | < 0.01 | < 0.01 | < 0.01 |
| 20 | 248.3 ± 21.3$^{b}$ | 1041.9 ± 94.4$^{a}$ | | | | | | | |
| 60 | 200.1 ± 12.8$^{b}$ | 766.0 ± 54.4$^{a}$ | | | | | | | |
| 120 | 111.6 ± 17.3$^{c}$ | 761.8 ± 59.4$^{a}$ | | | | | | | |
| 300 | 45.7 ± 10.4$^{d}$ | 133.7 ± 35.6$^{b,c}$ | | | | | | | |
| Proximal small intestine | | | | | | | | | |
| 20 | 0.51 ± 0.16$^{k}$ | 0.55 ± 0.05$^{k}$ | | | | | | | |
| 60 | 1.4 ± 0.35$^{h,i,j,k}$ | 4.9 ± 1.2$^{e,f,g,h}$ | | | | | | | |
| 120 | 1.2 ± 0.19$^{i,j,k}$ | 2.2 ± 0.53$^{f,g,h,i}$ | | | | | | | |
| 300 | 0.85 ± 0.40$^{j,k}$ | 2.4 ± 1.4$^{e,f,g,h,i}$ | | | | | | | |
| Distal small intestine + Terminal Ileum | | | | | | | | | |
| 20 | 0.14 ± 0.03$^{l}$ | 2.0 ± 0.5$^{g,h,i,j}$ | | | | | | | |
| 60 | 1.7 ± 0.4$^{h,i,j,k}$ | 5.9 ± 0.9$^{e,f,g}$ | | | | | | | |
| 120 | 2.0 ± 0.2$^{g,h,i}$ | 10.2 ± 2.7$^{e}$ | | | | | | | |
| 300 | 1.4 ± 0.2$^{i,j,k}$ | 7.2 ± 1.7$^{e,f}$ | | | | | | | |

*Calculated using the dry matter content present in each gastrointestinal tract location.
Values with different superscripts along the columns and rows are significantly different < 0.05.
E, enzyme; T, time; GIT, gastrointestinal tract location; E x T, interaction between enzyme and time; GIT x T, interaction between gastrointestinal tract location and time; E x GIT, interaction between gastrointestinal tract location and enzyme; E x T x GIT, interaction between enzyme, time, and gastrointestinal tract location. Values are means ± SEMs, n = 6.

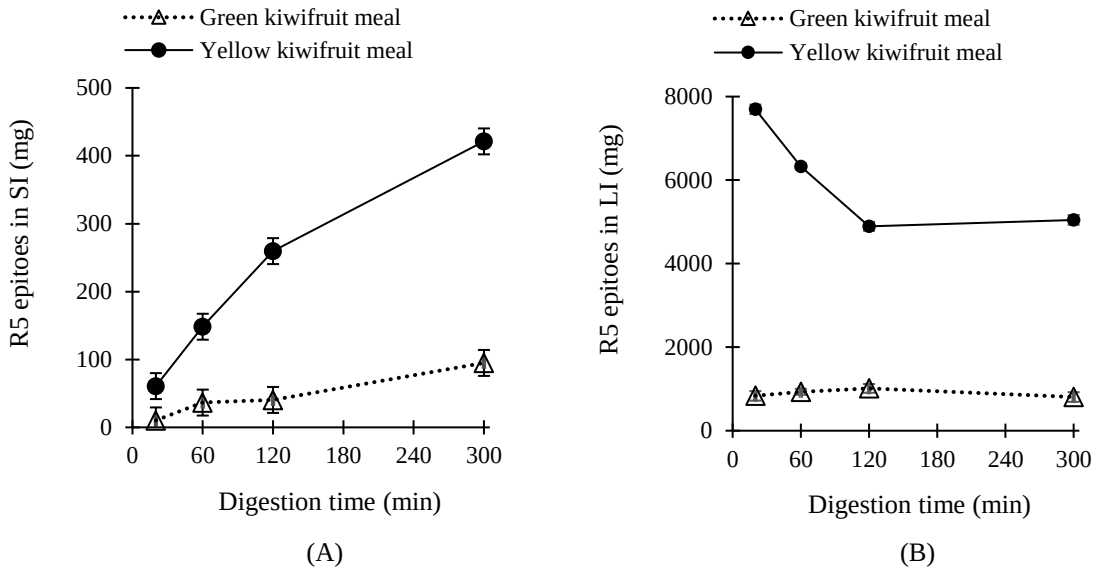


**Figure 6.4** Amount of residual R5 epitopes entering the small intestine (SI) (A) and amount of residual R5 epitopes present in the large intestine (LI) ((B) at different digestion times in growing pigs fed the green kiwifruit meal (with actinidin) and the yellow kiwifruit diet (no actinidin).

The P-values for the enzyme, time, and their interaction effects for the residual R5 epitopes entering the small intestine were < 0.01, 0.01 and < 0.05 respectively and the P-values for the enzyme, time, and their interaction effects for the residual R5 epitopes in the large intestine were < 0.01, 0.08Q and < 0.05Q respectively. Q, quadratic effect for the time factor. Values are means ± SEMs, n = 6.

The amount of R5 epitopes present in the large intestine is a combination of the R5 epitopes that reached the large intestine from the last meal and the diet from the previous meal. The amount of R5 epitopes present in the large intestine (caecum and colon) was affected by an interaction ($P < 0.01$) between treatment (with or without actinidin) and digestion time (Figure 6.4B). Actinidin increased ($P < 0.01$) the rate of hydrolysis of R5 epitopes in the large intestine by 0.6 mg/min on average during the 300 min of GIT digestion.

### 6.4.4 Immunogenic marker peptides in the gastrointestinal tract

All the nine immunogenic marker peptides were observed in the stomach chyme of pigs fed both meals. Among all peptides, the P5 (33-mer) was the most abundant. The interaction between treatment (presence or absence of actinidin) and digestion time had an effect ($P < 0.05$) on the amount of immunogenic marker peptides, P1-P6, and the treatment (presence or absence of actinidin) had an effect ($P < 0.05$) on the amount of P7 and P9 present in the stomach chyme of pigs (Figure 6.5). Actinidin reduced ($P < 0.05$) the amount of P1-P5, P7 and P9 present in the stomach chyme of the pigs. For instance, the amount of P5 (33-mer) was 4.4-fold lower in the stomach chyme of pigs fed the green kiwifruit meal at 20 min of digestion compared to that of pigs fed the yellow kiwifruit meal. For P6, the amount was significantly lower ($P < 0.05$) in the chyme of pigs fed the yellow kiwifruit meal.

The interaction between treatment (presence or absence of actinidin) and digestion time affected ($P < 0.05$) the amount of immunogenic marker peptides, P1, P4 – P6, that entered the small intestine at different gastric digestion times (Figure 6.6). The treatment (presence or absence of actinidin) had an effect ($P < 0.05$) on the amount of P3, P8 and P9 entering the small intestine. Except for P6, P7 and P8, actinidin significantly reduced the amounts of all the other immunogenic marker peptides that entered the small intestine during gastric digestion. For example, at 60 min, the total amount of P5 entering the small intestine was 280.4 µg in the pigs fed the green kiwifruit meal whereas it was 754.7 µg in the pigs fed the yellow kiwifruit meal, which is 9.4 µg/min higher compared to the pigs fed the green kiwifruit meal. The immunogenic marker peptides were not detected in any of the proximal small intestinal samples.

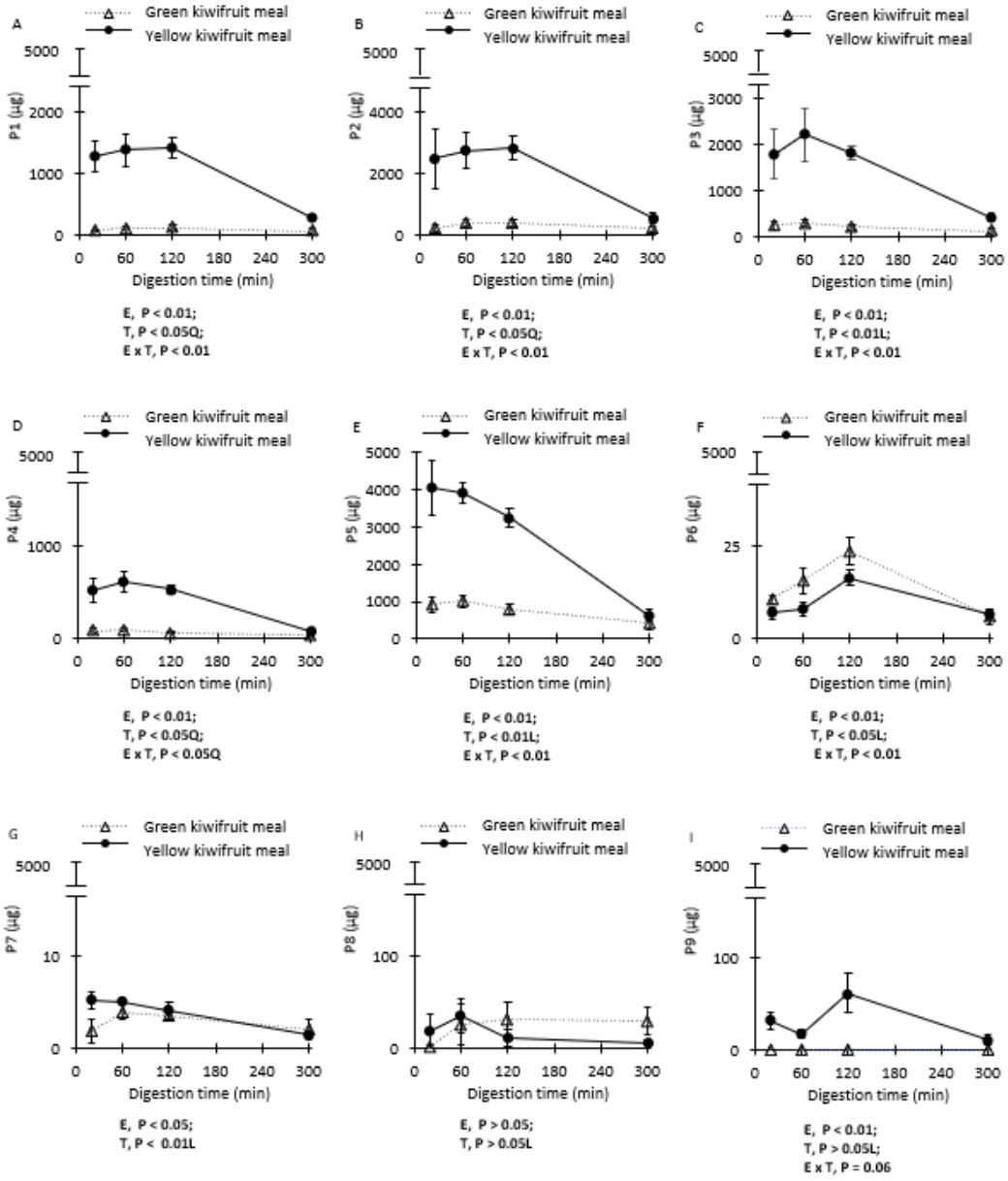


**Figure 6.5** Total amount of nine immunogenic marker peptides (A (P1) – I (P9)) present in the stomach chyme of growing pigs fed the green kiwifruit meal (with actinidin) and the yellow kiwifruit meal (no actinidin).

