Evaluation of Production of Opioid Peptides from Wheat Proteins

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This thesis is submitted in total fulfilment of the requirements for the degree of

Doctor of Philosophy

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I dedicate this thesis to my father-in-law

Late Sh. Puran Chand Goel

& my family

Abstract

Opioids such as morphine and codeine are the most commonly clinically used drugs for pain management, but have associated side-effects. Food-derived opioid peptides can be suitable alternative due to less side-effect and are relatively inexpensive to produce. So, wheat protein (gluten) was tested as source for production of opioid peptides. The thesis reports results of investigations carried out on production of opioid peptides from wheat gluten using enzymatic hydrolysis and fermentation by selected lactic acid bacteria and characterisation of bioactivity (opioid) of prepared peptides and gluten hydrolysates. Gluten protein sequences were accessed using *in silico* approach (Biopep database and PeptideRanker) to predict presence of opioid peptides. The search was based on presence of tyrosine and proline. This led to selection of three peptides for which opioid activity was measured by cAMP (cyclic adenosine monophosphate) assay. The EC_{50} values of YPG, YYPG and YIPP were 1.78 mg/mL, 0.74 mg/mL and 1.42 mg/mL for μ-opioid receptor, respectively.

Hydrolysates from gluten were produced using two different approaches, fermentation using lactic acid bacteria (LAB) and by commercial proteases. Six LAB (*Lb. acidophilus, Lb. alimentarius, Lb. brevis, Lb. fermentum, Lb. plantarum* and *Lb. hilgardii)* were selected based on their proteolytic activity and fermentation was carried out at 37°C for 24 h. After fermentation, hydrolysis was assessed by o-phthalaldehyde (OPA) assay and gel electrophoresis. Reverse phase HPLC and Size exclusion HPLC were used for peptide profiling. *Lb. acidophilus, Lb. brevis, Lb. fermentum* and *Lb. plantarum* showed more proteolysis. EC_{50} values of <3 kDa

hydrolysate fractions after fermentation were 6.3, 7.2, 4.9 and 4.3 mg/mL, respectively.

Effect of selected enzymes (pepsin, trypsin, alcalase and flavourzyme) on production of opioid peptides was assessed through similar approach. The hydrolysates fraction produced by flavourzyme was the best with EC_{50} value of 0.43 mg/mL. The number of peptides showing > 0.5 ranking were 19, 48 and 61 for pepsin, alcalase and flavourzyme fractions, respectively. Peptides, PQQPFPL from pepsin hydrolysate and QQPPFW and QPFPQPQPFP from flavourzyme hydrolysate showed ranking ≥ 0.9 and should be further tested for bioactivity.

Difficulty in dispersion of gluten due to clumping was observed during experiments involving enzymatic hydrolysis. This was investigated by characterising gluten dispersion as a function of acidic pH (1, 2 and 3) and hydrothermal treatment in terms of structural changes by scanning electron microscopy, fourier transform infra-red (FTIR) spectroscopy, changes in functional groups (free amino and thiol), and size distribution (sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and SDS extractability). Decrease in the extractability and free thiol and free amino group at pH-2 and 3 confirmed gluten complex formation. Also, hydrothermal treatment at pH-1 can be used as pre-treatment before enzymatic hydrolysis to improve peptide yield. The study concludes that wheat gluten can be used for the production of opioid peptides.

Certificate

This is to certify that the thesis entitled "EVALUATION OF PRODUCTION OF OPIOID PEPTIDES FROM WHEAT PROTEINS" submitted by Swati Garg in total fulfilment of the requirement for the award of the Doctor of Philosophy degree in Food Technology at Victoria University is a record of bonafide research work carried out by her under my personal guidance and supervision and the thesis has not previously formed the basis for the award of any degree, diploma or other similar title.

Dr. Vijay Kumar Mishra (MSc, PhD), PhD supervisor

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Date: 03/05/2019

Declaration

I, Swati Garg, declare that the PhD thesis entitled “EVALUATION OF PRODUCTION OF OPIOID PEPTIDES FROM WHEAT PROTEINS” is no more than 100,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work.

Swati Garg Date: 03/05/2019

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List of Publications from this thesis

Peer reviewed publications

1. **Garg, S**., Nurgali, K., Mishra, V. K. (2016). "Food proteins as source of opioid peptides-A review." Current Medicinal Chemistry 23(9): 893-910. **doi**: 10.2174/0929867323666160219115226
2. **Garg, S**., Apostolopoulos, V., Nurgali, K., Mishra, V. K. (2018). "Evaluation of in silico approach for prediction of presence of opioid peptides in wheat." Journal of Functional Foods 41: 34-40. https://doi.org/10.1016/j.jff.2017.12.022
3. **Garg, S**., Cran, M. J., Mishra, V. K. (2019). "Effect of heating and acidic pH on characteristics of wheat gluten suspension." International Journal of Food Science and Technology 54(5): 1892-1900. https://doi.org/10.1111/ijfs.14097

Papers under review

1. **Garg, S**., Apostolopoulos, V., Nurgali, K., Mishra, V. K. (2019). “Opioid activity of enzymatic hydrolysate of wheat gluten”. Journal of Functional Foods.
2. **Garg, S**., Apostolopoulos, V., Nurgali, K., Mishra, V. K. (2019). “Proteolytic and opioid activities of wheat flour fermented by selected lactic acid bacteria”. International Journal of Food Science and Technology.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

ACE-I	Angiotensin converting enzyme inhibitory
ATR	Attenuated total reflectance
BSA	Bovine serum albumin
BBB	Blood brain barrier
cAMP	cyclic Adenosine mono phosphate
CEPs	Cell envelope proteinases
CFU	Colony forming unit
CG	Control gluten
CHO	Chinese hamster ovary
DAMGO	D-Ala_2,N-$MePhe_4$, $Gly\text{-}ol_5$)-enkephalin
DPP-IV	Di-peptidyl-peptidase -IV
DMEM	Dulbecco's modified eagle's medium
DTNB	5,5-dithio-bis-(2-nitrobenzoic acid)
DY	Dough yield
EC	Effective concentration
ELISA	Enzyme linked immune sorbent assay
ENS	Enteric nervous system
FBS	Foetal bovine serum
FTIR	Fourier transform infrared spectroscopy
GI	Gastrointestinal
GPCRs	G Protein coupled receptors
GPI	Guinea pig ileum
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMW	High molecular weight
HMW-GS	High molecular weight glutenins
IBMX	Isobutyl-1-methylxanthine
IC	Inhibitory Concentration
kDa	kilo Dalton
LAB	Lactic acid bacteria
LMW	Low molecular weight

LMW-GS	Low molecular weight glutenins
MVD	Mouse *vas deferens*
NOP	Nociceptin opioid peptide
OPA	o-Phthalaldehyde
PBS	Phosphate buffer saline
PTFE	Polytetrafluoroethylene
QIT-MS	Quadrupole ion-trap mass spectrometry
RP-HPLC	Reverse phase-high performance liquid chromatography
RUBISCO	D-ribulose-1,5-bisphosphate carboxylase/oxygenase
SDSEP	Sodium dodecyl sulphate extractability of protein
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy
SE-HPLC	Size exclusion-high performance liquid chromatography
TFA	Trifluoroacetic acid
TOF-MS	Time of flight-mass spectrometry
TRF	Time-resolved fluorescence
UV/Vis	Ultraviolet/visible
WG	Wheat gluten
WSE	Water/salt soluble extract

Chapter 1. Introduction

This chapter gives general introduction, research gaps and significance of the project. Thesis aims and outline are also presented.

1.1. Background

Sedentary lifestyle and improper eating habits such as consumption of junk food have increased the risk of chronic diseases such as obesity, cardio-vascular disease, hypertension, diabetes and cancer. Available drugs used in the treatment of diseases are associated with serious side-effects. Hence alternative treatment options that have comparatively lesser side-effects and safe are very much in demand. “Nutraceuticals” or functional foods are promising as they are derived from food and provide medical or health benefits beyond basic nutrition. Changing consumer behaviour is expected to increase the consumption of functional food and beverages and further accelerate the growth of functional food market growth in the upcoming years. The global market for functional food ingredients is US $64.871 billion in 2018 and is expected to grow to US $99.975 billion by 2025 as quoted by Zion market research (Joel 2019). Australia alone is expected to see an increase in market growth in functional food product categories by US$ 5.7 million per year until 2020 (South Australian Food Innovation Centre 2018). This is because these functional foods aid in improving health and reducing the risk of various diseases and contribute to ever-increasing cost of Medicare. The medicinal effects of these functional foods are due to presence of bioactive components such as polyphenols, anti-oxidants, probiotics, tannins, polyunsaturated fatty acids and peptides.

Food-derived bioactive peptides are promising ingredients for designing future foods because of their health benefits. These peptides are inactive within native proteins and must be released to exert their benefits on cardio-vascular, endocrine, immune, digestive and nervous systems. Depending on their amino acid sequence, these peptides may possess opioid, anti-oxidant, anti-thrombotic, anti-

hypertensive, anti-cancerous and immune-modulatory activities, thus benefiting health directly or indirectly (García, Puchalska et al. 2013). These peptides can be released from protein sources by proteolysis using commercial enzymes (Kong, Zhou et al. 2008), during fermentation by bacteria and fungi (Coda, Rizzello et al. 2012), during germination of grains by hydrolysis of proteins (Mamilla and Mishra 2017) and during gastrointestinal digestion *in situ* or under simulated conditions (Stuknytė, Maggioni et al. 2015, Ul Haq, Kapila et al. 2015). The suitability of a protein for production of bioactive peptides depends on composition and sequence of amino acids present in protein sourced from animal and plant proteins.

A peptide must contain tyrosine and proline within their sequence in order to be opioid peptide (Yoshikawa 2013). These opioid peptides can bind to opioid receptors (μ, δ and κ) within central and peripheral system (Waldhoer, Bartlett et al. 2004). Hydrolysis of milk proteins (animal based) have been shown to release number of bioactive peptides including those having opioid activity. Depending on the protein subunit from which these are derived from, opioid peptides from milk protein are α-casein exorphins, β-casomorphins, casoxins, α-lactorphins, β-lactorphin and lactoferroxins from α casein, β-casein, κ-casein, α-lactalbumin, β-lactoglobulin and lactoferrin, respectively. Out of these, β-casomorphins are the most studied opioid peptides owing to their low EC_{50} values found using guinea-pig ileum assay. β-casomorphin-5 and -7 have EC_{50} values of 6.5 μM and 57 μM, respectively (Brantl, Teschemacher et al. 1981). Plant proteins also show the presence of opioid peptides within their sequence. Well known opioids from plant proteins are exorphins from wheat gluten, rubiscolin from spinach Rubisco (Yoshikawa, Takahashi et al. 2003) and soymorphin from soybean (Ohinata, Agui

et al. 2007). The EC_{50} values of these opioids are 0.017 µM and 24.4 µM for exorphin B5 and rubiscolin-6, respectively, as obtained by mouse *vas deferens* assay (Yoshikawa, Takahashi et al. 2003). The EC_{50} value of soymorphin-5 is 6.0 µM using guinea-pig ileum assay (Ohinata, Agui et al. 2007). The EC_{50} values of opioid peptides derived from plant proteins are lower than those derived from animal proteins. Additionally, there are differences in the way these opioid peptides bind to various pain receptors which have different pharmacological effects contributing to pain relief. Most of animal protein derived peptides bind to µ receptor, and those from plant proteins bind to δ receptor, except soymorphins (Yoshikawa, Takahashi et al. 2003). Plant proteins are less expensive than animal proteins and hence the cost of production of bioactive opioid peptides is expected to be low using plant proteins (García, Puchalska et al. 2013). Out of different plant proteins, wheat offers an excellent choice as it is one of the most important food crops grown worldwide and Australia is one of the leading producers of wheat in the world. According to the Australian Bureau of Agricultural and Resource Economics and Sciences (ABARES), the wheat production during 2018-19 seasons is estimated to be 16.95 million tonnes. Based on an average of 10-12% protein (which is mainly gluten) present in wheat, roughly 1.695 million tonnes of gluten is available, thus offering a renewable source of protein for the production of bioactive peptides. Using wheat protein as a source for production of bioactive peptides, including opioid, will add value to wheat produced and increase profitability of local Agriculture business.

Gluten consists of two main fractions: gliadins and glutenins. The amino acid composition of these subunits shows high occurrence frequency of opioid peptides

within their sequences (Cavazos and Gonzalez de Mejia 2013) due to the higher amounts of tyrosine, proline and other aromatic amino acids (Mohsen, Yaseen et al. 2010) which are key to opioid activity. The challenge is to develop methods of producing functionally active opioid peptides from gluten in significant amounts for food and pharmaceutical uses before strategies for their use can be devised for delivering functional benefits.

Protein hydrolysis is widely used for the improvement of functional and nutritional properties of food proteins. Protein hydrolysates can be used as nutritional, pharmaceutical and functional ingredients in improving quality of food. The enzymatic hydrolysis has shown to release opioid peptides from gluten protein sequences to various extents and generally, the opioid activity of hydrolysates were low due to the presence of interfering components present in the hydrolysates. In the absence of compositional data of the hydrolysates it was difficult to correlate the activities and subsequently application of hydrolysate for food and pharmaceutical use. Being specific in their activity to a substrate, enzymatic hydrolysis offers several advantages in producing opioid peptides from gluten. The use of flavourzyme for the release of opioid peptides from gluten needs investigation as flavourzyme is a mixture of endopeptidase and exopeptidase and is expected to hydrolyse the complex gluten protein leading to higher yields of opioid peptides compared to when pure enzymes, such as pepsin and trypsin are used.

Lactic acid bacteria are used in production of various fermented foods. Proteolytic activities of these bacteria is responsible for unique colour, texture and flavour of fermented foods. There is no research work conducted on the release of opioid peptides by lactic acid bacteria, although the proteolytic system of these

bacteria has shown the potential to release peptides with other bioactivities and these bacteria find use in sourdough fermentation.

These opioid peptides from wheat proteins are natural and should have lesser side-effects when used for pain relief as compared to traditional opioid alkaloids (morphine and codeine derived from poppy seeds). Despite their effectiveness, these alkaloids may cause sedation, dizziness, nausea, vomiting, constipation, physical-dependence, addiction and respiratory depression (Benyamin, Trescot et al. 2008), thus limiting their use. Hence, these food-based opioid peptides can be suitable alternatives and can be used as complimentary medicine with other drugs for the clinical management of pain, or part of the diet (Stefanucci, Mollica et al. 2018) for the management of pain in patients suffering from various diseases such as cancer.

1.2. Research Aims

This study aimed to evaluate the hypothesis whether wheat proteins can be used for the production of opioid peptides. The hypothesis was tested by formulating the following specific objectives:

- To assess the presence of opioid peptides in wheat gluten by using *in silico* approach on the basis of presence of tyrosine and proline.
- To assess use of lactic acid fermentation for the production of opioid peptides from wheat.
- To investigate enzymatic proteolysis of wheat gluten using pepsin, trypsin, alcalase and flavourzyme and predict the presence of most probable opioid peptides in the hydrolysate fractions.

- To investigate the effects of different pH treatments with and without heating on the characteristics of wheat gluten suspensions.

1.3. Thesis Outline

The results of this research are presented in the following chapters which form the basis of series of publications included in the appendix.

Chapter 1 discusses the objectives and scope of the thesis.

Chapter 2 of this thesis is a literature review on opioid peptides sourced from food proteins and part of it has been published. The chapter consists of information on the classification of opioid ligands and receptors, aspects of production of peptides by hydrolysis, methods used for the determination of opioid activity and the techniques used to characterize and identify opioid peptides. The review also discusses the fate of opioid peptides during digestion, absorption and transportation in human body.