Q, L, quadratic, and linear effect for the time factor. E, T, and E x T, P-values for the enzyme, time, and their interaction respectively. Values are means ± SEMs, n = 6. Amino acid sequence denoted in one-letter code: P1, LQLQPFPQPQLPY; P2, LQLQPFPQPQLPYPQPQPF; P3, LQLQPFPQPQLPYPQPHLPYPQPQPF; P4, LQLQPFPQPQLPYPQPQLPYPQPQPF; P5, LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF (33-mer); P6, RPQQPYPQPQPQY; P7, RPQQPYPQSQPQY; P8, FQPSQQNPQAQGSFQPQQLPQF; P9, VRVPVPQLQPQNPSQQQPQEQVPLVQQQ

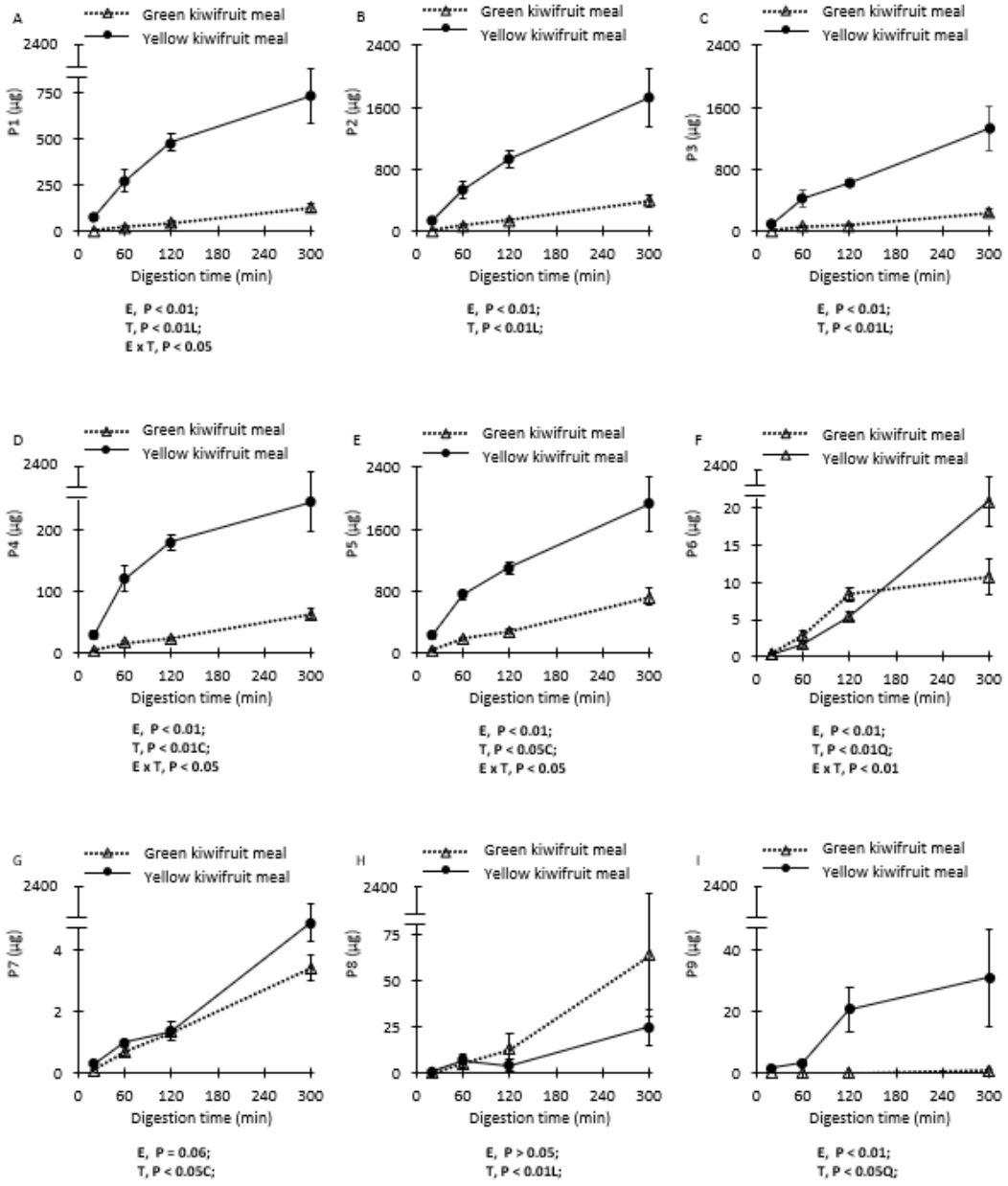


**Figure 6.6** Total amount of nine immunogenic marker peptides (A (P1) – I (P9)) entering the small intestine of growing pigs fed green kiwifruit meal (with actinidin) and yellow kiwifruit meal (no actinidin) at different gastric digestion times.

Q, L, C, quadratic, linear and cubic effect for the time factor. E, T, and E x T, P-values for the enzyme, time, and their interaction respectively. Values are means ± SEMs, n = 6. Amino acid sequence denoted in one-letter code: P1, LQLQPFPQPQLPY; P2, LQLQPFPQPQLPYPQPQPF; P3, LQLQPFPQPQLPYPQPHLPYPQPQPF; P4, LQLQPFPQPQLPYPQPQLPYPQPQPF; P5, LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF (33-mer); P6, RPQQPYPQPQPQY; P7, RPQQPYPQSQPQY; P8, FQPSQQNPQAQGSFQPQQLPQF; P9, VRVPVPQLQPQNPSQQQPQEQVPLVQQQQF

### 6.4.5 Immunogenic peptide profiles in the gastrointestinal tract

Results of the untargeted peptidomics revealed that the stomach chyme of pigs fed the green kiwifruit meal had a lower number of identified immunogenic peptides than the stomach chyme of pigs fed the yellow kiwifruit fed meal during the first 120 min post-feeding (Table 6.6). A small number of immunogenic peptides was identified in the proximal small intestinal samples from both diets.

The full list of the peptides is given in the google drive folder: https://drive.google.com/drive/folders/1JPm_499OsfVS1LNsPYUxb_0yKTHRedSd?usp=sharing.

**Table 6.6.** Number of immunogenic gluten peptides identified from untargeted LC-MS/MS in the stomach chyme and the proximal small intestine of growing pigs fed the green kiwifruit diet (with actinidin) and yellow kiwifruit diet (no actinidin)[1].

| | Green kiwifruit diet | Yellow kiwifruit diet |
|---|---|---|
| Stomach chyme | | |
| 60 min | 28 | 57 |
| 120 min | 32 | 59 |
| Proximal small intestine | | |
| 60 min | 0 | 5 |
| 120 min | 2 | 0 |

[1]Peptides were identified across six pooled replicates.

## 6.5 Discussion

In the present study, the hydrolysis of wheat proteins and gluten-derived immunogenic peptides was determined in the GIT of growing pigs in the presence or absence of actinidin. To our knowledge, this is the first study conducted with an animal model to evaluate the efficacy of an enzyme to hydrolyse gluten and its immunogenic peptides along the GIT.

The length of the immunogenic epitopes that can create innate or adaptive immune responses can be as short as four amino acids (Juhász et al., 2015). To determine the range of immunogenic epitopes, this study used several analytical techniques. The ELISA was used to determine the common gluten epitopes containing five amino acids and untargeted peptidomics determined the immunogenic peptides having six or more than six amino acids. The targeted mass spectrometry was used to quantify nine commonly found gluten-derived immunogenic peptides (Ogilvie et al., 2020; van den Broeck et al., 2015) having different chain lengths of up to 33 amino acids.

### 6.5.1 Gastric digestion of immunogenic peptides

Actinidin was active in the stomach of pigs fed the green kiwifruit meal even at 300 min and the actinidin activity observed in the chyme was lower than that observed in the meal. In contrast to the present observation, Montoya & Rutherfurd et al. (2014) observed a greater actinidin activity (~90 μmol-o-nitrophenol/min per g of dry matter) after 180 min in the stomach chyme of pigs when actinidin was consumed in the form of fresh green kiwifruit than that present in the original diet (36 μmol-o-nitrophenol/min per g of dry matter). The actinidin activity of the small intestinal samples could not be determined as the substrate used in the study, cbz-l-Lys-*o*-nitrophenol hydrochloride, was also hydrolysed by small intestinal proteases. However, considering the amount of actinidin in the gastric chyme, it can be expected that actinidin is active in the small intestine as has been shown *in vitro* (Grozdanovic et al., 2014). Actinidin has been found to be active around pH 7 (Sun et al., 2016).

The rate of gastric hydrolysis of wheat proteins was 3.2-fold higher at 20 min of gastric digestion when actinidin was added to the bread meal and this increased rate of hydrolysis was observed throughout the gastric digestion of 300 min in the pigs fed the

green kiwifruit meal. The effect of actinidin on wheat protein hydrolysis was rapid where the highest rate of hydrolysis was observed, between 20 – 60 min of digestion. Similarly, Montoya, & Hindmarsh, et al. (2014) observed a 3.2-fold enhanced gastric hydrolysis of wheat gluten in rats at around 60 min post-feeding when actinidin was present in the diet. Rutherfurd et al. (2011) observed a significantly higher gastric degradability (or disappearance of intact protein observed by gel electrophoresis) of specific gluten protein fractions (ω-gliadin and three low molecular weight subunits; 35, 37, and 41 kDa of glutenin) in rats fed a diet containing actinidin.

During the gastric digestion of wheat proteins, the amount of immunogenic marker peptides, the number of immunogenic peptides and the amounts of residual R5 epitopes were reduced over time when actinidin was added to the meal. For instance, actinidin was able to hydrolyse the highly immunogenic 33-mer peptide (P5) and significantly reduced the amount during gastric digestion.

In the present study, the rate of gastric emptying observed was similar between the two meals. In other words, actinidin did not increase the gastric emptying rate of the bread meal. This is in contrast to results previously found in rat studies.[21] This signifies that the wheat proteins/gluten proteins could remain in the stomach for gastric digestion without being emptied faster into the small intestine, providing time for actinidin to act on gluten proteolysis. Actinidin increased the amount of digested wheat protein that entered the small intestine by two-fold during the 300 min of gastric digestion. This enhanced digestion with actinidin corresponded to a 4-fold reduction in the amount of R5 epitopes that entered the small intestine at 300 min. On average, actinidin reduced the amount of all the immunogenic marker peptides that entered the small intestine by 70% at 300 min postprandial.

### 6.5.2 Intestinal digestion of immunogenic peptides

In the present study, the amounts of immunogenic peptides along the small intestine and large intestine were determined based on the residual R5 epitopes. The immunogenic marker peptides and other immunogenic peptides were not detected in the small intestine.

Whole wheat bread chyme usually takes approximately 20 min to start to release into the small intestine (Darwiche et al., 2001; Grimes & Goddard, 1977). This chyme is exposed to a large pool of small intestinal enzymes where the rest of the protein digestion takes place. However, the amount of digested protein entering the small intestine plays a role in the rate of digestion of protein in the small intestine (Montoya et al., 2018). Therefore, an exogenous enzyme (like actinidin) that rapidly acts on gluten is required to complete gluten proteolysis before the chyme enters the small intestine, where immunogenic reactions can occur. Some of the small intestinal brush-border enzymes, such as dipeptidylcarboxypeptidase and dipeptidylaminopeptidase can hydrolyse proline-containing peptide bonds (Gray, 2010; Hausch et al., 2002). In addition, actinidin can also help in hydrolysing proline-containing peptide bonds in the small intestine and appeared to cleave gluten in the small intestine (Jayawardana et al. unpublished data). The actions of both small intestinal enzymes and actinidin can therefore further reduce the accumulation of immunogenic peptides in the small intestine, as observed in the present study.

It has been proposed that the increase in the hydrolysis of gluten by gastric digestion can increase the gluten peptide immunogenicity by the generation of small immunogenic gluten peptide fragments that can more easily enter the small intestinal lamina propria than the larger immunogenic peptides produced by the human gastrointestinal proteases (Krishnareddy et al., 2017). However, this did not happen

with actinidin in the present study. The enhanced gastric hydrolysis of gluten by actinidin did not increase the amount of immunogenic peptides in the small intestine, which was confirmed by the ELISA results, where the amount of R5 epitopes present in the small intestinal digesta along the proximal small intestine, distal small intestine and terminal ileum of the pigs fed the green kiwifruit meal showed a significant reduction at all digestion time points.

The amount of R5 epitopes present in the large intestine of the pigs fed the green kiwifruit meal was significantly lower at all time points, signifying that the amount of immunogenic peptides reaching the large intestine would also have been lower when actinidin was present. In gluten-related health disorders, investigation of the effect of immunogenic peptides has always been focussed on the small intestine (primarily the duodenum) (Chander et al., 2018), but the present results indicate an accumulation of immunogenic peptides can occur in the large intestine and it would be worth investigating if these peptides in the large intestine also have an effect on any inflammation in the large intestinal epithelium of sensitive individuals.

In the pigs fed the yellow kiwifruit meal (absence of actinidin), due to poor peptic proteolysis of wheat proteins, a relatively low amount of digested proteins entered the small intestine. Due to the action of small intestinal enzymes, these proteins would be partially digested into larger peptides, creating a pool of immunogenic peptides in the small intestine. This was clearly revealed from the ELISA results of the present study where the R5 epitopes accumulated in the small intestine after 20 min of digestion, and throughout the 300 min postprandial. In agreement with the present results, poor gastric digestion of gluten proteins and the release of immunogenic peptides during the small intestinal digestion has been observed in other *in vitro* studies (Ogilvie et al., 2020; Prandi et al., 2012).

From a pathological point of view, one of the most important factors in the development of gluten-related health disorders is the amount of immunogenic peptides present in the small intestine, which can trigger immune responses. As discussed above, both gastric and small intestinal digestion of gluten proteins are important in reducing or eliminating immunogenic peptides in the small intestine. The present study used a normal meal size (4 slices of bread with two fresh kiwifruit) which contains around 13 g of gluten per meal. Based on the ELISA results, the actinidin provided from two fresh kiwifruit was adequate to reduce the R5 epitopes in this meal during gastric and small intestinal digestion to a level of < 2 mg throughout the intestine of pigs. It has been identified that the safe limit of gluten intake could be a daily intake of less than 10 mg of gluten for subjects with coeliac disease (Akobeng & Thomas, 2008; Catassi et al., 2007), as definite effects on the mucosal histology of coeliac patients could be observed even with 10 mg of gluten. The minimum daily intake of gluten to initiate wheat allergy has been reported to be higher than that for coeliac disease, around 1-2 g of wheat proteins (Hischenhuber et al., 2006). A clinical study is warranted to test the effect of actinidin with a moderate or low amount of gluten in the diet. Moreover, these results suggest that following unintentional consumption of gluten-containing products by sensitive subjects, consumption of green kiwifruit may alleviate adverse effects.

### 6.6 Conclusion

Lower gastric hydrolysis of wheat proteins and immunogenic gluten peptides was observed during 300 min postprandial, when a normal bread meal was fed to growing pigs, which resulted in a higher amount of undigested proteins and immunogenic peptides that entered the small intestine of these pigs when compared to the pigs fed the same bread meal with actinidin. This was corresponded to a higher amount of

immunogenic peptides present along the intestine of the growing pigs fed a normal bread meal throughout the digestion until 300 min postprandial than the pigs fed the same meal with actinidin. Actinidin hydrolysed wheat proteins available in whole wheat bread rapidly during gastric digestion and reduced the amount of immunogenic peptides in the GIT postprandially when fed to growing pigs as fresh green kiwifruit. Actinidin is able to survive peptic proteolysis and gastric pH conditions until 300 min postprandial. In this promising scenario, green kiwifruit probably could reduce the risk of the unintentional (or intentional) consumption of small amounts of gluten-containing products in those who have gluten-related health disorders and is probably a promising candidate for an oral enzyme therapy to supplement a gluten-free diet. As opposed to microbial origin enzymes, actinidin is a natural food-based route to solve the problem. Further preclinical and clinical studies to assess the effect of kiwifruit on gluten immunogenicity are warranted.

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# Chapter Seven

# 7. Overall discussion, conclusions, and future directions

Gluten intolerance has become a growing health issue worldwide. At present, there is only one solution to overcome this, to follow a strict gluten-free diet. Alternative therapies are being proposed and one of them is the dietary supplementation of exogenous enzymes to increase gluten hydrolysis. Exogenous enzyme therapy has been identified as one of the effective alternatives or adjuncts to gluten-free diet. This therapy requires proteases that can digest glutamine- and proline-rich gluten peptides. These enzymes must be resistant to proteolysis by host and microbial enzymes (e.g., pepsin in the stomach) and active/stable in the conditions of the stomach and/or upper small intestine (e.g., low pH of the stomach). Several proteases have been proposed (e.g., prolyl endopeptidases (Benucci et al., 2020; Moreno Amador et al., 2019), endopeptidase 40 (Cavaletti et al., 2019), glutamine-specific cysteine endoprotease (Gass et al., 2007)). However, most of the proteases are of microbial origin and are not yet validated for safe long-term human use. It is, therefore, timely to identify natural, readily available, and cost-effective enzymes that may be suitable for safe long-term usage. However, natural enzymes have not been widely tested as therapeutic enzymes for gluten detoxification. In the present study, actinidin, the predominant protease present in green kiwifruit, has been proposed as a natural enzyme that can hydrolyse immunogenic gluten peptides.

The proteolytic activity of actinidin with different protein sources has previously been cited (e.g., casein, beef muscle protein, soy protein isolate, gelatin, gluten) (Kaur et al., 2010; Lewis & Luh, 1988; Montoya et al., 2014; Puglisi et al., 2012; Rutherfurd et al.,

2011). However, to determine whether actinidin is a suitable exogenous enzyme to treat or alleviate gluten-related health disorders, it is vital to determine its capacity to hydrolyse glutamine- and proline-rich immunogenic gluten peptides.

The present study investigated whether actinidin from green kiwifruit was able to enhance gastric and small intestinal digestion of gluten proteins or gluten-containing foods and thereby reduce or eliminate the gluten peptides thought to be involved in immunogenic reactions. To test this, the present research study used a hierarchical approach combining several digestion models with different gluten (purified gluten and whole wheat soda bread) and actinidin sources (purified actinidin and kiwifruit) (Figure 7.1).