Chapter 3 describes general materials and methods used in the experiments designed specifically to test the objectives of this thesis.

An *in silico* approach was used to predict the presence of opioid peptides in wheat gluten. The results have been published and presented in **Chapter 4** with minor amendments. Opioid activity of peptides selected based on the presence of tyrosine and proline was determined and reported.

Chapter 5 discusses the fermentation of wheat flour by selected lactic acid bacteria (*Lactobacillus acidophilus, Lactobacillus alimentarius, Lactobacillus brevis, Lactobacillus fermentum, Lactobacillus hilgardii* and

Lactobacillus plantarum) for the production of opioid peptides. The hydrolysate were assessed for peptide profile and fractionated into low molecular weight fractions. The opioid activity of fractions was assessed by using cAMP assay and constituent peptides were characterised.

Chapter 6 describes the proteolysis of wheat gluten by enzymes (pepsin, trypsin, alcalase and flavourzyme) for production of opioid peptides. The hydrolysates obtained by these enzymes were characterised by a peptide profiling and fractionated into low molecular weight fractions. The opioid activity of fractions was assessed and peptides were characterised by LC/MS/MS and ranked for bioactivity.

Chapter 7 describes the characteristics of gluten dispersion as affected by acidic pH (1, 2 and 3) and hydrothermal treatment.

The general conclusion of the work based on use of in silico, enzymatic hydrolysis, and fermentation approaches for production of opioid peptides along with the limitations and suggested future directions for the further research are presented in **Chapter 8**.

Chapter 9 consist list of references used in this thesis.

Chapter 2. Literature Review

The material presented in this chapter is published and has been reproduced here with the permission of the publisher 'Bentham Science' with minor alterations.

S. Garg, K. Nurgali and V. K. Mishra (2016) Food proteins as Source of Opioid Peptides- A review. *Current Medicinal Chemistry*, Vol 23, Issue 9, p 893-910.

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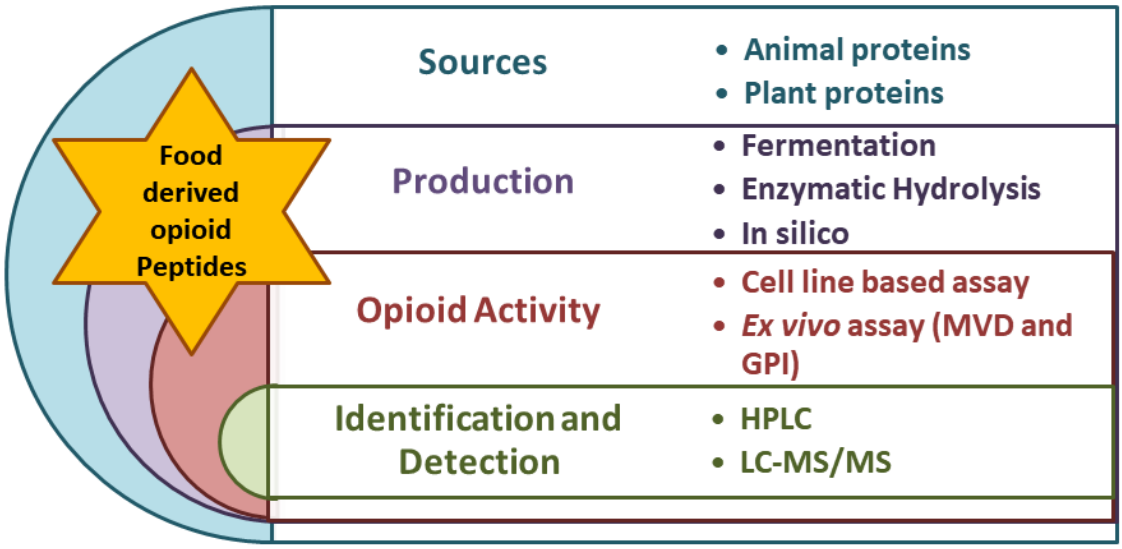


2.1. Summary

Traditional opioids, mainly alkaloids, have been used in the clinical management of pain for a number of years but are often associated with numerous side-effects including sedation, dizziness, physical dependence, tolerance, addiction, nausea, vomiting, constipation and respiratory depression which prevent their effective use. Opioid peptides derived from food provide significant advantages as safe and natural alternative due to the possibility of their production using animal and plant proteins as well as comparatively less side-effects. This review aims to discuss the current literature on food-derived opioid peptides focussing on their production, methods of detection, isolation and purification. The need for screening more dietary proteins as a source of novel opioid peptides is emphasized in order to fully understand their potential in pain management either as a drug or as part of diet complimenting therapeutic prescription.

2.2. Introduction

Food provides energy and essential nutrients to the body in the form of carbohydrates, proteins, fats, vitamins and minerals which are necessary for proper growth, development and functioning of the body. Improper eating habits and lifestyle changes increase the risk of a number of disorders, including obesity, heart disease, hypertension, cancer, osteoporosis and arthritis. Currently available clinical treatments are often associated with serious side-effects. Search for natural, healthy and safe alternatives with minimum side-effects is required for beneficial effects to patients and improving quality of life. Neutraceuticals are promising in this regard, as they are derived from food, hence are part of diet and should have minimal side-effects. The term “neutraceutical” is coined from the words "nutrition" and "pharmaceutical", and is defined as a food (or part of a food) that provides medical or health benefits, including the prevention and/or treatment of a disease (Kalra 2003). “Let food be thy medicine and medicine be thy food”, quoted by Hippocrates 2,500 years ago is certainly the need of today (Das, Bhaumik et al. 2012). These foods have potential health benefits beyond basic nutrition and are called functional foods. The term functional foods was first introduced in Japan in the mid-1980s (Hasler 1998). These medicinal effects of foods are predominantly due to the presence of polyphenols, antioxidants, probiotics, melatonins, tannins, polyunsaturated fatty acids or bioactive peptides. There has been considerable increase in interest in the functional foods market in recent years and as a result, some of these functional foods are available globally for specific health conditions.

Bioactive peptides are inactive within native proteins but exert physiological functions upon release from source proteins, animal or plant (dairy,

cereals, vegetables, meat and their products) during gastrointestinal (GI) digestion and/or after food processing. They may exhibit opiate, anti-oxidant, anti-thrombotic, anti-hypertensive, anti-cancerous or immune-modulatory activity thus benefiting cardiovascular, nervous, digestive and immune systems as shown in Figure 2.1 (Kitts and Weiler 2003, Shahidi and Zhong 2008, Adil 2012, Choi, Sabikhi et al. 2012, Udenigwe and Aluko 2012, García, Puchalska et al. 2013). As per Table 2.1, a functional food containing these peptides are available for sale mainly as supplements and used for management of various medical conditions including hypertension, obesity and type-2 diabetes. Hence, food can contribute to health, modulate immunity and prevent and help in clinical management of specific diseases. It may be possible to use these peptides as part of complimentary therapy along with other drugs.

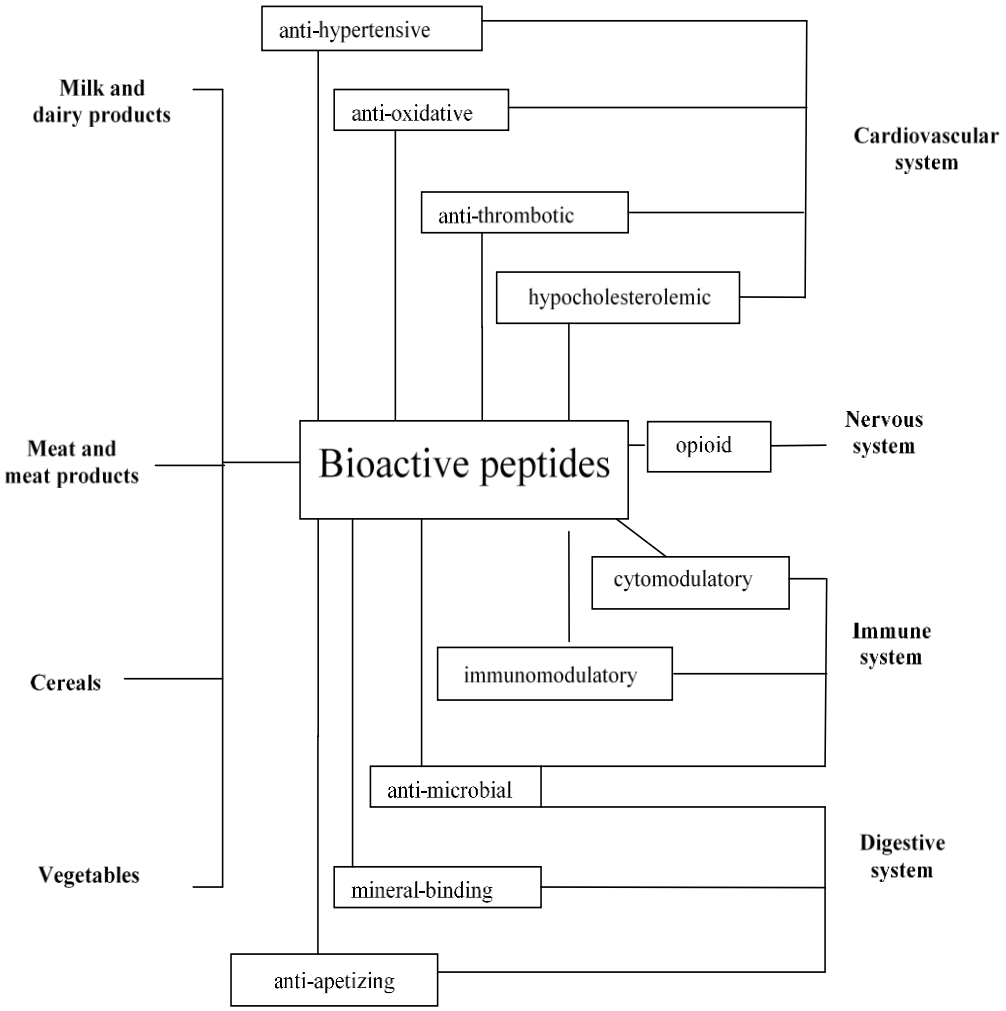


Figure 2.1: Effects of food derived bioactive peptides on various systems

Table 2.1: Some functional foods available on the global market (Adapted from El Sohaimy (2012))

Food containing functional ingredients	**Target**
Phyto-sterol stanolesters	Low density lipoprotein cholesterol
Bioactive peptides	Blood pressure
Melatonin	Quality of sleep
Omega-3 fatty acids	Depression
β glucan	Blood sugar values
Insulin	Hypoglycaemic drugs
Prebiotics	Bowel frequency
Probiotics	Immune functioning diarrhoea
Extra calcium or vitamin D or both	Bone health
Protein or bioactive peptides	Obesity and type-2 diabetes

Pain is an unpleasant sensation by the body that result from nerve stimulation. It is symptom that is treated by common analgesics. Most commonly clinically used drugs for severe pain management are opioids such as morphine and codeine (Teschemacher 2003, Janecka, Fichna et al. 2004, Trescot, Datta et al. 2008). They are very effective for management of pain but are often associated with side-effects like sedation, dizziness, nausea, vomiting, constipation, physical dependence, addiction, tolerance and respiratory depression (Benyamin, Trescot et al. 2008, Liu and Wang 2012). Opioids can also suppress the immune response and thereby increase patient's vulnerability to infections (Plein and Rittner 2018). An alternative to these opioids is actively sought in clinical management of pain. Food derived opioid peptides are generally characterised as having weaker activity making them less likely to cause side-effects generally associated with traditional opioids. Herein we summarize research on opioids, their receptors and classification with particular emphasis on food derived opioid peptides, methods of production, detection, isolation and purification from food, as well as their fate after digestion in order to fully understand their potential use.

2.3. Opioid receptors

Opioid receptors belong to the superfamily of G-protein coupled receptors and are distributed within the central and peripheral nervous system (Waldhoer, Bartlett et al. 2004, Cox, Christie et al. 2014). International Union of Basic and Clinical Pharmacology Receptor Nomenclature Committee (NC-IUPHAR) approved nomenclature for opioid peptide receptors as μ (mu or MOP), δ (delta or DOP) and κ (kappa or KOP) (Cox, Christie et al. 2014). A fourth opioid peptide

receptor is selectively activated by endogenous ligand, nociceptin and termed as NOP (nociceptin opioid peptide) and it is not antagonized by naloxone unlike the other three receptors (Cox, Christie et al. 2014). All of these receptors have close structural homology and are members of one family of proteins (Granier, Manglik et al. 2012, Manglik, Kruse et al. 2012, Thompson, Liu et al. 2012, Wu, Wacker et al. 2012), with differences between the receptor types arising due to gene duplication events during evolution (Cox, Christie et al. 2014). The affinity for the specific receptors influences the differences in activity of opioids (Trescot, Datta et al. 2008). Activation of MOP receptors mediate the most potent antinociceptive effects, however, they are prone to induce dependence; DOP receptors have lower efficacy in pain relief; and KOP receptors exert analgesic effects mainly in peripheral tissues (Janecka, Fichna et al. 2004). Activation of these receptors by opioid ligands leads to change in Ca^{++} and K^{+} channel conductance and protein phosphorylation via inhibition of cyclic AMP (cAMP) (Trescot, Datta et al. 2008). cAMP acts as a second messenger activating protein kinases and gene transcription which can result in effects including analgesia, respiratory depression, euphoria, release of hormones, and reduction of GI transit (De Noni, FitzGerald et al. 2009).

2.4. Classification of opioid ligands

Opioid ligand is any substance that binds specifically to opioid receptor to produce morphine like effects and the activity is reversed by non-specific antagonist, naloxone (Baldo and Pham 2013). Opioids were initially restricted to be alkaloids by nature (morphine, codeine and thebaine), but now peptides having

opioid activity (enkephalin, dynorphin, exogenous peptides) have also been identified (Figure 2.2).

2.4.1. Opioid alkaloids

Naturally occurring opioids are obtained from opium poppy plant, *Papaver somniferum*, and include morphine and structurally related alkaloids including codeine, noscapine (narcotine), thebaine, and papaverine (Baldo and Pham 2013). Out of them, morphine and codeine are most widely used opioid analgesic. Morphine is an agonist ligand primarily binding to μ receptors and has less affinity for δ and κ receptors (Janecka, Fichna et al. 2004). Codeine has weak affinity for μ opioid receptors and its analgesic activity is attributed to its principal metabolite, morphine (Kirchheiner, Schmidt et al. 2007, MacDonald and MacLeod 2010). Semisynthetic opioids are produced from naturally occurring alkaloids and include heroin, hydromorphone, oxymorphone, hydrocodone and oxycodone. Synthetic opioids are chemically synthesized in the laboratory from compounds unrelated to natural alkaloids and include fentanyl, tramadol or methadone (Figure 2.2) (Baldo and Pham 2013).