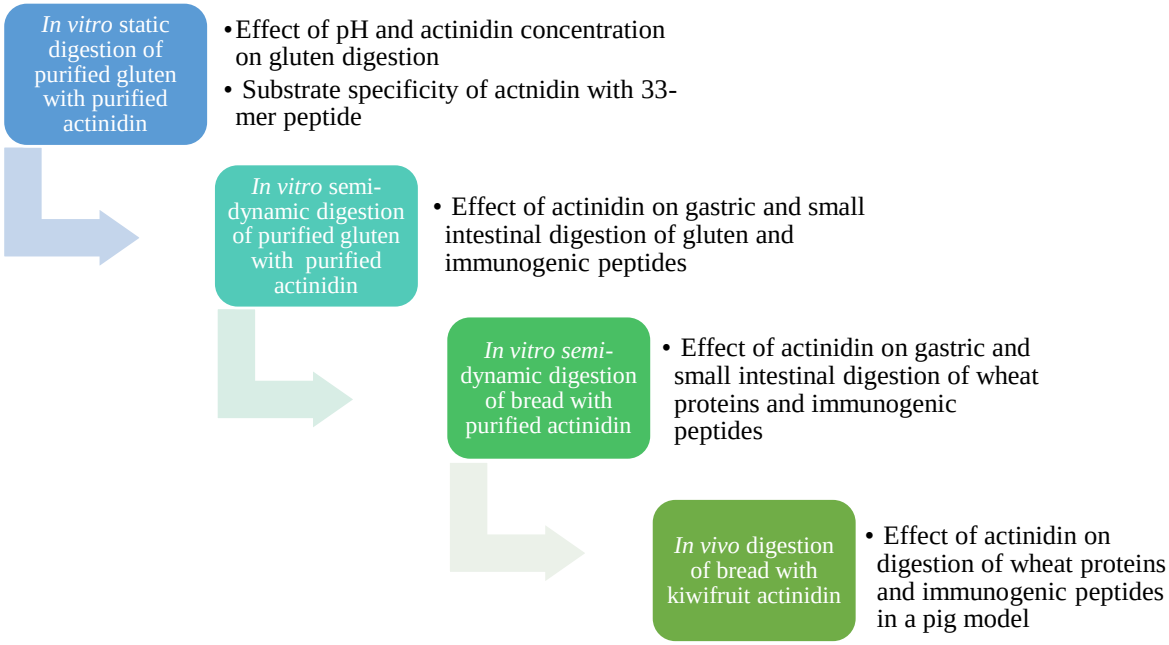


**Figure 7.1.** Schematic overview for testing the effect of actinidin on hydrolysis of gluten and its immunogenic peptides in the present research.

### 7.1 Digestion models

The present study used four different digestion models to understand the effect of actinidin on the hydrolysis of gluten and its immunogenic peptides. The first one was an *in vitro* closed and static digestion model where the gastric digestion continued for 120 min and the small intestinal digestion started after gastric digestion. The gastric pH was maintained at 4.0 throughout the gastric digestion. This model was useful to provide some basic mechanistic understanding of the effect of actinidin on the digestion of gluten proteins and their resultant peptides. This model did not consider parameters such as gradual acidification in the stomach and the continual release of chyme to the small intestine (i.e., gastric emptying).

The second model was an *in vitro* semi-dynamic digestion model which considered those dynamic parameters. This model allowed me to calculate important information such as the amount and number of different types of immunogenic peptides released into the small intestine during GIT digestion.

For the first two models, purified gluten was used as the source of gluten. The ability of actinidin to digest gluten could be affected by a food matrix like bread or pasta etc. Nutrients such as carbohydrates, fats, other proteins, and their interactions in gluten-containing foods could create specific matrixes that can limit the access of actinidin to gluten proteins (Mat et al., 2016). Therefore, in the third digestion model whole wheat soda bread was used as the source of gluten since bread is one of the commonly consumed gluten-containing foods around the world. Soda bread was used to avoid dietary proteins other than the wheat proteins. The digestion model used was a semi-dynamic model where oral digestion of bread was conducted using a human panel to get real oral physical disintegration of bread before gastric digestion. To simulate gastric digestion, a HGS which mimicked dynamic parameters of gastric digestion such

as reduction of pH, temperature, gastric secretions, peristaltic mixing, and gastric emptying was used, while a static digestion model was used to simulate small intestinal digestion. This model allowed me to understand the effect of actinidin on the digestion of wheat proteins and gluten immunogenic peptides in a food matrix when a normal amount of gluten was consumed.

The fourth digestion model was a pig model, where pigs were fed whole wheat soda bread and kiwifruit to understand the effect of actinidin on the digestion of a normal amount of gluten and its immunogenic peptides in an *in vivo* system. Pigs were used as an adult human model due to the anatomical and physiological similarities between the mouth and the terminal ileum (the last part of the small intestine) (Deglaire & Moughan, 2012).

### 7.2 Substrate specificity, optimum pH, and gastric stability of actinidin

As a starting point for the research in this PhD, a detailed mass spectral analysis of the hydrolysis of a synthetic peptide, the 33-mer peptide, which has been widely reported as one of the most digestion-resistant and immunogenic peptides from α-2 gliadin (residues 56–88) (Qiao et al., 2004), by actinidin was conducted. This analysis revealed that actinidin cleaved multiple peptide bonds including most of the N- and C-terminal bonds of the prolines in the 33-mer peptide. Actinidin hydrolysed the 33-mer peptide at a wide range of sites in a broad specific manner, including Q-X (X=L, P), L-Q, F-P, P-X (X=Q, Y, F) (Q= glutamine, L= leucine, P=proline, F= phenylalanine, Y= tyrosine), and only one Y-P bond. This highlights the potential of actinidin to hydrolyse immunogenic gluten peptides as these peptide motifs are commonly present in immunogenic gluten peptides.

The pH and actinidin concentration affected the level of hydrolysis of gluten proteins during gastric digestion. In general, the hydrolysis of gluten increased when the actinidin concentration and pH increased. However, at pH equal to or lower than 2, the level of actinidin concentration reduced effect on the hydrolysis of gluten. Actinidin at a concentration of > 2.7 U/mL and pH > 2 (optimum pH of actinidin for gluten ranges between 2 and 7) is considered ideal for gluten hydrolysis. This minimum amount of actinidin concentration of 2.7 U/mL corresponds to having about half a kiwifruit (15 U/mL and ~85 mL of juice per kiwifruit) per 250 mL of stomach volume. The stomach pH (~2 - 5.1) ~1 h after consuming bread in healthy individuals (Hoebler et al., 2002) is in this active pH range of actinidin. Therefore, it can be assumed that after consuming a gluten-containing meal, the pH in the stomach would support the hydrolysis of gluten proteins by actinidin.

Actinidin was stable and active under gastric conditions *in vitro* and *in vivo* and actinidin activity decreased with postprandial time. Under *in vitro* conditions, actinidin remained active until 90 min of gastric digestion. However, *in vivo* actinidin was active even at 300 min postprandial. The *in vitro* studies used purified actinidin powder whereas in the *in vivo* study, fresh kiwifruit was used. The prolonged actinidin activity observed with the fresh kiwifruit could be due to the activation of actinidinogen, a pro-enzyme of actinidin, with time, as significant amounts of precursor are known to be present (Hopsu-Havu et al., 1997). This suggests that actinidin is stable for peptic proteolysis and active under stomach conditions, which enables actinidin to hydrolyse gluten proteins for a prolonged period. In addition, actinidin did not affect the stomach emptying of bread in pigs, which means that gluten (and its immunogenic peptides) remained in the stomach for hydrolysis, without getting emptied faster.

### 7.3 Gastric digestion of gluten/wheat proteins and gluten derived immunogenic peptides

The effect of actinidin on gastric hydrolysis of gluten and wheat proteins is significantly higher compared to the gastric hydrolysis of gluten and wheat proteins in the control with pepsin only both under *in vitro* and *in vivo* conditions (Table 7.1). Actinidin accelerated the hydrolysis of gluten and wheat proteins during the first 90 min of gastric digestion (e.g., 0.19 and 0.16%/min during *in vitro* and *in vivo* respectively).

This is likely to be due to the broad amino acid specificity of actinidin, as discussed above, that enables it to hydrolyse a wide range of peptide bonds. This could also be due to the pH of the gastric medium supporting the action of actinidin.

**Table 7.1** Degree of hydrolysis (DH) of gluten and wheat proteins at 60 min of gastric digestion under different digestion models with and without actinidin.

| | | DH (%) | |
|---|---|---|---|
| **Digestion model** | **Gluten source** | **Actinidin** | **No actinidin** |
| *In vitro* static | Purified gluten | 9.2 ± 0.4 | 0.8 ± 0.4 |
| *In vitro* semi-dynamic | Purified gluten | 14.5 ± 0.7 | 0.5 ± 0.1 |
| *In vitro* semi-dynamic | Wheat protein | 3.3 ± 1.0* | 1.0 ± 0.5* |
| *In vivo* pig | Wheat protein | 14.6 ± 0.4 | 4.2 ± 0.3 |

Values are means ± SEMs, n = 3 6 for *in vitro* or *in vivo* studies respectively.
*Calculated based on the amount of free amino groups present in chewed bread as follows:
DH% = (Free amino groups present in the sample at 60 min - Free amino groups present in the sample at 0 min)/ Free amino groups present in chewed bread.

The rapid gastric hydrolysis of gluten and wheat proteins in the presence of actinidin resulted in significantly lower amounts of residual R5 epitopes in the gastric conditions both *in vitro* and *in vivo*. The lower amount of R5 epitopes with actinidin in the samples reaching the small intestine (*in vitro* and/or *in vivo*) indicates that protein fragments and/or peptides containing these motifs were hydrolysed when actinidin was present

during gastric digestion. In other words, actinidin was able to hydrolyse the R5 motifs containing peptide sequences and reduce the amount of R5 epitopes reaching the small intestine.

**Table 7.2** Amount of residual R5 epitopes reaching the small intestine at 60 min under different digestion models with and without actinidin.

| | | R5 epitopes (µg/g) | |
|---|---|---|---|
| **Digestion model** | **Gluten source** | **Actinidin** | **No actinidin** |
| *In vitro* semi-dynamic | Purified gluten | 47.1 ± 4.9 | 446.5 ± 30.0 |
| *In vitro* semi-dynamic | Wheat protein | 271.8 ± 83.9 | 395.2 ± 97.8 |
| *In vivo* pig | Wheat protein | 11.3 ± 0.6 | 53.2 ± 2.9 |

Values are means ± SEMs, n = 3.

Aligned to rapid hydrolysis of R5 epitopes during gastric digestion, the number of identified immunogenic peptides released during gastric digestion by untargeted peptidomics analysis was markedly lower with actinidin both *in vitro* and *in vivo*. However, contradictory results were observed when a few marker immunogenic peptides were quantified in the *in vitro* and *in vivo* gastric digestion of bread. *In vitro*, the amount of most of the marker immunogenic peptides was higher in the bread digesta released into the small intestine with actinidin than *in vivo*. This could be due to the lower pepsin activity in the simulated gastric conditions. Pepsin can help to release some of the cryptic peptides for actinidin to act on. In addition, the shape of the stomach compartment, differences in the gastric motility and gastric emptying mechanism, and high variability due to poor mixing in the gastric compartment could also contribute to the differences observed in the immunogenic marker peptide concentration *in vitro* and *in vivo*.

### 7.4 Small intestinal digestion of gluten/wheat proteins and gluten derived immunogenic peptides

The effect of actinidin on the small intestinal digestion of gluten proteins during *in vitro* static digestion was negligible. It was assumed that this could be due to the static nature of the digestion model with actinidin hydrolysing all potential peptide bonds during prolonged gastric digestion. However, in the semi-dynamic digestion model, actinidin was able to hydrolyse gluten during small intestinal digestion, but the magnitude of the DH was smaller than in the gastric digestion. This could be due to the lower activity of actinidin under small intestinal conditions since the upper limit of the actinidin activity falls around pH 7. Unlike pepsin, pancreatic enzymes can partially hydrolyse actinidin at pH ~7.0 (Bublin et al., 2008), which could also explain the lower hydrolysis determined during small intestinal digestion.

**Table 7.3** Amount of residual R5 epitopes at 60 min in the small intestine under different digestion models with and without actinidin.

| Digestion model | Gluten source | GIT location | R5 epitopes (μg/g) | |
|---|---|---|---|---|
| | | | Actinidin | No actinidin |
| *In vitro* semi-dynamic | Purified gluten | SI | 10.01 ± 1.7 | 54.6 ± 5.0 |
| *In vitro* semi-dynamic | Wheat protein | SI | 42.9 ± 24.7 | 65.4 ± 37.8 |
| *In vivo* pig | Wheat protein | PSI | 2.1 ± 0.9 | 8.2 ± 0.9 |
| | | DSI | 0.5 ± 0.1 | 2.0 ± 0.2 |

Small intestine, SI; PSI, Proximal small intestine (first half of the SI); DSI, Distal small intestine (second half of the SI). Values are means ± SEMs, n = 3.

The amount of residual R5 epitopes observed in the small intestine during digestion was substantially lower with actinidin both *in vitro* and *in vivo*. In the *in vitro* semi-dynamic digestion, the amount of residual R5 epitopes present in the small intestine

throughout the small intestinal digestion was markedly lower when actinidin was included in the digestion (Table 7.3) though, it was not significant when the soda bread was digested *in vitro*. Importantly, actinidin decreased the amount of residual R5 epitopes *in vivo* over time throughout the whole small intestine (proximal small intestine to terminal ileum).

The number of identified immunogenic peptides (by untargeted peptidomics) in the small intestine was consistent with the results of residual R5 epitopes where a lower number of immunogenic peptides was identified with actinidin both *in vitro* and *in vivo* compared to the control.

Similarly, the quantity of most of the immunogenic marker peptides showed a significant reduction in the small intestine during the small intestinal digestion *in vitro*. For example, the amount of the marker peptides P1 – P5 including the highly immunogenic 33-mer peptide (P5) was markedly reduced up to 90% during the first 20 min of small intestinal digestion with actinidin. However, *in vivo*, marker immunogenic peptides were not detected in the small intestinal samples. A marker peptide cannot be detected if a single peptide bond is broken, and *in vivo*, host (brush border) and microbial proteases can cleave proline-containing peptide bonds in gluten immunogenic peptides (Duar et al., 2015; Gray, 2010; Hausch et al., 2002).

### 7.5. Physiological relevance of rapid gluten digestion with actinidin

Both the amount of immunogenic peptides present in the small intestine and the time of exposure of the small intestinal epithelium to these immunogenic peptides are important factors responsible for triggering an immunological response in those individuals with gluten-related health disorders (Hausch et al., 2002). To avoid (or reduce) this response, it is essential to increase the hydrolysis of gluten immunogenic

peptides in the stomach. In the stomach, pepsin alone cannot hydrolyse these gluten immunogenic peptides. Thus, peptic proteolysis results in partial hydrolysis of gluten and a high amount of immunogenic peptides reaching the small intestine. In the small intestine, the partially hydrolysed gluten proteins are further hydrolysed by small intestinal proteases (pancreatic and brush border enzymes) leading to the release of cryptic immunogenic peptides and their accumulation (Ogilvie et al., 2020; Prandi et al., 2012). Some of these immunogenic peptides can be hydrolysed by brush border enzymes (e.g., aminopeptidases and dipeptidylcarboxypeptidase) and microbial proteases (e.g., enzymes secreted by *Pseudomonas aeruginosa* several *Lactobacillus* spp) if present. However, in a compromised small intestinal epithelium of an individual with gluten-related health disorders, the capacity of the epithelium to produce brush border proteases is weakened (Mercer et al., 1990) and the time of exposure to gluten immunogenic peptides is expected to be longer. In the present *in vivo* study, commercial pigs that were exposed on the farm to gluten-containing ingredients were used. Thus, it could be expected that more immunogenic peptides were cleaved in this animal model than in an individual with a gluten-related health disorder. Thus, from this model, it is not possible to determine if the reduction of immunogenic peptides in the small intestinal digestion can be ascribed only to actinidin.

Actinidin enhanced the rate of gastric hydrolysis of both gluten and immunogenic gluten peptides *in vitro* and *in vivo*. This resulted in a lower amount of immunogenic peptides being released into the small intestine. This concentration remained low during subsequent small intestinal digestion both *in vitro* and *in vivo*. Thus, actinidin was able to reduce both the amount of and the time of exposure to immunogenic peptides in the small intestine. For example, when considering the 33 mer peptide, the amount of the

peptide was reduced 2.3-fold at 60 min of *in vivo* digestion with actinidin compared to the control.

The average Western diet contains roughly 5-20 g of gluten per day (Hoppe et al., 2017). For instance, a slice of whole-wheat bread (45 g with 8% protein) and a serving of pasta (75 g with 11% protein) contain around 3.6 and 8.3 g of gluten, respectively. It has been identified that the safe limit of gluten intake could be a daily intake of less than 10 mg of gluten for subjects with coeliac disease (Akobeng & Thomas, 2008; Catassi et al., 2007). The *in vivo* study used a normal meal size (4 slices of bread with two fresh kiwifruit) which contains around 13 g of gluten per meal. Based on the ELISA results, the actinidin provided from two fresh kiwifruit was adequate to reduce the R5 epitopes in this meal during gastric and small intestinal digestion to a level of < 2 mg throughout the intestine of pigs. Thus, it could be expected that kiwifruit intake might help to reduce the difficulties caused by accidental intake of gluten by people with gluten-related health disorders. A clinical study is warranted with individuals who have gluten-related disorders to test the effect of actinidin with a moderate or low amount of gluten in the diet. In addition, in this study, we used only two kiwifruit or purified actinidin content equivalent to consuming two kiwifruit to determine the efficacy of actinidin to hydrolyse gluten. However, the actinidin concentration has a positive correlation with the hydrolysis of gluten and immunogenic peptides. Therefore, actinidin might eliminate the immunogenic peptides if a higher concentration is used.

To summarise, a natural plant enzyme, actinidin, has been investigated for its ability to hydrolyse purified gluten and a gluten-containing model food (whole wheat soda bread) using an array of *in vitro* and *in vivo* GIT digestion models. This study answered all the research questions mentioned in the Chapter 1. Actinidin was resistant to peptic

hydrolysis and was active under physiological gastric conditions even after 300 min postprandial. Actinidin hydrolysed gluten and wheat proteins and gluten immunogenic peptides, including the highly resistant 33-mer gliadin peptide, and peptides containing QQQ/PFP present in gluten epitopes, by cleaving multiple peptide bonds specifically the peptide bonds at the N- and C-terminal of proline residues. The optimum pH of actinidin for gluten hydrolysis ranges between 2 and 7 pH. Actinidin at a concentration of $\geq$ 2.7 U/mL is considered ideal for gluten hydrolysis, which is equivalent to the consumption of two kiwifruit with a normal gluten-containing meal. The greater hydrolysis of gluten with actinidin in gastric conditions contributed to increase the hydrolysis of gluten peptides in the small intestine and to reduce the presence of immunogenic peptides in the small intestine in a normal gluten-containing diet. Actinidin was able to reduce both the amount of, and the time of exposure to immunogenic peptides in the small intestine *in vivo*, however, actinidin did not eliminate the immunogenic peptides present in the small intestine when a normal gluten-containing meal was consumed. A clinical study with individuals who have gluten-related disorders is warranted to determine the efficacy of actinidin to eliminate the immunogenic peptides and thereby the complications caused by these immunogenic peptides when gluten is accidentally ingested or when a lower amount of gluten is consumed.

Overall, the findings of this research might help for those who have gluten-related health disorders, who find it very difficult to ensure there is absolutely no contamination of their food from gluten. Fruit consumption is much more practical for consumers, as it forms part of a daily diet with added benefits of micronutrients and dietary fibre. Actinidin is a potential candidate to be considered in alternative therapies for gluten-related health disorders.

### 7.6 Limitations and future directions

The *in vitro* and *in vivo* models used in this study had several limitations. The static digestion model used in the first experimental chapter did not simulate the gastric secretions and dynamic parameters of gastrointestinal digestion. The semi-dynamic model used in the second experimental chapter simulated some of the dynamic parameters of gastrointestinal digestion, however, not the gastric contractions and secretions. The HGS used in the third experimental chapter had several disadvantages. The shape of the stomach compartments is different to the real human stomach and the gastric motility and gastric emptying mechanism were different from real gastric digestion, therefore a high variability was observed in technical replicates. In addition, we used a healthy pig model as our animal model which is not representative of the actual small intestine of an individual with gluten-related health disorders. Therefore, the digestion results we observed may not represent the actual digestion we could expect in an individual who has one of the gluten-related health disorders.