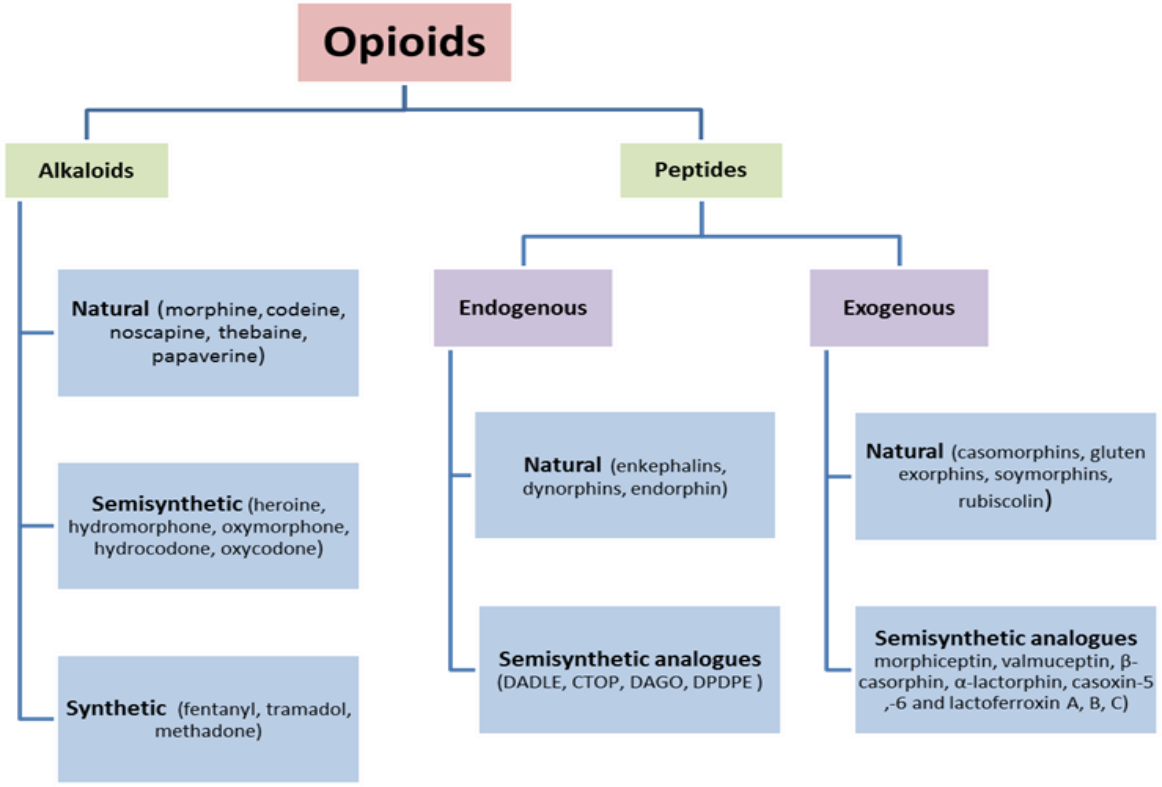


Figure 2.2: Classification of opioid ligands based on their structure

These opioid ligands have different affinity (strength of interaction) and efficacy (strength of activity) against opioid receptors and are classified into agonist, partial agonist or antagonist based on their action (Waldhoer, Bartlett et al. 2004). While an agonist has both affinity and efficacy (morphine, hydromorphone and fentanyl); an antagonist has affinity, but no efficacy (naloxone and naltrexone); and a partial agonist (agonist/antagonist) has affinity, but only partial efficacy (buprenorphine, pentazocine, nalbuphine, butorphanol, nalorphine) **(**Figure 2.3) (Trescot, Datta et al. 2008).

By chemical structure, opioid alkaloids are classified into phenanthrenes (morphine, codeine, hydromorphone, levorphanol, oxycodone, buprenorphine), benzomorphans (pentazocine), phenylpiperidines (fentanyl, meperidine, alfentanil, loperamide) and diphenylheptanes (propoxyphene, methadone) (Trescot, Datta et al. 2008).

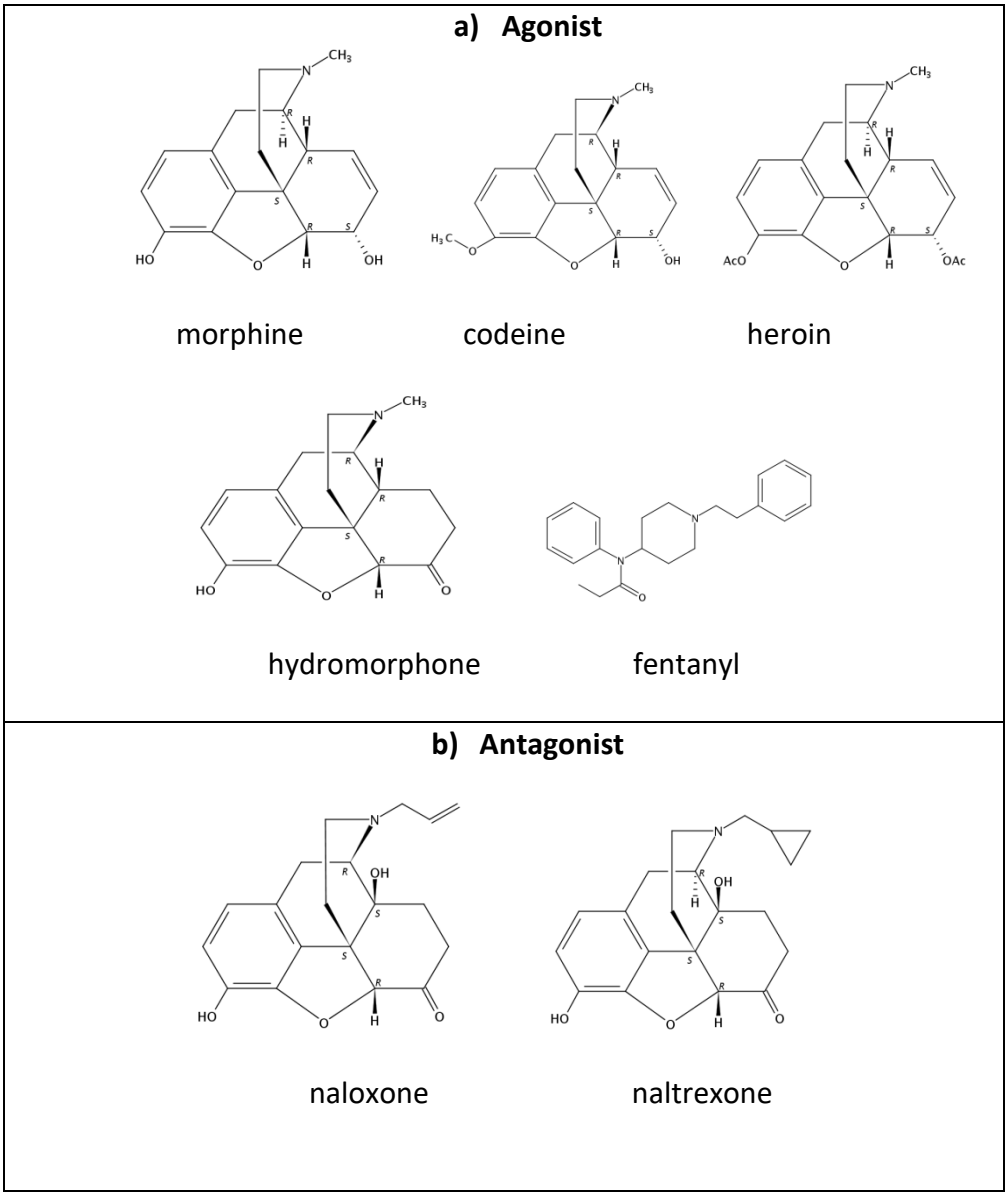


Figure 2.3: Chemical structures of opioid alkaloids

2.4.2. Opioid peptides

Opioid peptides were first identified in 1975 when met-enkephalin and leu-enkephalin from brain were found to have opioid activity (Hughes, Smith et al. 1975). Further investigations confirmed that endorphins, dynorphins and exogenous peptides from food also have opioid activity which opened a new era in the history of opioid ligands (Cox, Goldstein et al. 1976, Goldstein 1976, Goldstein, Tachibana et al. 1979, Zioudrou, Streaty et al. 1979, Goldstein, Fischli et al. 1981). Majority of peptide ligands are opioid agonists except casoxins and lactoferroxins which are opioid antagonist (Teschemacher, Koch et al. 1997, Meisel and FitzGerald 2000). Opioid peptides can be classified into endogenous or exogenous based on their origin as described (Figure 2.2).

2.4.2.1. Endogenous opioid peptides

Endogenous opioid peptides are produced naturally in the body and may function as hormones (secreted by gland and delivered to target tissues) or neuromodulators (secreted by nerve cells and act in central and peripheral nervous system) (Akil, Watson et al. 1984, Froehlich 1997, Janecka, Fichna et al. 2004). These peptides include β-endorphin, dynorphins and enkephalins which are hydrolysed by peptidases from precursor proteins, pro-opiomelanocortin, pro-dynorphin and pro-enkephalin, respectively (Goldstein, Fischli et al. 1981, Janecka, Fichna et al. 2004, Kyrkanides and Tallents 2004). These peptides have conserved YGGF sequence at their N terminus and are also called as typical opioid peptides (Table 2.2) (Janecka, Fichna et al. 2004). Apart from these, endomorphin-1 and -2 which lack conserved YGGF sequence have also been identified as endogenous

opioids (Martin-Schild, Gerall et al. 1999, Koneru, Satyanarayana et al. 2009) **(Figure 2.4)**

These endogenous peptides have been modified into semisynthetic analogues using amino acid substitution, addition, deletion, cyclisation or hybridization of two ligands to incorporate conformational constraints and make them more potent to be used as clinical analgesic (Janecka, Fichna et al. 2004, Liu and Wang 2012). Endomorphins have been modified into analogues which have increased stability against proteases by incorporation of unnatural amino acids followed by cyclisation (Okada, Tsuda et al. 2002, Gentilucci 2004, Janecka and Kruszynski 2005, Cardillo, Gentilucci et al. 2006, Liu and Wang 2012). Changes in leu-enkephalin (δ selective) by substitution and cyclisation have modified it to analogues which are μ selective with known analgesic effects (DiMaio and Schiller 1980). Modification of leu-enkephalin by amino acid substitution, addition and deletions have resulted in several agonists with enhanced δ receptor selectivity (Janecka, Fichna et al. 2004). Extensive research has been done to synthesize analogues with desired characteristics which are not discussed in this review; however, this information is available in more recent reviews (Liu and Wang 2012, Bodnar 2013)**.**

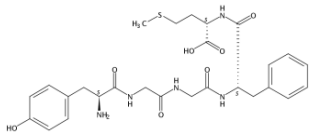
Table 2.2: Endogenous opioid peptides, their precursor, amino acid sequence and selectivity

Opioid peptide	Protein precursor	Amino acid sequence	Opioid receptor selectivity	References
β-endorphin	pro-opiomelanocortin	YGGFMTSEKSQTPLVTLFKNA IILNAYKKGE	μ	Goldstein (1976)
met-enkephalin leu-enkephalin	pro-enkephalin	YGGFM YGGFL	δ>> μ δ>> μ	Hughes, Smith et al. (1975) Kimura, Lewis et al. (1980) Koneru, Satyanarayana et al. (2009)
dynorphin A dynorphin B	pro-dynorphin	YGGFLRRIRPKLKWDNQ YGGFLRRQFKVVT	κ>> μ,δ	Goldstein, Fischli et al. (1981) Koneru, Satyanarayana et al. (2009)
nociceptin/orphanin FQ	pro-nociceptin/OFQ	FGGFTGARKSARKLANQ	ORL	Wang, Zhang et al. (1996)
endomorphin-1 endomorphin-2	pro-endomorphin	YPWF-NH2 YPFF-NH2	μ μ	Martin-Schild, Gerall et al. (1999)

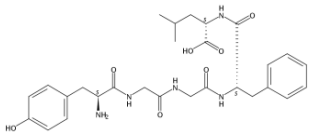
2.4.2.2. Exogenous opioid peptides

Exogenous peptides are hydrolysed from animal or plant precursor proteins and are also called as atypical opioid peptides since they carry various amino acids at their N terminus with conserved tyrosine residue (Janecka, Fichna et al. 2004, Fred Nyberg 2013). These exogenous opioid peptides were discovered in 1979, and term exorphin was used for food-derived peptide for the first time (exogenous origin and morphine like activity) (Zioudrou, Streaty et al. 1979). Majority of these atypical opioid peptides contain tyrosine residue at the amino terminal end and another aromatic amino acid in the 3rd or 4th position **(Figure 2.4)** (Meisel and FitzGerald 2000). This structural motif fits into the binding site of opioid receptors (Teschemacher, Koch et al. 1997, Pihlanto-Leppälä 2000). However, peptide lacking tyrosine at amino terminus RYLGYLE from bovine α-casein, has also shown opioid activity (Loukas, Varoucha et al. 1983, Teschemacher, Koch et al. 1997). Thus far, it has been identified that animal protein derived opioid peptides bind to μ receptors and those from plant proteins to δ receptors (Yoshikawa, Takahashi et al. 2003) except, soymorphins. This is further described below.

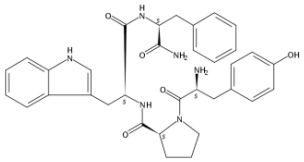
a) Endogenous Opioids



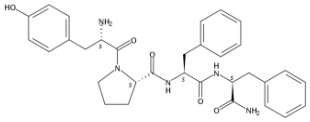
met-enkephalin



leu-enkephalin

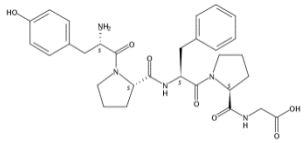


endomorphin-1

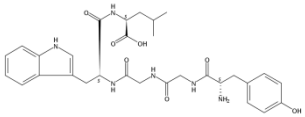


endomorphin-2

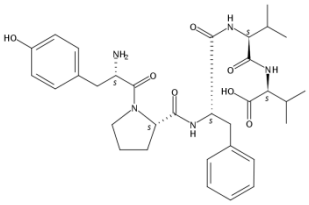
b) Exogenous opioids



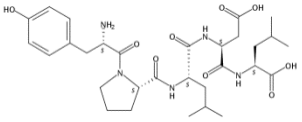
β casomorphin-5



gluten exorphinb5



soymorphin-5



rubiscolin-5

Figure 2.4: Chemical structures of a) endogenous and b) exogenous opioid peptides

2.4.2.2.1 Animal proteins as source of opioid peptides

Dietary proteins from animals (milk and their products, egg, poultry and fish) have been a source of bioactive peptides and opioid peptides have been identified in milk and dairy products Table 2.3).

Milk contains caseins (α, β and κ casein) and whey proteins (albumin and globulin). Hydrolysis of these proteins either inside or outside the body produces peptides that may have opioid activity. Depending on the protein they are derived from, opioid peptides have been named α-casein exorphins or casoxin D (α-casein), β-casomorphins or β-casorphin (β-casein), casoxins or casoxin A, B, or C (κ-casein), α-lactorphins (α-lactalbumin), β-lactorphin (β-lactoglobulin) or lactoferroxins (lactoferrin) (Teschemacher and Koch 1991, Teschemacher, Koch et al. 1997, Meisel and FitzGerald 2000).

In 1979, milk was proposed to have opioid activity (Brantl and Teschemacher 1979, Zioudrou, Streaty et al. 1979) and morphine like substance was isolated form milk at concentrations of 200 to 500 ng/L (Hazum, Sabatka et al. 1981). Opioid activity was due to the presence of peptides corresponding to f 60-66 (YPFPGPI, β-casomorphin-7) of β-casein (Henschen, Brantl et al. 1980) and f 90-96 (RYLGYLE) and f 90-95 (RYLGYL) of α-casein (Loukas, Varoucha et al. 1983). Sequence corresponding to f 91-96 (YLGYLE) and f 91-95 (YLGYL) amino acid residues also showed opioid activity and RYLGYLE being the most potent (Loukas, Varoucha et al. 1983).