The findings of the present study answered key research questions related to actinidin activity in the stomach, and the ability of actinidin to enhance gastric and small intestinal digestion of gluten or gluten-containing foods and thereby reduce the immunogenic gluten peptides. As highlighted in the previous section, the consumption of kiwifruit with a gluten-containing food might mitigate the immunogenic reactions that occur after ingestion of gluten in genetically predisposed individuals. However, the methodological techniques used in the present PhD did not directly evaluate the reduction of immunogenic reactions. The reduction of the immunogenicity could have been evaluated using different methodological approaches:

1. Use of *in vitro* models of epithelial cell lines such as Caco-2, IEC-6, monocytes, and dendritic cells to measure the cytoskeletal rearrangement and

disruption of the tight junction integrity, expression of inflammatory cytokines (IL-15, TNFα and IL-8) (Barone et al., 2011; Jelínková et al., 2004), which are common inflammatory reactions of gluten-related health disorders.

2. Use of *in vitro* mucosal biopsy cultures extracted from celiac patients to evaluate the T-cell stimulatory potential of gluten epitopes and how gluten epitopes are transported across the surface epithelium via the paracellular route or via the transcellular route (de Ritis et al., 1988; Lebreton et al., 2012).

3. Use of spontaneous animal models i.e., dog and monkey models, in which the gluten-related health disorders occur naturally, to measure gluten-related immunogenic responses such as villous atrophy, the skin manifestation of celiac disease (Batt et al., 1985; Bethune et al., 2008).

4. Use of animal models such as rat and mouse where induced enteropathy to measure gluten-related immunogenic responses such as inflammation and expression of inflammatory cytokines is possible (Laparra et al., 2012; Štěpánková et al., 1996).

5. Use of transgenic mice models to analyse pathologies related to gluten ingestion and expression of inflammatory cytokines (DePaolo et al., 2011; Marietta et al., 2004; Pinier et al., 2012).

6. Use of human clinical trials to assess clinical responses such as gastrointestinal symptoms, mucosal immunohistology, antibodies related to gluten epitopes (anti-gliadin antibodies, anti-endomysial antibodies) after a gluten challenge (Cornell et al., 2016; Ido et al., 2018; Tack et al., 2013).

Kiwifruit allergy is an issue associated with kiwifruit consumption. Several incidents of kiwifruit allergy were reported in different populations (Kerzl et al., 2007; Novembre et al., 1995; Palacin et al., 2008; Shimizu & Morikawa, 1995; Veraldi & Schianchi-

Veraldi, 1990). The major kiwifruit allergen is actinidin (Wang et al., 2019). The allergenicity of actinidin is caused by its resistance to both gastric and intestinal digestion (Grozdanovic et al., 2014). The clinical symptoms of the kiwifruit allergy range from localized symptoms confined to the oral mucosa to severe anaphylactic reactions (Gawrońska-Ukleja et al., 2013; Mancuso & Berdondini, 2001; Mempel et al., 2003). However, limited information is available in the literature on the prevalence of kiwifruit allergy in the general population. Based on the available information, it seems that kiwifruit allergy is not a common allergic condition among the general population and the symptoms of kiwifruit allergy are not severe among adults but can be in children (Lucas et al., 2004). There is no information available on any link between kiwifruit allergy and gluten intolerance, however, those who have allergies to birch pollen are likely to develop kiwifruit allergy (Gall et al., 1994). The cross-reactivity with European birch pollen is likely to be the main cause of this kiwifruit allergy (Bublin, 2013; Voitenko et al., 1997). Future work should consider the subjects' allergenicity to kiwifruit.

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# Appendix 1

**Chapter Four:** Calculations of the degree of hydrolysis

The degree of hydrolysis (DH) for 60 min gastrointestinal tract (GIT) digestion with actinidin is used as an example to show how different parameters were calculated with the observed data in this study and the gastric emptying values in Table 4.2.

Based on Figure 4.1, the DH at 60 min GIT digestion required the DH at 60 min gastric digestion, DH at 30 min gastric digestion followed by 30 min small intestinal digestion, and DH after only 60 min small intestinal digestion. These DH were calculated using the equation described in Calculations of Materials and Methods in Chapter four, and the following information was obtained:

- The DH of gluten at 60 min gastric digested sample ($DH_{60}$) was 14.83%
- The DH of gluten at 0 gastric and 60 min small intestinal digested sample was ($DH_{0/60}$) 8.47%
- The DH of gluten at 30 min gastric and 30 min small intestinal digested sample ($DH_{30/30}$) was 14.56%
- The gastric emptying at 30 min 8.55% ($GE_{30}$)
- The gastric emptying at 60 min was 19.16% ($GE_{60}$)

Assumptions:

1. A 1 mg of gluten contains 150 µg free amino groups (please see Calculations in Materials and Methods section).
2. The amount of gastric material that entered the small intestine during the initial postprandial minutes was assumed to be half of the material released at 30 min (i.e., 4.27%).
3. The calculation was done assuming one gram of gluten intake.

Gluten remaining in the stomach at 60 min (mg)

$= Gluten\ (mg)\ x\ \frac{(100\% - GE_{60}\%)}{100\%}$

$= 1{,}000\ x\ \frac{(100-19.16)}{100}$

$= 808.4\ mg\ gluten$

Free amino groups remaining in the stomach at 60 min (µg)

$= Amount\ of\ gluten\ in\ the\ stomach\ at\ 60\ \text{min}\ (mg)\ x\ \frac{DH_{60}\%}{100\%} x\ Total\ amino\ groups\ in\ 1\ mg\ of\ gluten\ (\mu g)$

$= 808.4\ x\ \frac{14.83}{100} x\ 150$

$= 17{,}988.9\ \mu g\ free\ amino\ groups$

Gluten released from the stomach into the small intestine at 60 min (mg)

$= Gluten\ (mg) - Gluten\ (mg)\ x\ (\frac{100\% - (GE_{60}\% - GE_{30}\%)}{100\%})$

$= 1{,}000 - 1{,}000\ x\ (\frac{100-(19.16-8.55)}{100})$

$= 106.1\ mg\ gluten$

Free amino groups released from the stomach into the small intestine at 60 min (µg)

$= Gluten\ released\ at\ 60\ min\ (mg)\ x\ \frac{DH_{60}\%}{100\%} x\ Total\ amino\ groups\ in\ 1\ mg\ of\ gluten\ (\mu g)$

$= 106.1\ x \frac{14.83}{100}\ x\ 150$

$= 2{,}360.19\ \mu g\ free\ amino\ groups$

Gluten released from the stomach at 0 min (mg) (please see assumption 2)

$= Gluten\ (mg) - Gluten\ (mg)\ \ x\ \frac{100\% - GE_{30/2}\%}{100\%}$

$= 1{,}000 - 1{,}000\ \ x\ \frac{100-4.275}{100}$

$= 42.75$ mg gluten

Free amino groups at 0 min gastric and 60 min small intestinal digested sample (µg)

$= \ Gluten\ released\ at\ 0\ min\ (mg)\ x\ \frac{DH_{0/60}\%}{100\%} x\ 150\ \mu g$

$= \ 42.75\ \text{mg}\ x\ \frac{8.47}{100} x\ 150$

$= 543.13\ \mu g\ free\ amino\ groups$

Gluten released into the small intestine at 30 min and went through small intestinal digestion for 30 min (mg)

$= Gluten\ (mg) - Gluten\ (mg)\ x\ \frac{100\% - GE_{30/2}\%}{100\%}$

$= 1{,}000 - 1{,}000\ x\ \frac{100 - 4.275}{100}$

$=$ 42.75 mg gluten

Free amino groups released after 30 min gastric and 30 min small intestinal digestion (µg)

$= \ Gluten\ released\ at\ 30\ min\ (mg)\ x\ \frac{DH_{30}\% DH_{30/30}\%}{100\%} x\ 150$ µg

$= \ 42.75$ mg $x\ \frac{14.56}{100} x\ 150$

$= 933.66\ \mu g\ free\ amino\ groups$

The DH% at 60 min GIT digestion

$= \frac{Sum\ of\ free\ amino\ groups\ at\ 60\ \text{min}\ gastric,\ \ 60\ min\ small\ intestinal,\ \ 30/30\ min\ gastric/small\ intestinal}{Total\ amino\ groups\ in\ 1{,}000\ mg\ gluten} x\ 100\%$

$= \frac{17{,}988.9 + 2{,}360.19 + 543.13 + \ + 933.66}{150{,}000} x\ 100\%$

$= 14.55\%$

The same equations were used to determine the amount of R5 epitopes at 60 min of GIT digestion.

Only the gastric emptying data were used to calculate the predicted number of immunogenic peptides in the small intestine at 60 min.