Table 2.3: Exogenous opioid peptides derived from animal and plant food proteins

Source	Opioid peptide	Protein precursor	Amino acid sequence	Opioid receptor selectivity	Agonist(A)/ Antagonist(AN)	Reference
Animal protein	β_b-casomorphin-4	β_b-casein (60-63)	YPFP	μ	A	Brantl, Teschemacher et al. (1981)
	β_b-casomorphin-5	β_b-casein (60-64)	YPFPG	μ	A	
	β_b-casomorphin-6	β_b-casein (60-65)	YPFPGP	μ	A	
	β_b-casomorphin-7	β_b-casein A2 (60-66)	YPFPGPI	μ	A	
	β_b-casomorphin-8	β_b-casein (60-67)	YPFPGPIP/H	μ>δ>>κ	A	Petrilli, Picone et al. (1984) Teschemacher, Koch et al. (1997)
	β_b-casomorphin-9	β_b-casein (59–68)	VYPFPGPIPN	μ	A	Jinsmaa and Yoshikawa (1999) Ebner, Arslan et al. (2015)

	α_b-casein exorphin (1-7) α_b-casein exorphin (2-7)	α_b-casein (90-96) α_b-casein (91-96)	RYLGYLE YLGYLE	$\mu/\delta<<\kappa$ μ/δ	A A	Loukas, Varoucha et al. (1983) Teschemacher, Koch et al. (1997)
	casoxin A casoxin B casoxin C	κ_b-casein (35-41) κ_b-casein (57-60) κ_b-casein (25-34)	YPSYGLN YPYY YIPIQYVLSR	$\mu>>\delta, \kappa$ μ μ	AN AN AN	Chiba, Tani et al. (1989)
	neocasomorphin-6	β_b-casein (114-119)	YPVEPF	μ	A	Jinsmaa and Yoshikawa (1999)
	morphiceptine	β_b-casein	YPFP-NH_2	$\mu>>\delta>>\kappa$	A	Chang, Lillian et al. (1981) Day, Freer et al. (1981)
	α_b-lactorphin β_b-lactorphin	α_b-lactalbumin (69-72) β_b-lactoglobulin (118-121)	YGLF-NH_2 YLLF-NH_2	μ μ	A A	Antila, Paakkari et al. (1991) Teschemacher, Koch et al. (1997)

	β_h-casomorphin-4	β_h-casein (51-53)	YPFV	$\mu>\delta>\kappa$	A	Yoshdcawa, Yoshimura et al. (1984)
	β_h-casomorphin-5	β_h-casein (51-55)	YPFVE	$\mu>\delta>\kappa$	A	Koch, Wiedemann et al. (1985)
	β_h-casomorphin-7	β_h-casein (51-57)	YPFVEPI	$\mu>\delta>\kappa$	A	Teschemacher, Koch et al. (1997)
	β_h-casomorphin-8	β_h-casein (51-58)	YPFVEPIP	$\mu>\delta>\kappa$	A	Koch, Wiedemann et al. (1985) Teschemacher, Koch et al. (1997)
	casoxin D	α_{S1}-casein (158-164)	YVPFPPF	μ/δ	AN	Yoshikawa, Tani et al. (1994)
	α_h-casomorphin α_h-casomorphin amide	α_h-casein (158-162) α_h-casein (105-111)	YVPFP YVPFP- NH_2	$\mu/\delta<<<\kappa$ $\mu<<\delta/\kappa$	A/AN A/AN	Kampa, Loukas et al. (1996)

	β_h-casorphin valmuceptin α_h-lactorphin	β_h-casein (41-44) β_h-casein (51-54) α_h-lactalbumin (50-53)	YPSF-NH_2 YPFV-NH_2 YGLF-NH_2	μ μ μ	A A A	Yoshikawa, Tani et al. (1986) and Teschemacher, Koch et al. (1997)
	lactoferroxin A lactoferroxin B lactoferroxin C	lactoferrin(318-323) lactoferrin (536-540) lactoferrin (673-679)	YLGSGY-OCH_3 RYYGY-OCH_3 KYLGPQY-OCH_3	μ μ μ	AN AN AN	Tani, Iio et al. (1990)
Plant proteins	gluten exorphin A5 gluten exorphin A4 gluten exorphin B5 gluten exorphin B4	HMW glutenin HMW glutenin HMW glutenin HMW glutenin	GYYPT GYYP YGGWL YGGW	μ<<<δ μ<<δ δ δ	A A A A	Fukudome and Yoshikawa (1992) and Yoshikawa, Takahashi et al. (2003)
	gluten exorphin C	HMW glutenin	YPISL	μ<δ	A	Fukudome and Yoshikawa (1993) and Yoshikawa, Takahashi et al. (2003)

	rubiscolin-5 rubiscolin-6	rubisco (103-108) rubisco (103-109)	YPLDL YPLDLF	δ δ	A	Yang, Yunden et al. (2001)
	rALP-2	rubisco (85-90)	YHIEPV	δ	A	Kimura, Uchida et al. (2018)
	soymorphin-5	soy β-conglycinin (323-327)	YPFVV	μ	A	Ohinata, Agui et al. (2007)
	soymorphin-6	soy β-conglycinin (323-328)	YPFVVN	μ	A	
	soymorphin-7	soy β-conglycinin (323-329)	YPFVVNA	μ	A	

β-casomorphin-7 (YPFPGPI), corresponding to f60-66 of bovine β-casein (β_b-casein) was the most potent opioid peptide among β_b-casomorphin-6, -5 and -4 (Brantl, Teschemacher et al. 1981). β_b-casomorphins-9, corresponding to f59-68 position has been detected in enzymatic hydrolysate of milk (Jinsmaa and Yoshikawa 1999) and in kefir (Ebner, Arslan et al. 2015). Based on primary structure of human β-casein (β_h-casein) and sequence comparison with β_b-casein, 10 residue shifted alignment relationship and 47 % identity have been revealed (Yoshdcawa, Yoshimura et al. 1984, Koch, Wiedemann et al. 1985). Accordingly, β_h-casomorphin-4, -5, -6 and -8 with Tyr-Pro-Phe-amino-termini were tested for opioid activities (Yoshdcawa, Yoshimura et al. 1984). β_h-casomorphin-7 (YPFVEPI) was found at position 51-57 and have Val-Glu in position 4-5 as opposed to β_b-casomorphins-7 which was found at position 60-66 and have Pro-Gly (Koch, Wiedemann et al. 1985, Fred Nyberg 2013). However, both β_h-casomorphins and β_b-casomorphins bind particularly to μ receptors, with highest affinity for μ receptors and lowest for κ receptors (Koch, Wiedemann et al. 1985). β_h-casomorphin-4 and -5, have higher affinity to δ receptors than corresponding β_b-casomorphins **(**Table 2.4) (Koch, Wiedemann et al. 1985). Enzymatic digestion of human κ-casein/α-casein complex released opioid antagonist peptide casoxin D (YVPFPPF) corresponding to f 158-164 of α_{S1}-casein (Yoshikawa, Tani et al. 1994, Teschemacher, Koch et al. 1997).

Table 2.4: Opioid activity of peptides derived from food proteins

Opioid Peptide	Opioid activity		μ/δ	References
	guinea pig ileum IC_{50} (μM)	mouse *vas deferens* IC_{50} (μM)		
gluten exorphin A5	1000	60	60.7	Fukudome and Yoshikawa (1992)
gluten exorphin A4	>1000	70	-	Fukudome and Yoshikawa (1992)
gluten exorphin B5	0.05	0.017	2.9	Fukudome and Yoshikawa (1992)
gluten exorphin B4	1.5	3.4	0.44	Fukudome and Yoshikawa (1992)
gluten exorphin C	110	30	3.7	Fukudome and Yoshikawa (1993)
β_b-casomorphin-9	3.3	n.d.	-	Jinsmaa and Yoshikawa (1999)
β_b-casomorphin-7	57±7.5	>200	<0.29	Brantl, Teschemacher et al. (1981)
β_b-casomorphin-6	27.4±1.7	>150	<0.18	Brantl, Teschemacher et al. (1981)
β_b-casomorphin-5	6.5	40	0.16	Brantl, Teschemacher et al. (1981)

β_b-casomorphin-4	22	84	0.26	Brantl, Teschemacher et al. (1981)
β_h-casomorphin-4	19	750	0.025	Yoshikawa, Tani et al. (1986)
β_h-casomorphin-5	14	n.d.	-	Yoshikawa, Tani et al. (1986)
β_h-casomorphin-6	25	350	0.071	Yoshikawa, Tani et al. (1986)
β_h-casomorphin-8	25	540	0.047	Yoshikawa, Tani et al. (1986)
rubiscolin-5	1110 ± 71	51 ± 6.6	21.8	Yang, Yunden et al. (2001)
rubiscolin-6	748 ± 207	24.4 ± 3.6	30.7	Yang, Yunden et al. (2001)
soymorphin-5	6	50	0.12	Ohinata, Agui et al. (2007)
soymorphin-6	9.2	32	0.2875	Ohinata, Agui et al. (2007)
soymorphin-7	13	50	0.26	Ohinata, Agui et al. (2007)

n.d.- not determined

A number of opioid peptide analogues have been identified by amidation or esterification (by methyl group) of carboxyl terminal of the peptide such as morphiceptin (β-casein amide, YPFP-NH_2), valmuceptin (β_h-casein 51-54 amide, YPFV-NH_2), β-casorphin (β_h-casein 41-44 amide, YPSF-NH_2) and α-lactorphin (α_h-lactalbumin 50-53 amide, YGLF-NH_2) and β-casomorphin-4 and β-casomorphin-5 amides (Brantl, Pfeiffer et al. 1982, Yoshikawa, Tani et al. 1986). Lactoferroxin A (YGSGY-OCH_3), B (RYYGY-OCH_3) and C (KYLGPQY-OCH_3) are the opioid antagonists derived from methyl esterified peptic digest of human lactoferrin (Tani, Iio et al. 1990).

Studies have identified release of β_h-casomorphin from human milk during different gestational stages. Large precursor fragments of β_h-casomorphin (51–73, 48–73 and 35–73) were present both in preterm and normal term milk but the shorter sequences (including β_h-casomorphin–8) were only detected in milk from women delivering after normal gestation of 39 and 40 weeks (Ferranti, Traisci et al. 2004). The concentration of β_h-casomorphin-5 and β_h-casomorphin-7 in colostrum was higher than in mature milk (Jarmołowska, Sidor et al. 2007). These studies confirm that the size of the peptide decreases with an increase in gestation and lactation periods (Ferranti, Traisci et al. 2004, Jarmołowska, Sidor et al. 2007, De Noni, FitzGerald et al. 2009).

Differences between genetic variants of β-casein have also been investigated. Three genetic variants (A1, A2 and B) of β-casein were tested for release of β_b-casomorphin-7 on simulated GI digestion, and concentration of peptide released was more from A1 and B variants as compared to A2 variant (De Noni 2008, Ul Haq, Kapila et al. 2015). This could be explained due to the

difference between variants at position 67; being histidine in A1 and B and proline in A2 (Cieslinska, Kaminski et al. 2007). Peptide bond between proline and isoleucine has more enzymatic resistance than that between histidine and isoleucine which gets hydrolysed by GI enzymes, resulting in the release of β-casomorphin-7 from A1 and B variants (Jinsmaa and Yoshikawa 1999).

The effects of administration of these opioid peptides in different doses in various animal models are presented in **Table 2.5**. It is difficult to compare the doses and effects as different animal models and routes of administration were used by various researchers. β-casomorphins have shown analgesic activity in mice which is reversed by naloxone, thus confirming that the effects are mediated by opioid receptors (Grecksch, Schweigert et al. 1981, Matthies, Stark et al. 1984). Intraperitoneal administration of β-casomorphins-5 and -7 has positive effects on learning and memory (Sakaguchi, Koseki et al. 2006, Dubynin, Malinovskaya et al. 2008). Orally administered opioid peptides from milk stimulated release of postprandial pancreatic polypeptide (Schusdziarra, Schick et al. 1983). β-casomorphins stimulate release of somatostatin and insulin (Schusdziarra, Schick et al. 1983). They have shown to prolong gastrointestinal transit time (Daniel, Vohwinkel et al. 1990, Defilippi, Gomez et al. 1994, Mihatsch, Franz et al. 2005) and modulate intestinal mucus secretion (Claustre, Toumi et al. 2002, Trompette, Claustre et al. 2003, Zoghbi, Trompette et al. 2006). Apart from these effects on central and peipheral nervous system mediated via opioid receptors, β-casomorphins-7 increased plasma insulin and superoxide dismutase and catalase activities in diabetic rats, thus, protecting them from hyperglycaemia and free radical-mediated oxidative stress (Yin, Miao et al. 2010). In most cases,

casomorphins have significant effects on central and peripheral nervous system and have role in regulation of blood sugar in diabetics (**Table 2.5**). There are also reports that indicate β-casomorphin-7 blocked the inhibitory effect of enterostatin on high fat intake leading to obesity (Lin, Umahara et al. 1998, White, Bray et al. 2000).

2.4.2.2.2 Plant proteins as source of opioid peptides

Despite a large variety of available dietary proteins from plants, release of opioid peptides has been reported in gluten from wheat, D-ribulose-1, 5-bisphosphate carboxylase/oxygenase (RUBISCO) from spinach, and β-conglycinin from soybean.

Gluten exorphins

Gluten is the storage protein of wheat and consists of two different proteins, gliadin and glutenin. The presence of opioid peptides was first described in peptic digest of wheat gluten based on adenylate cyclase and mouse *vas deferens* (MVD) assays (Zioudrou, Streaty et al. 1979, Morley, Levine et al. 1983). These peptides were identified as gluten exorphins A5 (GYYPT), A4 (GYYP), B5 (YGGWL) B4 (YGGW) and C (YPISL) and showed lower IC_{50} in MVD as compared to guinea-pig ileum (GPI) assay (**Table 2.4**) (Fukudome and Yoshikawa 1992, Fukudome and Yoshikawa 1993). So, these gluten exorphins bind specifically to δ opioid receptors and B5 is most potent having IC_{50} value 0.017 μM in MVD among the known sequences (Yoshikawa, Takahashi et al. 2003). The associated effects of the use of gluten exorphin A5 are mild anti-nociception and consolidation process of learning and memory (Takahashi, Fukunaga et al. 2000, Yoshikawa, Takahashi et al. 2003). Gluten exorphin B5 stimulated prolactin secretion (Fanciulli, Dettori et al. 2003, Fanciulli, Dettori et al. 2005) and gluten exorphin C increased exploratory activity,

decreased anxiety and improved learning (**Table 2.5)** (Belyaeva, Dubynin et al. 2009). These studies suggest the potential effect of these gluten exorphin on central and peripheral nervous system.

Rubiscolin

Rubiscolin-5 (YPLDL) and -6 (YPLDLF) peptides identified in spinach RUBISCO have opioid activity attributed to the presence of YP at amino end. The IC_{50} values of rubiscolin-5 and -6 using δ receptor binding assay were 2.09 μM and 0.093 μM, respectively, and are comparatively weaker agonist (Cassell, Mores et al. 2019). These are selective for δ opioid receptor with significant anti-nociceptive activity (Yang, Yunden et al. 2001). Rubiscolin–6 enhances memory consolidation (Yang, Kawamura et al. 2003), exerts anxiolytic (Hirata, Sonoda et al. 2007) and orexigenic effects (Kaneko, Lazarus et al. 2012, Miyazaki, Kaneko et al. 2014), supresses high fat intake (**Table 2.5**) (Kaneko, Mizushige et al. 2014) and improves glucose uptake in skeletal muscles (Kairupan, Cheng et al. 2019). Rubisco anxiolytic-like peptide rALP-2 (YHIEPV) was identified as δ opioid peptides in digests of RUBISCO hydrolysed under simulated GI digestion (Kimura, Uchida et al. 2018).

Soymorphins

Soymorphins are synthesized from spinach β-conglycinin. Soymorphin-5 (YPFVV), -6 (YPFVVN), and -7 (YPFVVNA) were found to have lower IC_{50} value in GPI as compared to MVD assay (Ohinata, Agui et al. 2007). They are selective for the μ opioid receptor and have shown anxiolytic activities (**Table 2.5**) (Ohinata, Agui et al. 2007). Soymorphin-5 suppresses food intake and small intestinal transit (Kaneko, Iwasaki et al. 2010), increases β-oxidation and energy expenditure

(Yamada, Muraki et al. 2012). Analogue synthesized by amidation of soymorphin-5 (YPFVV-NH_2), stimulated locomotion in adult rats and decreased anxiety in juvenile rats (Chesnokova, Saricheva et al. 2014). These activities have shown potential of soymorphin-5 in management of obesity, diabetes and anxiety (Kaneko, Iwasaki et al. 2010, Yamada, Muraki et al. 2012, Chesnokova, Saricheva et al. 2014).

Table 2.5: Effects of opioid peptides after parenteral administration to animals

Opioid Peptide	Dosage	Animal	Route of administration	Effect	Reference
β-casomorphin-5	0.166–166 nM	rat	i.v.	analgesic, naloxone reversible	Grecksch, Schweigert et al. (1981)
β-casomorphin-4,-5,-6,-7	60–2000 nM	rat	i.c.v.	analgesic, naloxone reversible	Brantl, Teschemacher et al. (1981)
β-casomorphin-5	1–100 mg/kg	rat pups	i.p.	no effect on walking, decreased active sleep at a dose of 100 mg/kg	Taira, Hilakivi et al. (1990)
β-casomorphin-7	0.1–20 nM	rat	i.c.v.	stimulated food intake of high fat meal	Lin, Umahara et al. (1998)
β-casomorphin-7	1 mg/kg	albino rat pups	chronic	negative effect on passive avoidance conditioning	Dubynin, Malinovskaya et al. (2008)
β-casomorphin-5	1 mg/kg	mice	i.p.	improves learning and memory	Sakaguchi, Koseki et al. (2006)
β-casomorphin-7	7.5×10^{-8} M/day	diabetic rats	oral	increased plasma insulin and superoxide dismutase and catalase	Yin, Miao et al. (2010)

gluten exorphin A5	30 & 300 mg/kg 30 & 300 µg/mouse	mice	oral i.c.v.	no antinociception and morphine analgesia effects mild antinociception but no effect on morphine analgesia	Takahashi, Fukunaga et al. (2000)
gluten exorphin B5	3 mg/kg	rats	i.v.	stimulated prolactin secretion	Fanciulli, Dettori et al. (2005)
gluten exorphin C	5 mg/kg		i.p.	increased exploratory activity, decreased anxiety, and improved learning	Belyaeva, Dubynin et al. (2009)
rubiscolin-5 rubiscolin-6	3 nM/mouse 1 nM/mouse	mice	i.c.v.	antinociception	Yang, Yunden et al. (2001)
rubiscolin–6	100 mg/kg 3 nM/mouse	mice	oral i.c.v.	enhanced memory consolidation	Yang, Kawamura et al. (2003)
rubiscolin–6	0.3–1 mg/kg	mice	oral	stimulated food intake (orexigenic effect) in non-fasted mice	Kaneko, Lazarus et al. (2012)
rALP-2	n.d.	mice	oral	anxiolytic effect	Kimura, Uchida et al. (2018)
rubiscolin–6	1 mg/kg	mice	oral	supressed high fat intake	Miyazaki, Kaneko et al. (2014)
soymorphin-5	10-30 mg/kg	mice	oral	anxiolytic effect	Ohinata, Agui et al. (2007)

soymorphin-5	30 mg/kg	mice	oral	suppressed food intake and small intestinal transit	Kaneko, Iwasaki et al. (2010)
soymorphin-5	10 mg/kg	mice	oral	increased β-oxidation and energy expenditure	Yamada, Muraki et al. (2012)
soymorphin-5 amide	5 mg/kg	rats	i.p.	stimulated locomotion in adults and decreased anxiety in juvenile rats	Chesnokova, Saricheva et al. (2014)

i.c.v.- intra-cerebroventricular; i.p.- intraperitoneal; i.v.- intravenous

2.5. Production of opioid peptides from food proteins

Food proteins themselves do not have opioid activity. In order to enhance their opioid activity they need to be hydrolysed through enzymes in the GI tract or during fermentation (Korhonen and Pihlanto 2006, Choi, Sabikhi et al. 2012, Udenigwe and Aluko 2012). To assess their formation during GI digestion, GI enzymes are required. Hence, fermentation and enzymatic hydrolysis is a requirement for the production of peptides.

2.5.1. Fermentation

Fermentation is used for the production of yogurt, cheese, sauerkraut, kimchi, salami, bread and beverages (wine, beer, cider). Depending on the type of fermentation, proteolytic enzymes of the microorganism contribute to the release of peptides which may have bioactivity. These peptides have known anti-cancerous (Rizzello, Nionelli et al. 2012, Sah, Vasiljevic et al. 2014), anti-oxidant (Galli, Mazzoli et al. 2018), anti-hypertensive (Ashar and Chand 2004, Muguerza, Ramos et al. 2006), anti-microbial (Rizzello, Cassone et al. 2011, Garofalo, Zannini et al. 2012), anti-inflammatory (Eom, Hwang et al. 2018, Galli, Mazzoli et al. 2018) and opioid (Jarmołowska, Kostyra et al. 1999) activities.

2.5.1.1. Fermentation for the production of opioid peptides

Fermentation by different bacteria has led to the release of opioid peptides from different food proteins. Fermentation of milk by *Pseudomonas aeruginosa* and *Bacillus cereus* leads to formation of β-casomorphin immunoreactive material (Hamel, Kielwein et al. 1985). Enzymatic (pepsin and trypsin) proteolysis of *Lactobacillus (Lb.) GG* fermented milk released peptide sequences YPFP,

RYLGYLE, YGLF, YPFPGPIPNSL which are opioids by nature (Rokka, Syvaoja et al. 1997). Whey fermentation by *Kluyveromyces marxianus var. marxianus* released YLLF which corresponds to β-lactorphin (Belem, Gibbs et al. 1999). β-casomorphin-4 was detected in milk fermented with PepX-deficient mutant strains of *Lb. helveticus L89* and absent in milk fermented with wild strain (Matar and Goulet 1996). This suggests possible degradation of β-casomorphin by the Pep X (X-prolyl dipeptidyl aminopeptidase) which digests the peptide bonds between proline and other amino acid residues (Liu, Bayjanov et al. 2010). The proteolysis of casein by proteolytic system of *Lb. helveticus* T105 has shown release of bioactive peptides including opioid peptides mainly from κ-casein, (Skrzypczak, Gustaw et al. 2017)

Yoghurt fermented with *Lb. delbrueckii ssp. bulgaricus* and *S. salivarius ssp. thermophiles* has been source of β-casomorphin precursors corresponding to f 57-68 and f 57-72 (Schieber and Brückner 2000), but not β-casomorphin (f 60-67). This can be due to incapability of these starter cultures to hydrolyse β-casein into β-casomorphin. Moreover, the stability of β-casomorphin in yoghurt may be influenced by the symbiotic growth of *Lb. delbrueckii ssp. bulgaricus* and *S. thermophilus.* β-casomorphin may be released by *Lb. delbrueckii ssp. bulgaricus* protease activity but are degraded by *S. thermophilus*, and vice versa. Earlier studies were not able to detect β-casomorphin-7 in Brie and Cheddar which could be due to lower threshold of analytical techniques employed for detection of these peptides (Muehlenkamp and Warthesen 1996). It was also proposed that β-casomorphins are degraded in these cheeses during ripening as suggested by the fact that enzymes derived from *Lb. lactic ssp. cremoris* can reduce concentration of

β-casomorphin-7 by 50 % (pH 5.0 and 1.5 % NaCl) after 6-15 weeks (Muehlenkamp and Warthesen 1996).

β-casomorphin-7 was later detected in Brie (Jarmołowska, Kostyra et al. 1999) and its concentration ranged from 6.48 µg/g (Sienkiewicz-Szłapka, Jarmołowska et al. 2009) to 0.15 µg/g (De Noni and Cattaneo 2010). β-casomorphin-7 was also detected in the range of 0.01 - 0.11 µg/g in Gorgonzola, Gouda, Fontina and Cheddar cheeses. Upon *in vitro* simulated GI digestion the concentration increased up to 15.22 µg/g and 21.77 µg/g in Cheddar and Gouda cheeses, respectively (De Noni and Cattaneo 2010). Along with β-casomorphin (opioid agonist), opioid antagonists, casoxin-6, casoxin-C and lactoferroxin A were also detected in semi-hard cheeses (Edamski, Gouda and Kasztelan) along with β-casomorphins (Sienkiewicz-Szłapka, Jarmołowska et al. 2009). β-casomorphin-9 was detected in kefir (Ebner, Arslan et al. 2015). These studies suggest possibility of formation of opioid sequences from food proteins fermented by fungus as compared to bacteria because of specificity of enzymes present within them and needs further investigation (Nguyen, Johnson et al. 2015).

2.5.2. Enzymatic hydrolysis

Enzymatic hydrolysis is conducted by the use of GI enzymes (pepsin, trypsin, chymotrypsin and pancreatin) or microbial enzymes thermolysin, alcalase and flavourzyme for production of bioactive peptides. Enzymes are specific so that peptides with specific bioactivity such as anti-hypertensive (Yang, Marczak et al. 2004, Jiang, Chen et al. 2007, Barbana and Boye 2010, Yang, Zou et al. 2011), anti-oxidant (Kong and Xiong 2006, Liu and Chiang 2008, Lin, Tian et al. 2012) and

opioid (De Noni 2008, Kong, Zhou et al. 2008) can be obtained after enzymatic hydrolysis of different food proteins.

2.5.2.1. Enzymatic hydrolysis for the production of opioid peptides

Opioid activity has been detected in the enzymatic hydrolysate of various food protein hydrolysate such as in pepsin hydrolysate of wheat gluten (Zioudrou, Streaty et al. 1979). Gluten exorphins A5, A4, B5 and B4 were identified in pepsin-thermolysin hydrolysate (Fukudome and Yoshikawa 1992) and gluten exorphin C in pepsin-trypsin-chymotrypsin hydrolysate (Fukudome and Yoshikawa 1993). Concentration of gluten exorphin A5 was higher in hydrolysate prepared with pepsin-elastase (250 µg/g) than pepsin-thermolysin (40 µg/g) (Fukudome, Jinsmaa et al. 1997). Recently, simulated *in vitro* GI digest (using pepsin-trypsin-chymotrypsin) of bread and pasta showed presence of gluten exorphins A5 (0.747–2.192mg/kg) and C (3.201–6.689 mg/kg) (Stuknytė, Maggioni et al. 2015). Opioid activity was detected in the hydrolysates produced by alcalase (6 hr) and pepsin (24 hr) with IC_{50} values of 1.21 and 1.57 mg/mL, respectively, and not in hydrolysate produced by protamex and neutrase (Kong, Zhou et al. 2008). Thus, release of these gluten exorphins has been confirmed during GI digestion. Similarly, release of casomorphins after simulated GI digestions by various enzymes have been studied in milk and their products (De Noni and Cattaneo 2010) and differences has been detected in genetic variants of β-casein (Cieslinska, Kaminski et al. 2007, De Noni 2008, Ul Haq, Kapila et al. 2015).

2.6. Determination of opioid activity

Opioid activity of the hydrolysate can be tested by using one of the several available assays. Commonly used assays for testing opioids from food are naloxone-reversible inhibition of adenylate cyclase activity (Sharma, Klee et al. 1975), naloxone-reversible inhibition of electrically stimulated contraction of isolated organ preparation either GPI or MVD (Hughes, Kosterlitz et al. 1975), receptor binding assay or radioreceptor assay (Pert, Pasternak et al. 1973, Pert and Snyder 1973).

Opioid receptors belong to the family of G protein-coupled receptors and their activation results in adenylate cyclase inhibition, K^+ channel activation or Ca^{2+} channel inactivation (Satoh and Minami 1995, Gupta, Décaillot et al. 2006). Inhibition of adenylate cyclase has been frequently used for identification of opioid peptide from wheat gluten and α-casein (Zioudrou, Streaty et al. 1979, Loukas, Varoucha et al. 1983, Yoshikawa, Takahashi et al. 2003). Binding of opioid ligand to opioid receptors in hybrid cells (tranformed with specific opioid receptor) or brain membrane inhibits adenylate cyclase which decreases intracellular cAMP which can be detected through availabale asays to find if binding ligand is opioid (Sharma, Klee et al. 1975, Satoh and Minami 1995, Gupta, Décaillot et al. 2006).

In addition opioid activity of the peptide involves binding of ligand to specific opioid receptor present on GPI longitudinal muscle myenteric plexus or MVD preparations (Teschemacher 2003, Janecka, Fichna et al. 2004). These tests are based on inhibition of electrically evoked contractions of the GPI and MVD and have been used for determination of opioid activities of food derived peptides Table 2.4. The opioid effect in the GPI and MVD preparations are mediated by μ and δ

receptors, respectively. Thus these tests are used to screen μ and δ receptor ligands from different sources (Zioudrou, Streaty et al. 1979, Janecka, Fichna et al. 2004).

The receptor binding assays may involve saturation and competition studies. While, in the saturation binding studies, the affinity of different compounds to opioid receptors is characterized, competition studies can be performed subsequently or independently to confirm these results (Janecka, Fichna et al. 2004). Binding assays require the use of radiolabeled ligands, which are expensive, may not be available for the receptors being studied and generate radioactive waste that needs to be carefully discarded. Also, molecules that will allosterically change the binding of a chosen compound will not necessarily lead to an increase in the signaling potency (Gupta, Décaillot et al. 2006). Moreover, data obtained in different laboratory are not comparable due to the differences in synaptosomal preparations, the concentration, type of radioligand used, and the method of reporting binding. Thus, bioassay involving adenylate cyclase and tissue preparation offer certain advantages over receptor binding assays since they are less expensive, more feasible and have minimum impact on environment.

2.7. Identification and detection of opioid peptides

Reversed-phase high performance liquid chromatography (RP-HPLC) is one of the most frequently used techniques for identification and purification of bioactive peptides (Thewissen, Pauly et al. 2011, Coda, Rizzello et al. 2012) because it is well-established, relatively inexpensive, and user-friendly. It has been used widely for identification, separation and quantification of β-casomorphins (Matar and Goulet 1996, Muehlenkamp and Warthesen 1996, Jinsmaa and

Yoshikawa 1999). Despite these advantages, RP-HPLC is comparatively less sensitive to detect the relatively small amount of opioid peptide present in food and it can lead to overestimation of the peptide concentration (Muehlenkamp and Warthesen 1996) because of co-elution of peptides with similar physico-chemical and spectrophotometric absorption properties.