# Appendix 2

**Chapter Four:** An example of the calculation of the number of immunogenic peptides present in the small intestine at each digestion time point

| Gastric digestion (1 g of gluten) → | | | | | |
|---|---|---|---|---|---|
| Gastric emptying time (min) | 0 | 30 | 60 | 90 | 120 |
| Gastric emptying (%) | 0 | 8.5 | 19.1 | 35.7 | 41.3 |
| Number of immunogenic peptides identified during gastric digestion | 0 | 107 | 122 | 72 | 89 |
| Number of immunogenic peptides entering the small intestine during gastric emptying | 0 | 9 | 13* | 12 | 5 |

| | | Number of immunogenic peptides identified during subsequent small intestinal digestion | | | | |
|---|---|---|---|---|---|---|
| Small intestinal digestion ↓ | Time (min) | | | | | |
| | 30 | * | 26 | * | * | * |
| | 60 | 15 | * | * | * | * |
| | 90 | * | * | * | * | * |
| | 120 | * | * | * | * | * |

| | |
|---|---|
| Number of immunogenic peptides released into the small intestine during gastric emptying at 60 min with actinidin | **13** |
| Calculated number of immunogenic peptides identified during gastrointestinal digestion along the small intestine at 60 min (30 min gastric + 30 min small intestinal) + (60 min small intestinal) | **15 + 26** |
| Total number of immunogenic peptides identified in the small intestine during gastrointestinal digestion at 60 min | **41 + 13** |

*Number of immunogenic peptides entering the small intestine during gastric emptying at 60 min = (19.1% - 8.5%) x 122 = 13

**Figure 1.** Schematic representation of the procedure used to calculate the number of immunogenic peptides in the small intestine after 60 min of gastrointestinal digestion with actinidin during semi-dynamic digestion of gluten

# Appendix 3

**Chapter Four:** Supplementary Tables

**Table 1.** Amino acid profile of pure gluten used in the study*.

| Amino acid | mg/g (dry matter basis) |
|---|---|
| Aspartic Acid | 25.2 |
| Threonine | 20.6 |
| Serine | 36.7 |
| Glutamic Acid | 316.6 |
| Proline | 117.1 |
| Glycine | 26.7 |
| Alanine | 19.1 |
| Valine | 35.6 |
| Methionine | 14.2 |
| Isoleucine | 32.1 |
| Leucine | 61.2 |
| Tyrosine | 31.2 |
| Phenylalanine | 48.3 |
| Histidine | 18.1 |
| Lysine | 11.5 |
| Arginine | 27.1 |
| Cysteine | 19.4 |
| Tryptophan | 7.3 |

*Analysed in the Nutrition Laboratory of the Riddet Innovations, Massey University, Palmerston North, New Zealand using RP HPLC separation using AccQ Tag derivatization; AOAC method: 994.12.

# Appendix 4

**Chapter Five:** Details for the chromatographic and mass spectrometric settings

**Table 1**. Inclusion list for parallel reaction monitoring.

| Peptide ID | Mass (*m/z*) | CS [*z*] | Polarity | Start (min) | End (min) | NCE |
|---|---|---|---|---|---|---|
| P7 | 808.8944 | 2 | + | 0.3 | 0.8 | 22 |
| P6 | 813.9051 | 2 | + | 0.3 | 0.8 | 22 |
| P8 | 1265.6046 | 2 | + | 2.2 | 2.9 | 16 |
| P9 | 1164.9482 | 3 | + | 2.8 | 3.9 | 16 |
| P1 | 784.9270 | 2 | + | 4.2 | 4.6 | 15 |
| P1H | 787.9303 | 2 | + | 4.2 | 4.6 | 15 |
| P3 | 1032.5437 | 3 | + | 4.45 | 4.8 | 15 |
| P2 | 1132.0989 | 2 | + | 4.5 | 4.85 | 16 |
| P4 | 1029.5433 | 3 | + | 4.65 | 5.05 | 14 |
| P5 | 1304.0170 | 3 | + | 4.85 | 5.2 | 15 |

Order of list based on their relative retention time.
P1, LQLQPFPQPQLPY; P2, LQLQPFPQPQLPYPQPQPF; P3, LQLQPFPQPQLPYPQPHLPYPQPQPF; P4, LQLQPFPQPQLPYPQPQLPYPQPQPF; P5, LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF (33-mer); P6, RPQQPYPQPQPQY; P7, RPQQPYPQSQPQY; P8, FQPSQQNPQAQGSFQPQQLPQF; P9, VRVPVPQLQPQNPSQQQPQEQVPLVQQQQF.

**Table 2.** Liquid chromatography configuration.

| Parameter | Description |
|---|---|
| LC system | Dionex UltiMate™ 3000 quaternary RS system (ThermoFisher Scientific) |
| Mass spectrometer | Q Exactive™ Focus (ThermoFisher Scientific) |
| Ionization source | HESI-II (ThermoFisher Scientific) |
| Analytical column | Phenomenex Aeris™ 2.6 µm PEPTIDE XB-C18 100 Å, 100 x 2.1 mm. |
| Flow rates | 0.35 mL/min |
| Column oven temperature | 45 °C |
| Buffers | A) 0.1% Formic acid, 5 mM ammonium formate in water<br>B) 0.1% Formic acid, 5 mM ammonium formate in acetonitrile |
| Gradient | 20-45% B in 3 minutes, 45-90% B in 0.5 minutes, 1-minute hold at 100% B, 90-20% B in 0.5 min, 5-minute equilibration at 20% B. |
| Sample tray temperature | 4 °C |

**Table 3.** Mass Spectrometer settings.

| Parameter | Description |
|---|---|
| Polarity | Positive |
| Sheath gas flow rate (L/min) | 40 |
| Aux gas flow rate (L/min) | 8 |
| Spray voltage (kV) | 4.0 |
| Capillary temperature (°C) | 350 |
| S-Lens RF level (%) | 50 |
| Aux gas heater temperature (°C) | 300 |

**Table 4.** Mass detection parameter settings.

| Parameter | Description |
|---|---|
| Resolution | 35,000 |
| Isolation window | 3.0 *m/z* |
| Normalised Collision energy | 15 |
| Default charge | 2 |
| AGC target | 2e4 |
| Maximum injection time (ms) | Auto |
| Spectrum data type | Centroid |

**Table 5.** Trace finder peptide quantitation ions settings.

| Peptide ID | Mass (*m/z*) | Target Peak 1 | Confirming peak (T1C1) | Target peak 2 |
|---|---|---|---|---|
| P7 | 808.8944 | 1210.5965 | 1182.5973 | 407.1925 |
| P6 | 813.9051 | 1220.6172 | 1192.6212 | 407.1925 |
| P8 | 1265.6046 | 2141.0195 | 857.4502 | 391.1969 |
| P9 | 1164.9482 | 1254.1615 | 1017.6191 | 987.5244 |
| P1 | 784.9270 | 1290.7208 | 617.3293 | 279.1341 |
| P1H | 787.9303 | 1293.7296 | 618.3329 | 279.1341 |
| P3 | 1032.5437 | 1417.7468 | 1305.1910 | 263.1391 |
| P2 | 1132.0989 | 2002.0605 | 713.3612 | 488.2505 |
| P4 | 1029.5433 | 713.3608 | 1550.8344 | 263.1389 |
| P5 | 1304.0170 | 713.3613 | 2374.2735 | 263.1390 |

Order of list based on their relative retention time.

**Table 6.** Gastric emptying (GE) rates at different digestion times.

| Time | GE, % |
|---|---|
| 10 | 7.1 |
| 20 | 22.9 |
| 30 | 35.7 |
| 60 | 59.3 |
| 90 | 77.6 |
| 120 | 85.5 |
| 150 | 88.5 |

Adapted from (Malagelada, 1977; Malagelada et al., 1976).

## Reference

Malagelada, J.-R. (1977). Quantification of Gastric Solid-Liquid Discrimination During Digestion of Ordinary Meals. *Gastroenterology*, *72*(6), 1264-1267.

Malagelada, J.-R., Longstreth, G. F., Summerskill, W. H. J., & Go, V. L. W. (1976). Measurement of Gastric Functions During Digestion of Ordinary Solid Meals in Man. *Gastroenterology*, *70*(2), 203-210.

# Appendix 5

**Chapter Five:** Calculations for the amount of free amino groups present in the small intestine

The calculation of the amount of free amino groups present in the small intestine at 30 min with actinidin is used as an example to show how the observed data in this study and the gastric emptying values in Appendix 4, Table 6 were used in the calculation.

Based on Figure 5.1, the amount of free amino groups present in the small intestine at 30 min are the free amino groups in the 10 min gastric and 20 min small intestinal digested sample and free amino groups in the 20 min gastric and 10 min small intestinal digested sample.

The free amino groups of each sample were calculated using the equation described in Calculations of Materials and Methods in chapter 5, and the following information was obtained:

- Amount of free amino groups in the 10 min gastric and 20 min small intestinal digested sample was 5,603 µg/g of bread
- Amount of free amino groups in the 20 min gastric and 10 min small intestinal digested sample was 5,794 µg/g of bread
- The gastric emptying at 10 min 7.1% ($GE_{10}$)
- The gastric emptying at 20 min 22.9% ($GE_{20}$)

The calculation was done based on one gram of bread (dry matter).

Amount of bread (mg) released from the stomach at 0-10 min

$$1{,}000\ x\ \frac{GE_{10}}{100}$$

$$1{,}000\ x\ \frac{7.1}{100} = 71.0$$

Amount of bread (mg) released from the stomach at 10-20 min

$$1{,}000\ x\ \frac{GE_{20} - GE_{10}}{100}$$

$$1{,}000\ x\ \frac{(22.9-7.1)}{100} = 158.8$$

Amount of free amino groups present in the bread released from the stomach at 10 min

= 0.071 g x 5,603 µg/g of bread = 397.81 µg/g of bread

Amount of free amino groups present in the bread released from the stomach at 20 min

= 0.158 g x 5,794 µg/g of bread = 915.45 µg/g of bread

Total free amino groups present in the small intestine at 30 min

= 397.81 + 915.45 = 1,313.26 µg/g of bread

The same equations were used to determine the amount of epitopes in the small intestine at 30 min.

# Appendix 6

**Chapter Si**x: Supplementary methods

**Supplementary methods 1. Pig sample collection**

The whole GIT was dissected out. The stomach, small intestine, caecum, and colon including the rectum were ligated before dividing these sections with minimal handling. The stomach was immediately dissected out. The weight of the stomach full and empty was recorded to determine the total chyme dry matter content. The contents were collected into a plastic container and thoroughly mixed. One aliquot of 2 mL was taken into a tube for actinidin activity analysis. All the other chyme was taken into pre-weighed plastic bags. The small intestinal digesta samples were collected using autoclaved saline solution to individually flush out the whole digesta of the proximal, and distal jejunum (without the first ~20 cm of the small intestine (duodenum), remaining small intestine divided into equal lengths), terminal ileum (last 30 cm), caecum, and proximal and distal colon (two equal lengths) were collected into pre-weighed plastic bags. One aliquot of 2 mL from each place was taken into a tube for actinidin activity analysis. The faeces after feeding the final meal (and cleaning the cages) were collected to determine if any indigestible marker was excreted. All the samples were immediately frozen on dry ice and stored at -20 °C and -80 °C (samples for actinidin activity assay). Digesta samples in the plastic bags were freeze-dried before any analysis.

**Supplementary methods 2. Extraction of gluten**

Briefly, 0.25 g of freeze-dried sample was transferred to a 15 mL falcon tube and 2.5 mL of the UPEX solution (5 mM TCEP, 2% N-lauroylsarcosine in PBS, pH 7), which

was prepared immediately before use, was added. The samples were vortexed and then incubated at 50 °C for 40 min. After cooling for 10 min, 7.5 mL of 80% ethanol/water (v/v) was added, and vortexed for ~30 s, and incubated for 1 h at 23 °C in a rotary shaker (Ecotron, INFORS HT, Switzerland) at 45 turns/min. The tubes were then centrifuged (Heraeus Multifuge 3SR Plus, England) for 10 min at 2,500 g at 23 °C. The supernatant was used to determine free amino groups and residual R5 epitopes using the o-phthaldialdehyde (OPA) method and enzyme-linked immunosorbent assay (ELISA) respectively as described below in detail.

**Supplementary methods 3. OPA method**

An OPA solution containing 25 µL of 20% sodium dodecyl sulphate (SDS), 2 µL of β-mercaptoethanol and 0.02 mL of methanol-OPA (40 mg of OPA in 1 mL of methanol), and 953 µL of 0.1 M sodium tetraborate decahydrate solution (pH = 9.3) was prepared just before the analysis. The assay was carried out by the addition of 100 µL of the UPEX extracted supernatant or the standard to 1 mL of OPA solution. The mixture was mixed and allowed to stand for 2 min before measuring the absorbance at 340 nm with a UV-VIS spectrophotometer (GENESYS™, Thermo Fisher Scientific, USA). A standard curve was prepared using glycine (0–500 µg/mL). The total amino groups in the experimental diets were determined after acid hydrolysis with 6 M HCl for 24 h at 110 °C (7). Then, HCl was removed using a centrifugal concentrator and replaced by 0.1 M sodium tetraborate buffer to measure the amino groups as described above.

**Supplementary methods 4. ELISA**

The UPEX extracted supernatants in all stomach chyme samples and selected small and large intestinal digesta samples were analysed in duplicate to quantify the residual R5

epitopes (QQPFP, QQQFP, LQPFP, and QLPFP) using a commercially available competitive ELISA based on R5 antibody. All the procedures were carried out according to the manufacturer's instructions. The absorbance was measured at 450 nm using a microplate reader (SPECTROstar Nano, BMG LABTECH, Germany).

**Supplementary methods 5. Sample preparation for liquid chromatography-tandem mass spectrometry**

Freeze-dried stomach chyme or proximal small intestinal digesta (200 mg) were mixed with 300 µL of 100 mM ammonium bicarbonate containing trypsin (0.1 mg/mL), vortexed and incubated overnight at 37 °C to release cryptic immunogenic peptides in the digested bread matrix. Pure methanol (500 µL) and isotopically labelled internal standard, P1H (l-Phenylalanine-$^{13}C_9$,$^{15}N$ at N terminal residue six) at 3 ppm were added. Samples were vortexed and incubated at -20 °C for 30 min prior to being centrifuged at 13,300 rpm for 15 min. Supernatants (500 µL) were loaded to polymeric reversed-phase solid extraction (SPE) Strata™-XL 100 µm, 30 mg/1 mL cartridges (Phenomenex® Inc.), washed with 2 x 0.2 mL 40% methanol, followed by drying under full vacuum for 10 min and eluted with 200 µL of 70% acetonitrile/2% formic acid. Samples were concentrated to 50 µL and reconstituted to 100 µL with 5% acetonitrile/0.1% formic acid. Samples were centrifuged at 13,300 rpm for 15 min before MS analysis.

**Supplementary methods 6. Quantitative peptidomics**

Heated-electrospray ionization was used on a Dionex UltiMate™ 3000 quaternary RS system (Thermo Fisher Scientific, Waltham, MA USA). Reversed-phase chromatography was conducted with a capillary analytical column (Phenomenex

Aeris™ 2.6 µm PEPTIDE XB-C18 100 Å, 100 x 2.1 mm) at 45°C on a Q Exactive™ Focus (Thermo Fisher Scientific, Waltham, MA USA) mass spectrometer.

Parallel reaction monitoring tandem MS acquisition method with an inclusion list of ions was used. Fragment ion spectra produced via higher-energy collision-induced dissociation were acquired with a resolution setting of 35,000. Sample (5 µL) was injected onto the pre-equilibrated column, then eluted by buffer A (0.1% Formic acid, 5 mM ammonium formate in water) and buffer B (0.1% Formic acid, 5 mM ammonium formate in acetonitrile with a gradient of 20-45% B in 3 minutes, 45-90% B in 0.5 minutes, 1-minute hold at 100% B, 90-20% B in 0.5 min, 5-minute equilibration at 20% B). Data were collected using spray voltage = 4.0 kV; aux gas flow rate = 8 L/min; sheath gas flow rate = 40 L/min; scan range = 3 *m/z*; capillary temperature = 350°C; normalised collision energy = 15. The peak areas of target peptides were integrated and analysed by Trace Finder version 4.1 SP5 (ThermoFisher Scientific, Waltham, MA USA). A standard curve with concentrations of 0.05, 0.1, 0.5, 1, 5, 10, 50 and 100 ppm/µL for each peptide was established and was used to calculate concentrations of peptides in each unknown sample. Samples (1 µL) were injected per run and repeated five times. Three technical replicates were undertaken at each concentration.

**Supplementary methods 7. Discovery proteomics**

The SPE-extracted samples were separated by online reversed-phase chromatography using a Dionex UltiMate™ RSLCnano system (ThermoFisher Scientific, Waltham, MA USA) with Acclaim™ PepMap™100 C18, 3 µm particle size, 75 µm inner diameter, 2 cm length trapping column (ThermoFisher Scientific, Waltham, MA USA) and Acclaim™ PepMap™ C18, 2 µm particle size, 75 µm inner diameter, 50 cm length (ThermoFisher Scientific, Waltham, MA USA) reversed-phase C18 peptide analytical

column. The liquid chromatography system was coupled online to a Q Exactive™ Plus mass spectrometer equipped with a higher-energy collision-induced dissociation collision cell, an Orbitrap mass analyser and a Nanospray Flex™ ion source (ThermoFisher Scientific, Waltham, MA USA). Data-dependent tandem MS acquisition method (Top10) was employed where the top ten most intense ions in each scan cycle were selected for fragmentation. In all experiments, MS1 scans were acquired over a mass range of 375-1,600 *m/z* with detection in the Orbitrap mass analyser at a resolution setting of 70,000. dd-$MS^2$ spectra produced via higher-energy collision-induced dissociation were acquired with a resolution setting of 17,500. The column oven temperature was 50 °C and a gradient of 3-30% acetonitrile in 0.1% formic acid/water over 60 min was used. Ion exclusion conditions were optimized according to observed chromatographic peak width (typically 12s). The raw data files were searched using Proteome Discoverer™ version 2.4 search engine (ThermoFisher Scientific, Waltham, MA USA). The following search parameters used matched the specifications of the Q Exactive instrument, and variables resulting from chemical treatment.

The database was from NCBI (1 July 2020), taxonomy *Triticum aestivum*, proteins α-gliadin and glutenin with custom synthetic peptide sequences; enzyme specificity none; max number of missed cleavages 2; minimum peptide length 6; precursor mass tolerance 10 ppm; fragment mass tolerance 0.02 Da; static modification none; variable modifications included oxidation of methionine and N-terminal protein acetylation; target false discovery rate for peptide spectrum matches and peptides ≤ 0.01. The validation node of choice was percolator and target decoy. Only for gastric chyme samples, peptides having high confidence values and having at least 10 peptide spectrum matches were considered when identifying immunogenic peptides. For

proximal small intestinal samples, all the peptides generated were considered. The immunogenic gluten peptides were recognized by the presence of at least one epitope within their primary peptide sequence using the online database ProPepper (Juhász et al., 2015).

**Supplementary methods 8. Actinidin activity assay**

Briefly, 100 µL of the substrate Cbz-l-Lys-o-nitrophenol hydrochloride (1.2 g/L of deionised distilled water) was added to 2.85 mL of phosphate buffer solution (0.05 M, pH 6.0) directly into a cuvette placed in a UV-Vis-spectrophotometer (GENESYS™, Thermo Fisher Scientific, USA). After 20 s, allowing spontaneous hydrolysis of the substrate, 50 µL of a mixture containing the sample and 0.1 m-dithioerythritol (1:1, v/v) were added. The rate of change in absorbance was measured for 100 seconds at 348 nm to calculate the actinidin activity. The enzyme activity was calculated using the following formula.

$$\text{Actinidin activity (U/mg)} = \frac{\Delta\text{A348 test} - \Delta\text{ A 348 blank x 1,000 x 3}}{(5{,}400 \text{ x X})}$$

$\Delta A_{348}$: slope of the initial linear portion of the curve [unit absorbance/minute] for the test [with enzyme] and blank.
5,400: molar extinction coefficient (L/(mol x cm) of CBZ at 348 nm
3: volume of reaction mix [mL]
X: quantity of actinidin in the final reaction mixture (in the cuvette) [mg]

**Reference:**

Juhász, A., Haraszi, R., & Maulis, C. (2015). ProPepper: a curated database for identification and analysis of peptide and immune-responsive epitope composition of cereal grain protein families. *Database, 2015,* 1-16.

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Isuri Jayawardana has contributed to develop the methodology, has done the data curation, and has written the original draft

○ It is intended that the manuscript will be published, but it has not yet been submitted to a journal

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| Candidate's Signature: | Isuri Jayawardana Digitally signed by Isuri Jayawardana Date: 2021.11.27 08:56:15 +13'00' |
| Date: | 27-Nov-2021 |
| Primary Supervisor's Signature: | Warren McNabb Digitally signed by Warren McNabb Date: 2021.11.29 15:07:10 +13'00' |
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# STATEMENT OF CONTRIBUTION
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We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

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## Chapter 2



## Chapter 3