To overcome the sensitivity problem during identification and quantification, HPLC has been coupled to mass spectrometry such as tandem mass spectrometry (MS/MS), quadrupole ion-trap mass spectrometry (QIT-MS) and time of flight mass spectrometry (TOF-MS). Using tandem mass spectrometry, several potentially bioactive peptides, including β-casomorphin-7 have been identified from an enzyme-modified cheddar cheese prepared using a neutral protease produced from *Bacillus subtilis* (Haileselassie, Lee et al. 1999). It has been used for detection of β-casomorphin-9 in water-soluble extracts of an Italian goat cheese (Rizzello, Losito et al. 2005), gluten exorphins A5 and B5 in cerebro-spinal fluid (Fanciulli, Azara et al. 2006, Fanciulli, Azara et al. 2007) and gluten exorphins B4 and B5 in blood (Pennington, Dufresne et al. 2007). It has been used for detection of β-casomorphins -5 and -7 from infant milk and casein variants (De Noni 2008) and dairy products after simulated GI digestion (De Noni 2008). QIT-MS is sensitive, easy and time efficient technique that allows structural elucidation of peptides. This technique gives information about the major product ions obtained which can be used to construct fragmentation pathways and has been used for detection of β-casomorphin in cheese and milk (Juan-García, Font et al. 2009). TOF-MS has high resolving power and is highly accurate technique for identification and confirmation of unknown compounds in a complex matrix. It

combines elemental formula information from high mass accuracy experiments with structural information from fragmentation experiments. It has been used for detection of β-casomorphins in the water-soluble extract of a matured Gouda cheese (Toelstede and Hofmann 2008) and opioid fragment from bovine β-casein (Schmelzer, Schöps et al. 2004, Schmelzer, Schöps et al. 2007). Apart from these chromatographic techniques, enzyme–linked immunosorbent assay (ELISA) is more precise and specific for detection of low quantities of peptide from different sources. It has been used for determination of β-casomorphin-7 and -5 from cheese (Sienkiewicz-Szłapka, Jarmołowska et al. 2009) and milk (Cieslinska, Kostyra et al. 2012).

Thus, HPLC coupled to different ranges of mass spectrometry technologies can be used successfully for identification and purification of opioid peptides, but more advancement is still needed to screen complex food material since interferences from proteins, lactose and lipids can lead to misleading quantitative and qualitative results.

2.8. *In silico* approach for predicting opioid sequences

In order to screen bioactive peptides, protein is hydrolysed and hydrolysate is tested for the presence of desired bioactivity, followed by isolation and purification of peptide and the whole process is time consuming and tedious. Moreover, hydrolysates will also have additive and synergistic effect of various components (Udenigwe 2014). So, there is need for more precise and direct methods for identification of bioactive peptides. Recently, computer-based simulation has been applied towards the discovery of bioactive peptides encrypted

in food proteins (Carrasco-Castilla, Hernández-Álvarez et al. 2012, Lacroix and Li-Chan 2012, Holton, Pollastri et al. 2013, Udenigwe, Gong et al. 2013). In this approach bioinformatics peptide databases BIOPEP and PEPBANK are used to predict the presence of bioactive peptides in the primary structure of food protein, which can be obtained from databases UniProtKB, SwissProt and TrEMBL (Udenigwe, Gong et al. 2013). Proteolysis tools such as BIOPEP, ExPASy PeptideCutter (http://web.expasy.org/peptide_cutter) and PoPS (http://pops.csse.monash.edu.au) are then used to find the specificities of various enzymes to liberate desired peptide from food. This approach has been used for prediction of angiotensin converting enzyme inhibitory peptides (Cheung, Nakayama et al. 2009, Dellafiora, Paolella et al. 2015) and dipeptidyl peptidase-IV inhibitors (Lacroix and Li-Chan 2012, Nongonierma, Mooney et al. 2014) from different food proteins and confirmed using *in vitro* experiments. Cereal proteins (wheat, oat, barley and rice) sequence clearly shows high occurrence frequencies of peptides having angiotensin-converting enzyme-inhibitory, dipeptidyl peptidase-inhibitory, antithrombotic, antioxidant, hypotensive, and opioid activities (Cavazos and Gonzalez de Mejia 2013) Yet the sequences corresponding to opioid peptides and their bioactivity needs further research (Cavazos and Gonzalez de Mejia 2013). This approach offers inexpensive and time efficient way for detecting novel bioactive peptides from various food proteins and aids in screening of various proteins as a substrate for production of bioactive peptides.

2.9. Absorption and fate of opioid peptides in the body

During digestion, food proteins are hydrolysed to peptides and amino acids by digestive enzymes. Several factors affect the transport and absorption of peptides in GI tract including pKa and size of the peptide and pH microclimate. Gastric emptying and intestinal transit affect site where peptide is present along the GI tract thereby impacting absorption. In healthy adults, peptides larger than di-tripeptides are not readily absorbed except during stress, certain diseases or aggression when increased intestinal permeability occurs (De Noni, FitzGerald et al. 2009).

Peptides formed during digestion can cross the intestinal epithelium via transcellular or paracellular pathways. During transcellular transportation, peptides are subjected either to carrier mediated transport or transcytosis and/or endocytosis and during paracellular transportation, peptides are transported by passing through intercellular spaces. It has been shown that intestinal mucosa does not present an absolute barrier and various peptides including gluten exorphins A5 and A4 can cross the intestinal epithelium (Shimizu, Tsunogai et al. 1997, Chabance, Marteau et al. 1998, Roumi, Kwong et al. 2001, Dorkoosh, Broekhuizen et al. 2004, Quirós, Dávalos et al. 2008, Stuknytė, Maggioni et al. 2015). However, the mechanisms of transfer of opioid peptides across the intestinal epithelium have not been clearly established. Transportation of DADLE (a synthetic opioid) occurs through sodium coupled intestinal transporter (Ananth, Thakkar et al. 2012). It has been reported that opioid peptides are not transported by translocation across cellular membranes (Patel, Zaro et al. 2007), diffusion or paracellular pathways (Iwan, Jarmołowska et al. 2008). .

Intestinal absorption of opioid peptides has been studied using *in vitro* systems and *in vivo* animal models. *In vitro* systems use monolayer of Caco-2 cell line or intestinal mucosa mounted in Ussing-type chambers. Umbach *et al*. have shown the presence of β-casomorphin-7 immunoreactive material in the plasma of newborn calves after milk intake (Umbach, Teschemacher et al. 1985). Morphiceptin activates opioid receptor only when added to serosal side of rabbit ileum as compared to mucosal addition due to mucosal degradation of morphiceptin by di-peptidyl-peptidase-IV (DPP-IV) and pre-treatment of ileum with diisopropylfluorophosphate (inhibitor of DPP-IV) prevents mucosal degradation (Mahe, Tome et al. 1989). The trans-epithelial transport of μ opioid receptor agonists, human β-casomorphin-5 and -7 and antagonist, lactoferroxin A have been confirmed (Iwan, Jarmołowska et al. 2008).

Food-derived opioids absorbed in the GI tract first interact with receptors present on the enteric nervous system (ENS) thus affecting GI functions. The ENS is a network of nerve cells found in the wall of the GI tract controlling motility and secretion and regulating digestion, absorption and immunomodulation (Furness 2008). β-casomorphin fragments modulate mucus secretion by acting on the intestinal mucin producing cells (Claustre, Toumi et al. 2002, Trompette, Claustre et al. 2003, Zoghbi, Trompette et al. 2006), gluten exorphins (Morley, Levine et al. 1983) and β-casomorphin prolong GI transit time (Daniel, Vohwinkel et al. 1990, Defilippi, Gomez et al. 1994). β-casomorphins have been shown to stimulate insulin release (Schusdziarra, Schick et al. 1983), inhibit gastric motility and emptying (Froetschel 1996) and attenuate the suppression of fat intake via enterostatin (White, Bray et al. 2000).

2.10. Transport in the blood stream and across the blood brain barrier (BBB)

Half-life of opioid peptides in blood is short due to peptidase activity. Endogenous opioids, dynorphin (1-13) and leu-enkephalin have half-life of less than one minute and 6.7 minutes, respectively (Brugos and Hochhaus 2004, Wang, Hogenkamp et al. 2006). Dermorphin displays a longer half-life as compared to enkephalins (Negri, Lattanzi et al. 1998). Half-life of these peptides can be extended by binding them to carrier proteins such as albumin (Dennis, Zhang et al. 2002) or transferrin (Kim, Zhou et al. 2010). To our knowledge, *in vivo* half-lives of exogenous opioid peptides in blood have not been measured and needs investigation.

Transfer of peptides from peripheral circulation to the central nervous system via the BBB can be done by four different peptide transport systems PTS-1, PTS-2, PTS-3 and PTS-4 (Ganapathy and Miyauchi 2005). PTS-1 is responsible for the transport of opioid peptides including Tyr-MIF-1, met-enkephalin and leu-enkephalin (Ganapathy and Miyauchi 2005). Transport of opioid peptides (DPDPE and deltorphin II) can also be done through organic anion transporting polypeptides which are expressed at BBB (Gao, Hagenbuch et al. 2000). Transport of biphalin, a potent opioid analgesic involves neutral amino acid carrier (Brasnjevic, Steinbusch et al. 2009). Glycosylation of peptides has shown promising result for transportation of peptides via glucose carrier GLUT1 including glycosylated analogues of met-enkephalin and dermorphin (Brasnjevic, Steinbusch et al. 2009).

Stefanucci, Mollica et al. (2018) suggested the potential use of food derived opioid peptides as dietary supplement. These peptides exert nociception, emotion

and memory when administered via oral, intra-cerebral-ventricular or intra-peritoneal route. This indicates that the peptides may have crossed the blood brain barrier or acted peripherally. These food-derived opioid peptides influence gastrointestinal functions such as gut motility, hormone release, appetite, mucus production, and local immunity and can benefit both the nervous and digestive systems in healthy adults. (Stefanucci, Mollica et al. 2018, Liu and Udenigwe 2019).

2.11. Conclusion

Opioids are well known analgesic used in clinical practice, but serious side-effects are known with traditional opioids like morphine. Peptides derived from food having opioid activity might have less side-effect thus, offering significant advantages over traditional opioids. Amino acid profiles of most plant and animal food proteins suggest these to be potential source for production of these bioactive peptides. Presence of tyrosine at N terminus is required for these peptides to demonstrate opioid activity. α-casein exorphins, casomorphins, casoxins, lactorphins, and lactoferroxins from milk and gluten exorphins, rubiscolin and soymorphins from plant proteins have been described as peptides with opioid activity. Fermentation and enzymatic hydrolysis of food proteins can be used to release these peptides in varying quantities and opioid bioactivity. There is a lack of information available on using these approaches for production of opioid peptides from readily available food proteins such as gluten which is a by-product of the starch manufacturing industry. Research on adding value to wheat gluten will

certainly increase the profitability of Australian agriculture benefiting national economy.

The activity of opioids depends on their affinity for specific receptor, μ, δ, and κ. This suggests that opioid activity assessment needs to be very specific and the end result of pain relief using these opioids are rather complex. To date, opioid peptides from animal proteins appear to mainly bind to μ receptors while those from plant proteins to δ receptors with an exception of soymorphin. Most investigations about food opioids have been based on commonly used tissue preparations, GPI and MVD, are specific for μ and δ receptors, respectively. Binding to κ receptor by food derived opioid peptides is not known. There is no evidence in the literature of the use of rabbit *vas deferens*, which is rich in κ opioid receptor, and therefore can be used to confirm whether food opioids bind to this receptor (Aldrich, Kulkarni et al. 2011). The complexity of using different assays for determination of opioid activity makes it impossible to compare the activity of different opioids. From pharmacological perspective therefore there is an urgent need for development of a standard method for assaying opioid activity.

When released in GI tract, peptides interact with ENS receptors or transported across the intestinal epithelium, into blood stream and across the blood brain barrier to interact with receptors present on central nervous system. Investigation of transportation and interaction of these food derived peptides with receptors present on central and peripheral nervous system will open possible uses of these peptides for treatment of various diseases. Some studies have shown the effect of these peptides on nervous system (analgesia, antinociception, enhanced memory), GI functions (increased intestinal transit time, enhanced appetite and suppression of

high fat intake), and increase β-oxidation and energy expenditure. Thus, suggesting that they can be used as nutraceuticals for management of pain, blood sugar levels and reduction in obesity. Further research is needed to develop methods of producing functionally active opioid peptides from food proteins in significant amounts for food and pharmaceutical uses and to devise strategies to assure their targeted delivery.

Chapter 3. General materials and methods

This chapter describes general materials and methods used in the thesis. Specific descriptions of the procedures are presented in **Chapters 4 –7**.

3.1. Materials

Wheat flour with a protein content of 12.5% was obtained from Allied Mills (Australia). The composition of the flour as supplied by the company was: 14% moisture, 69.4% carbohydrate, 12.5% protein, 1.2% fat and 0.55 % ash. Peptides were custom synthesized from Mimotopes (Melbourne, Australia) with > 95% purity guaranteed (**Chapter 4**). Enzymes used for hydrolysis: pepsin (P77160), trypsin (T1426) and alcalase (30079) were from Sigma Aldrich while flavourzyme was from Novozyme (Bagsværd, Denmark) (**Chapter 6**).

Hydrochloric acid was purchased from Merck (Melbourne, Australia); mini-PROTEAN TGX gel (12%), 10X Tris/Glycine/SDS buffer and 2X Laemmli sample buffers were obtained from Bio-Rad (Melbourne, Australia); o-phthalaldehyde (OPA), methionine, 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB), glutathione and all other chemicals were from Sigma Aldrich (Steinheim, Germany).

3.1.1. Bacterial cultures and media

Lactic acid bacteria cultures: *Lactobacillus acidophilus* was acquired from Victoria University culture collection, *Lactobacillus alimentarius (*ATCC 29643), *Lactobacillus brevis* (ATCC 13648), *Lactobacillus hilgardii* (ATCC 8290) and *Lactobacillus plantarum* (ATCC 8014) were acquired from ATCC culture collection and *Lactobacillus fermentum* (LMG 26741) and *Lactobacillus plantarum* (LMG 6907) were acquired from BCCM/LMG bacteria collection. de Man, Rogosa, and Sharpe (MRS) broth was obtained from Oxoid (Basingstoke, Hampshire, England) (**Chapter-5**).

3.1.2. Cell lines and media

FlpIn CHO (Chinese hamster ovary) cells stably transfected with pOG44 vector encoding Flp recombinase and pDEST vector encoding either human μ or κ receptors were a kind gift from Dr. Meritxell Canals, Monash Institute of Pharmaceutical Sciences, Melbourne, Australia. The cells were transfected using polyethylenimine as transfection reagent, and hygromycin-B (200 μg/mL) was used as a selection agent (Burford *et al.*, 2015) (**Chapters 4, 5 and 6**). Cell culture media, Dulbecco's Modified Eagle's Medium (DMEM) containing 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), fetal bovine serum (FBS), antibiotic-antimycotic (100X) and hygromycin-B and phosphate buffer saline (PBS) - pH 7.2, were acquired from Life Technologies (Carlsbad, California, US). Lance cAMP detection reagents, and bovine serum albumin (BSA) stabiliser and optiplate were from Perkin Elmer Life Sciences (Cambridge, MA). 3-Isobutyl-1-methylxanthine (IBMX), trypsin-EDTA, forskolin, [D-Ala^2, N-MePhe^4, Gly^5-ol]-enkephalin (DAMGO), dynorphin A and all other chemicals were purchased from Sigma Aldrich (Australia).

3.2. Methodology

3.2.1. Preparation of gluten

Gluten was extracted from wheat flour by hand-washing using AACC method 38-10 (Approved Methods of the American Association of Cereal Chemists, 2000). The extracted gluten was freeze dried using Alpha 1–4 LSC freeze dryer (Martin Christ, Germany) and powdered using a coffee grinder. The gluten powder was passed through a 212 μm sieve before using and had protein

concentration of 75% (correction factor N * 5.7) as measured using Kjeldahl method (Qiu, Sun et al. 2014). Please refer to **Chapters 6 and 7**.

3.2.2. Preparation of fermented slurry

The wheat flour slurry was prepared by mixing 120 g flour and 280 g of tap water with a dough yield (DY; dough weight * 100/flour weight) of 330 (DY330) (Coda, Rizzello et al. 2012) **(Chapter 5).** Selected LAB were inoculated and fermentation was carried out under stirring conditions (120 rpm) at 37°C for 24. Control doughs (DY 330) was prepared without any bacterial inoculum and incubated under the same conditions. Water/salt-soluble extracts (WSE) containing albumins/globulins was being prepared. Briefly, 10 mL of fermented sample were extracted with 10 mL of Tris-HCl buffer (50 mM, pH 8.8) for 30 min with vortexing in between, followed by centrifugation (20,000 g, 20 min at 4°C) and the supernatant was collected and filtered through 0.45 µm filter and used for further analysis.

3.2.3. Preparation of enzymatic hydrolysates

Gluten suspension (50mg/mL) was made in Milli-Q water and pH of the suspension was adjusted to optimum pH and temperature as specified in **Chapter 6**. Hydrolysis was carried out using enzyme: substrate ratio of 1:100 (Kong, Zhou et al. 2008) for 2 hours at optimum pH. Hydrolysates were kept in boiling water bath for 10 min to inactivate the enzyme, cooled to room temperature, and centrifuged at 3274 g for 20 min. The supernatant were filtered through 0.45µM PTFE membranes and freeze dried using Alpha 1–4 LSC Christ freeze dryer (Martin Christ, Germany).

3.2.4. Determination free amino group concentration using o-phthalaldehyde (OPA) reagent

This method is based on protocol published by Church *et al*., 1983. The OPA reagent was prepared fresh by combining 25 mL of sodium phosphate buffer (50 mM; pH 8.3), 2.5 mL of sodium dodecyl sulphate (20%, w/v), 40 mg of OPA (dissolved in 1 mL of methanol), and 100 µL of β-mercaptoethanol and diluting to 50 mL with water. Samples (50 µL) were added to a test tube containing 3 mL of the OPA reagent and mixed gently for 5 sec. The absorbance was measured at 340 nm after two min using a Nova Spec-II spectrophotometer (Pharmacia, England, UK). Buffer was used as a blank and concentration of free amino group was determined using methionine as a standard. The specific method used for preparation of samples and analysis can be found in **Chapters 5, 6 and 7.**

3.2.5. Peptide profiling

3.2.5.1. Size exclusion high performance liquid chromatography (SE-HPLC)

SE-HPLC was conducted on Prominence-i, LC-2030C (Shimadzu, Kyoto, Japan) with automatic injection. 10 µL of sample was loaded onto a Yarra SEC 3000 column (Phenomenex, Torrance, United States). Samples were eluted with 50 mM sodium phosphate buffer (pH 6.8) containing 300 mM NaCl at flow rate of 0.4 mL/min and detected at 214 nm using UV/Vis detector (Jansens, Lagrain et al. 2013). The specific details for the preparation of samples and analysis are presented in **Chapters 5, 6 and 7**. Albumin (67 kDa), β-lacto globulin (18.4 kDa), RNAse (13.7 kDa) and vitamin B_{12} (1.3 kDa) were used to prepare a standard curve by plotting the molecular weights and the corresponding retention times determined

by running these proteins under the same conditions of chromatography. This standard curve was used to determine the molecular weights of the proteins/ peptides present in samples (**Chapter-5)**.

3.2.5.2. Reverse phase high performance liquid chromatography (RP- HPLC)

The samples were profiled on a Prominence-i, LC-2030C (Shimadzu, Kyoto, Japan) liquid chromatography equipped with C-18 monomeric column (5mm, 300A˚, 250 mm*4.6 mm; Grace Vydac, Hesperia CA, USA). Samples were eluted by a linear gradient from 95% to 5% solvent A (0.1% trifluoroacetic acid (TFA) in deionised water) in solvent B (0.1% TFA in acetonitrile) over 90 min. Separations were conducted at room temperature at a flow rate of 0.75 mL/min and eluents were detected at 214 nm using UV/Vis detector. All samples and solvents were filtered through a 0.45 mm membrane filter (Schleicher & Schuell GmbH, Germany). The specific details for the preparation of samples and analysis are presented in **Chapters 5 and 6.**

3.2.6. Liquid chromatography/ mass spectrometry (LC/MS) analysis

For LC/MS analysis, samples were dissolved in 2% acetonitrile containing 0.1 % TFA and analysed using an Ultimate 3000 nano HPLC (ThermoFisher scientific) through MicroTOFq quadrupole TOF (Bruker Daltonics) mass spectrometer. The samples were separated over a 30 min gradient on a pepmap 100, 75 µm id, 100 Å pore size, reversed phase nano column with 95% buffer A (0.1% formic acid) to 70% B (80% acetonitrile and 0.1% formic acid), at a flow rate of 300 nL/min. The eluent was nebulised and ionised using the Bruker nano ESI source with a capillary voltage of 4000 V. Peptides were selected for MS/MS

analysis in auto MSn mode based on peak intensity selecting 4 precursor ions and active exclusion released after 2 min. Prior to analysis, the qTOF mass spectrometer was calibrated using tune mix as standard. Data from LC/MS/MS run were exported in Mascot generic file format (*.mgf) and searched against wheat database downloaded from UniProt using the MASCOT search engine (version 2.4). The search parameters used were: missed cleavages, 1; peptide mass tolerance, ± 20 ppm; peptide fragment tolerance, ± 0.04 Da; peptide charge, 2+, 3+ and 4+; fixed modifications, carbamidomethyl; variable modification, Methionine oxidation. Please refer to **Chapter 6** for specific details.

3.2.7. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Samples were dissolved in the SDS buffer and mixed with a volume of 2× Laemmli sample buffer containing β-mercaptoethanol followed by heating at 95°C for 5 min for the reducing SDS-PAGE analysis. For the non-reducing SDS-PAGE analysis, all the conditions were the same except that the sample buffer did not contain β-mercaptoethanol and it was not heated. Samples were then loaded on the gel and electrophoresis was conducted at 200 V for 45 min. The gels were stained using 0.15% Coomassie Brilliant Blue R250 dye dissolved in 72% isopropanol and 3% acetic acid for 1 h followed by overnight destaining (10% isopropanol, 10% acetic acid) on a shaker. The gel images were captured using a Chemidoc MP imager (Bio-Rad). More specific details about method and sample preparation are presented in **Chapters 5 and 7**.

3.2.8. Fractionation of hydrolysate to <3 kDa

To obtain fraction of <3 kDa from the hydrolysates, the hydrolysates were fractionated by using centrifugal ultrafiltration membrane (Z740200, Sigma Aldrich, Australia). The hydrolysates were centrifuged at 3200 X g for 120 minutes at 4 °C to collect filtrates. The collected fractions were lyophilised and subsequently used for determination of opioid activity. The specific details are presented in **Chapters 5 and 6**.

3.2.9. Opioid activity measurement

Opioid activity was determined on the basis of inhibition of adenylate cyclase using transfected cell lines expressing μ and κ opioid receptors. The protocol developed by (Burford, Livingston et al. 2015) was adopted. Cells were grown and maintained in DMEM media (containing 10% FBS, 1% antibiotic-antimycotic and 200 μg/mL hygromycin-B) in a humidified incubator containing 5% CO_2 at 37°C. Confluent cells (90%) were harvested and resuspended at 2 x 10^6 cells/mL in media and 100 μL of cell suspension was transferred into sterile 96 well plates and incubated at 37°C and 5% CO_2 overnight. The culture media in all the wells were replaced with stimulation buffer (PBS, 50 mM IBMX and BSA stabiliser) and incubated for 30 min. Cells were treated with different concentrations of hydrolysate in the presence of forskolin (10μM) for 30 min. The stimulation buffer was removed by decantation and 50 μL of 100% ethanol (ice cold) was added to each well. Ethanol was evaporated and of 75 μL of lysis buffer (0.3% tween-20, 5 mM HEPES and 0.1% BSA) was added. Change in the concentration of cAMP in the lysate was estimated using Lance cAMP detection

kit (Perkin Elmer, Melbourne) using kit instructions provided by the supplier. Time-resolved fluorescence (TRF) was detected using an Envision plate reader (PerkinElmer, Melbourne) with excitation at 337 nm and emission was read at 615 nm and 665 nm. Data were analysed and EC_{50} values determined using nonlinear regression analysis to fit a logistic equation to the dose response curve using Graph Pad Prism, version 7 (Graph Pad San Diego, CA). DAMGO was used as a positive control. The details are presented in **Chapters 4, 5 and 6.**

3.2.10. Sequences of wheat gluten proteins

The sequences of wheat storage proteins high molecular weight (HMW) and low molecular weight (LMW) glutenins and gliadins (alpha, gamma and omega) were accessed from UniProt database at http://www.uniprot.org/uniprot/ ((Boutet, Lieberherr et al. 2007). These sequences were then searched for the presence of tri and oligo-peptides containing tyrosine and proline amino acids either consecutively or separated by a single amino acid. The details are presented in **Chapter 4.**

3.2.11. Peptide ranking and bioactivity prediction

Occurrence of predicted sequences and their structural motif thereof were analysed in known opioids using BIOPEP database (Minkiewicz, Dziuba et al. 2008). Please refer to **Chapter 4** for specific details. The ranking of the peptides is based on the fact that different functional classes of bioactive peptides share structural features. The ranking of the peptides produced by selected enzymes was predicted by PeptideRanker (http://bioware.ucd.ie/~compass/biowareweb/Serverpages/peptideranker.php). The

peptides with a score of more than 0.5 were considered bioactive (Qiao, Tu et al. 2018). The details are presented in **Chapter 4**.

3.2.12. Heat treatment of gluten

Gluten powder (400 mg) was suspended in 5 mL aqueous HCl solutions at pH 1, 2 and 3 in glass tubes to prepare 8% gluten dispersions. The tubes were sealed and shaken thoroughly to obtain homogenous dispersions. Another set of samples at pH 1, 2 and 3 were heated in an oil bath set at 80ºC for 15 min to study the effect of heat on gluten. After treatment, the samples were freeze-dried using an Alpha 1–4 LSC freeze dryer (Martin Christ, Germany) before further analysis. Please refer to **Chapter 7** for specific details.

3.2.13. Colour measurement of gluten

A portable Konika Minolta (Tokyo, Japan) Chroma Meter CR-300 with illuminant D65 and a 2° standard observer was used to measure the colour of gluten. A measuring head area of 8 mm diameter was used with diffuse illumination and 0º viewing angle, and a white chromameter standard tile (L = 97.47, a = 0.13, b = 1.83) was used for calibration. Samples were placed on the white standard plate to measure the CIE $L^*a^*b^*$ colour values which were taken in triplicate. Please see **Chapter 7** for specific details.

3.2.14. Scanning electron microscopy (SEM) of treated gluten

Images were obtained using a JOEL NeoScope SEM (JCM-5000, Frenchs Forest, NSW, Australia). Freeze-dried samples were mounted on an aluminium sample holder and coated with gold for 6 min using JOEL NeoCoater

(MP19020NCTR). The images were acquired at a scale of 10 µm under high vacuum using an accelerating voltage of 10 kV. Please see **Chapter 7** for specific details.

3.2.15. Fourier transform infrared (FTIR) analysis of treated gluten

The FTIR absorbance spectra were acquired using Perkin Elmer Frontier™ FTIR spectrophotometer (Waltham, USA) in attenuated total reflectance (ATR) mode using a diamond ATR crystal. Background spectra were recorded at the beginning of the measurements with a blank diamond ATR cell. For each sample, 16 scans were collected at a resolution of 4 cm^{-1}. For determination of the secondary structure of the proteins, deconvolution of spectrum was performed using Origin Pro 8.5 software. The spectra were analysed by taking second derivatives and applying the fit peak function (option available within peak analyser feature of the software) in the amide I region (1700-1600 cm-1). Assignment of bands in the de-convoluted spectra was as follows: the bands located at 1611-1600 cm^{-1} were assigned to hydrated extended chains, 1640-1619 cm^{-1} and 1690-1670 cm^{-1} to β-sheets, 1670-1660 cm^{-1} to β-turns, 1660-1650 cm^{-1} to α-helix, and 1645 cm^{-1} to random coil (Ukai, Matsumura et al. 2008, Wang, Luo et al. 2016). The secondary structures were determined from the relative areas of the individual assigned bands according to Ukai et al. (2008). Please see **Chapter 7** for specific details.

3.2.16. Free thiol group analysis of treated gluten

Samples prepared for primary amine analysis were also used for the determination of free-thiol using DTNB (Wang, Luo et al. 2016). The reagent was prepared by mixing 4 mL DTNB (2 mg/mL in n-propanol) and 20 mL of sodium

phosphate buffer (pH 8). A sample of 100 µL was mixed with an equal volume of DTNB reagent, shaken for 1 hour and the absorbance was measured at 415 nm using iMark™ Microplate Absorbance Reader (Bio-Rad, Australia). Buffer containing DTNB was used as a blank and the concentration of free-thiol was determined using glutathione as a standard. Please see **Chapter 7** for specific details.

Chapter 4. Evaluation of in *silico* approach for prediction of presence of opioid peptides in wheat gluten

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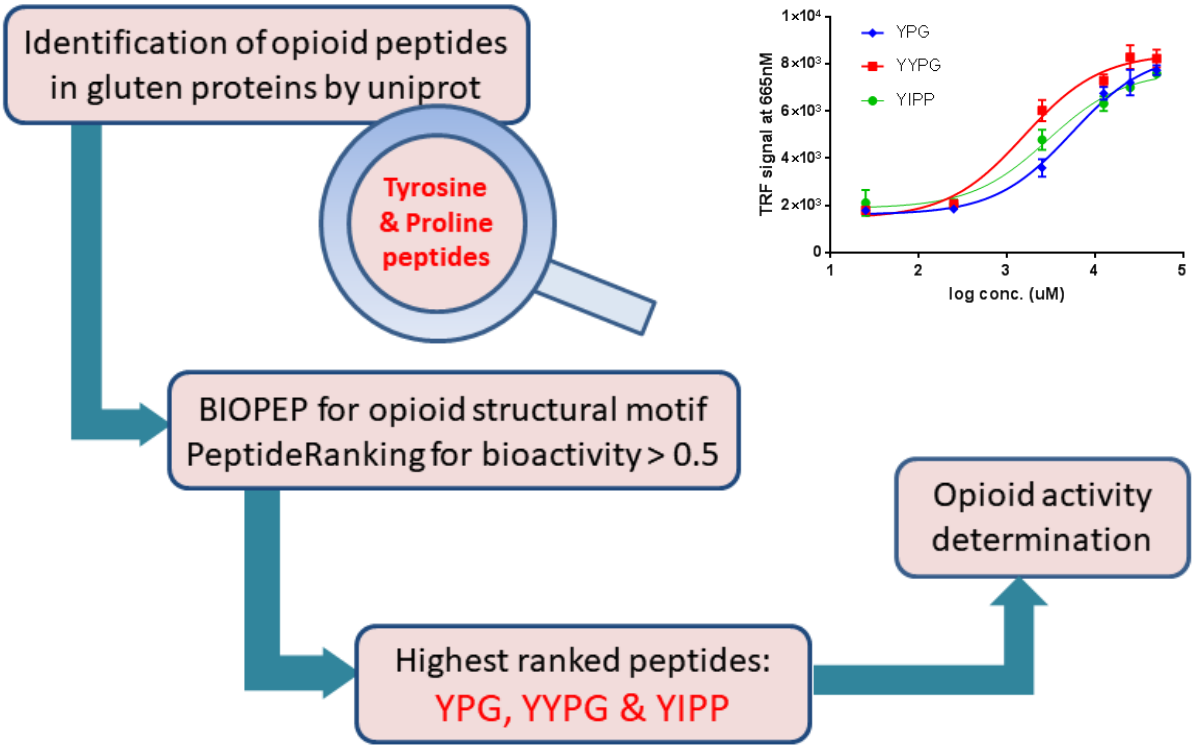


4.1. Summary

Peptides containing tyrosine and proline are widely accepted to be opioids in nature and therefore their presence in the protein indicates suitability of the protein for production of bioactive opioid peptides. In this chapter, an *in silico* approach was used to assess wheat gluten proteins as potential source of opioid peptides. *In silico* evaluation of protein sequences offer several advantages as it is precise, cost effective and time-saving method for evaluation of presence of bioactive peptides in food proteins. Eleven peptides were identified and occurrence of predicted sequences or their structural motifs were analysed using BIOPEP database and ranked using PeptideRanker for bioactivity. Based on higher peptide ranking, three sequences YPG, YYPG and YIPP were selected and their opioid activity was measured by cAMP assay against μ and κ opioid receptors. Three peptides identified inhibited the production of cAMP to varied degree with EC_{50} values of YPG, YYPG and YIPP were 5.3 mM, 1.5 mM and 2.9 mM for μ-opioid receptor, and 1.9 mM, 1.2 mM and 3.2 mM for κ-opioid receptor, respectively. The study showed that *in silico* approach can be used for the prediction of opioid peptides from gluten.

4.2. Introduction

Opioids, such as morphine and codeine, are the most common clinically used drugs for pain management (Teschemacher 2003, Janecka, Fichna et al. 2004, Trescot, Datta et al. 2008). These opioids bind to opioid receptors present in the central and peripheral nervous system. However, they are often associated with side-effects like sedation, dizziness, nausea, vomiting, constipation, addiction, tolerance and respiratory depression (Benyamin, Trescot et al. 2008). Opioids were considered to be alkaloid (derived from opium) only until discovery of endogenous opioid peptides in 1975 (Goldstein, Goldstein et al. 1975, Hughes, Smith et al. 1975). These endogenous peptides and their modified forms have shown activity similar to alkaloids (Mollica, Davis et al. 2005, Giordano, Sansone et al. 2010, Mollica, Pinnen et al. 2011, Mollica, Pinnen et al. 2013, Mollica, Carotenuto et al. 2014). However, exogenous opioid peptides or exorphins are naturally derived from food proteins (Yoshikawa 2013, Stefanucci, Mollica et al. 2016). These exogenous peptides are of particular interest as they are naturally derived from food, have possibly less side-effects (compared to synthetic drugs) and are inexpensive to produce (Udenigwe, Gong et al. 2013, Garg, Nurgali et al. 2016). Most known bioactive peptides are small and non-immunogenic, as compared to larger peptides (6-25 amino acids) (Wang, Mejia et al. 2005). Hence, small peptides are researched more for their bioactivity and considered safe (Shahidi and Zhong 2008).

Generally, bioactive peptides, including opioids, are produced by hydrolysis of food protein during food processing (ripening, fermentation), storage (Choi, Sabikhi et al. 2012) and during gastrointestinal (GI) digestion as reviewed by Garg, Nurgali et al. (2016) and Stefanucci, Mollica et al. (2016). The protein hydrolysate

is then tested for bioactivity using *in vitro* and *in vivo* methods. Since these hydrolysates are mixtures of several peptides, their bioactivity results from the additive and synergistic effect of various components present. Bioactive hydrolysates containing mixtures of peptide needs to be fractionated, purified and then tested for bioactivity (Udenigwe and Aluko 2012). The whole process of preparing bioactive peptides from native proteins by hydrolysis, separation and fractionation, is tedious, time consuming and the yields are low (Udenigwe 2014), limiting and/or delaying their use in clinical applications.

Alternatively, bioinformatics tools can be used for predicting the presence of bioactive peptides in proteins (*in silico* approach) (Carrasco-Castilla, Hernández-Álvarez et al. 2012, Lacroix and Li-Chan 2012, Holton, Pollastri et al. 2013). Using this approach, one can search for potential precursors of bioactive peptides and develop efficient proteolytic enzymes for their release from native protein sequences (Carrasco-Castilla, Hernández-Álvarez et al. 2012, Udenigwe, Gong et al. 2013). In this approach, protein databases, such as, UniProtKB, SwissProt and TrEMBL can be used to access sequences of a food protein, and presence of bioactive peptides can be predicted using peptide databases BIOPEP and Pepbank (Udenigwe 2014). The BIOPEP application contains a database of biologically active peptide sequences and a program enabling construction of profiles of the potential biological activity of protein fragments, calculation of quantitative descriptors as measures of the value of proteins as potential precursors of bioactive peptides, and prediction of bonds susceptible to hydrolysis by endopeptidases in a protein (Minkiewicz, Dziuba et al. 2008). In fact, it has been successfully used for prediction of bioactive peptides from different food proteins having angiotensin

converting enzyme inhibitory (ACE-I) activity (Cheung, Nakayama et al. 2009, Dellafiora, Paolella et al. 2015) and dipeptidyl peptidase-IV inhibitors (DPP-IV) (Lacroix and Li-Chan 2012, Nongonierma, Mooney et al. 2014). PeptideRanker is a web based application and can predict the probability of a peptide being bioactive according to their score between 0 and 1 and can assist in the discovery of new bioactive peptides across many functional classes. Generally, any peptide over 0.5 threshold is labelled to be bioactive (Mooney, Haslam et al. 2012, Mooney, Haslam et al. 2013). Increasing the threshold from 0.5 to 0.8 reduces the number of false positive prediction from 16 to 6 %, however, true positive rates also decrease (Mooney, Haslam et al. 2012). If predicted probability is closer to 1, the probability of peptide to be bioactive is significantly high (Mooney, Haslam et al. 2012).

Bioinformatics approach is used for identification of structural patterns of peptides of known bioactivities. Presence of tryptophan in a peptide is associated with antioxidant activity (Chuan-Hsiao, Yin-Shiou et al. 2014) and carboxyl terminal alanine or proline containing peptides are DPP-IV inhibitors (Lacroix and Li-Chan 2012). However, there is general lack of information for screening opioid peptides using bioinformatics approach. The opioid peptides present in wheat gluten are exorphins; A5, A4, B5, B4 and C having sequences GYYPT, GYYP, YGGW, YGGWL and YPISL, respectively (Zioudrou, Streaty et al. 1979, Fukudome and Yoshikawa 1992). These opioids have tyrosine and proline within their sequence and are key determinant of opioid peptides (Yoshikawa 2013). Tyrosine (Y) is present either at the amino terminal or at the second position (as in gluten exorphins GYYPT and GYYP) and acts as part of the message domain to anchor the opioid peptide within the receptor (Heyl, Schullery et al. 2003, Li, Fujita

et al. 2005). At position 1, Y acts as a dual hydrogen bond donor/acceptor with less acidic hydroxyl groups exhibiting stronger binding to opioid receptors. Moreover, steric bulk in the Y strengthens receptor binding by either a ligand conformational effect or enhanced van der Waals interactions with a loose receptor site (Heyl, Schullery et al. 2003). Proline (P) acts as a spacer that fixes the peptide shape and induces other residues to assume proper spatial orientation for interacting with the opioid receptor (Cardillo, Gentilucci et al. 2002). Peptides containing P also exhibit enhanced resistance to hydrolysis by enzymes of GI tract (Cardillo, Gentilucci et al. 2002, Trivedi, Shah et al. 2014) and are therefore more likely to be active upon oral administration (Yang, Yunden et al. 2001).

These peptides must bind to opioid receptors present within the central and enteric nervous systems. Opioid receptors belong to the superfamily of G protein coupled receptors (GPCRs) and on activation by opioid ligands, they inhibit adenylate cyclase enzyme (Gupta, Décaillot et al. 2006, Garg, Nurgali et al. 2016) thus decreasing the production of cyclic adenosine monophosphate (cAMP) in the cells (Gupta, Décaillot et al. 2006). This decrease in concentration of cAMP in cells can be used for screening opioid ligands (Huang, Kehner et al. 2001). This forms the basis of using cell lines transfected with opioid receptors for assaying the activity of peptides and using it for confirmation of peptides selected using bioinformatics approach.

The objective of this study was to search for opioid peptides in wheat proteins based on the presence of tyrosine and proline and use bioinformatics tools, BIOPEP and PeptideRanker to identity and rank these peptides for likelihood of

having opioid activity. The identified peptides were then assayed for opioid activity by cAMP assay for confirmation of their bioactivity.

4.3. Materials and methods

4.3.1. Chemicals and reagents

Cell culture media, Dulbecco's Modified Eagle's Medium (DMEM) containing 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), fetal bovine serum (FBS), antibiotic-antimycotic (100X) and hygromycin-B and phosphate buffer saline (PBS) - pH 7.2, were acquired from Life Technologies (Carlsbad, California, US). Lance cAMP detection reagents, and bovine serum albumin (BSA) stabiliser and optiplate were from Perkin Elmer Life Sciences (Cambridge, MA). 3-Isobutyl-1-methylxanthine (IBMX), trypsin-EDTA, forskolin, [D-Ala^2, N-MePhe^4, Gly^5-ol]-enkephalin (DAMGO) and dynorphin A and all other chemicals were purchased from Sigma Aldrich (Australia) unless otherwise stated. Custom peptides were synthesized from Mimotopes (Melbourne, Australia) at > 95% purity.

4.3.2. Cell lines

FlpIn CHO (Chinese hamster ovary) cells stably transfected with pOG44 vector encoding Flp recombinase and pDEST vector encoding either human μ or κ receptors were a kind gift from Dr. Meritxell Canals, Monash Institute of Pharmaceutical Sciences, Melbourne, Australia. The cells were transfected using polyethylenimine as transfection reagent and hygromycin-B (200 μg/mL) was used as a selection agent (Burford, Livingston et al. 2015).

4.3.3. *In silico* analysis

4.3.3.1. Sequences of wheat gluten proteins

The sequences of wheat storage proteins high molecular weight (HMW) and low molecular weight (LMW) glutenins and gliadins (alpha, gamma and omega) were accessed from UniProt database at http://www.uniprot.org/uniprot/ (Boutet, Lieberherr et al. 2007). These sequences were then searched for the presence of tri and oligo-peptides containing Y and P amino acids either consecutively or separated by a single amino acid.

4.3.3.2. Peptide ranking and bioactivity prediction

Occurrence of predicted sequences or their structural motif thereof were analysed in known opioids using BIOPEP database (Minkiewicz, Dziuba et al. 2008). The PeptideRanker (Bioware.ucd.ie) was used to rank the predicted sequences according to bioactivity. A peptide having ranking closer to 1 increases its chances to be bioactive so they were selected to be tested for opioid activity.

4.3.4. Opioid activity assay

Opioid activity of the peptides was determined on the basis of cAMP assay. Cells were grown and maintained at 37°C in a humidified incubator containing 5% CO_2 in DMEM, 10% FBS, 1% antibiotic-antimycotic and 200 μg/mL hygromycin-B (Burford, Livingston et al. 2015). Cells were grown to 90% confluency, harvested and resuspended at $2x10^6$ cells/mL in the media (DMEM + FBS + hygromycin B) and 100 μL of cells were seeded into sterile 96 well plates and incubated at 37°C and 5% CO_2 overnight. The culture media in all the wells were replaced with stimulation buffer consisting of PBS, 50 mM IBMX and BSA stabiliser and

incubated for 30 minutes before stimulation. Cells were stimulated at different concentrations of peptides in the presence of 10 µM forskolin for 30 minutes. The stimulation buffer containing peptides was then removed and 50 µL of ice cold 100% ethanol was added to each well. Ethanol was then evaporated and 75 µL of lysis buffer (0.3% tween-20, 5 mM HEPES and 0.1 % BSA) was added to each well and the change in concentration of cAMP in the lysate was determined using Lance cAMP detection kit. 5 µL of lysate containing cAMP was mixed with 5 µL of Alexa flour-647 anti-cAMP antibody (stock antibody diluted at 1:100 in detection buffer provided in kit). Detection mix containing Europium W8044 labelled streptavidin and biotin-cAMP was prepared according to kit instructions and kept at room temperature for 15 minutes. The detection mix (10 µL) was added to each well and incubated for 1 hour before reading. Time-resolved fluorescence (TRF) was detected using an Envision plate reader (Perkin Elmer, Cambridge, MA) with excitation at 337 nm and emission read at 615 nm and 665 nm. DAMGO and dynorphin A were used as positive controls for µ and κ opioid receptors, respectively. Data were analysed and EC_{50} values determined using nonlinear regression analysis to fit a logistic equation using Graph Pad Prism, version 7 (Graph Pad San Diego, California, US).

4.4. Results and discussion

4.4.1. Prediction of opioid peptide sequences in wheat gluten

Wheat is one of the most important cereals consumed globally providing protein and carbohydrates to the diet. The main storage protein of wheat gluten consists of glutenins and gliadins. **Table 4.1** shows the sequence of gluten proteins

and the relevant sequences containing Y and P have been highlighted in each of the wheat proteins. Peptides sequences YPG, YPTSP, YYPG (from HMW glutenin), YIPP, YPH, YPQ, YPS (from alpha-gliadin), YPH, YVPP (from gamma gliadin) and YPN (from omega gliadin) were identified to have opioid activity **(Table 4.1**). The occurrence frequency of these opioid peptides are 17, 7, 2 and 1 in HMW glutenin, alpha gliadin, gamma gliadin and omega gliadin, respectively. Based on this observation, HMW glutenin is by far a superior source of opioid peptides than the rest of tested proteins. This has also been reported by Cavazos and Gonzalez de Mejia (2013) in terms of the occurrence frequency of opioid peptide which was more in HMW glutenin (A=0.206) as compared to LMW glutenin (0.003).

Table 4.1: Sequence of wheat storage proteins as obtained by UniProt

Wheat protein	UniProt accession number	Amino acid (AA) sequence	AA residue
HMW glutenin subunit	Q41553	MTKRLVLFAAVVVALVALTAAEGEASGQLQCER ELQEHSLKACRQVVDQQLRDVSPECQPVGGGPVA RQYEQQVVVPPKGGSFYPGETTPPQQLQQSILWGI PALLRRYYLSVTSPQQVSYYPGQASSQRPGQGQQ EYYLTSPQQSGQWQQPGQGQSGYYPTSPQQSGQK QPGYYPTSPWQPEQLQQPTQGQQRQQPGQGQQL RQGQQGQQSGQGQPRYYPTSSQQPGQLQQLAQG QQGQQPERGQQGQQSGQGQQLGQGQQGQQPGQ KQQSGQGQQGYYPISPQQLGQGQQSGQGQLGYY PTSPQQSGQGQSGYYPTSAQQPGQLQQSTQEQQL GQEQQDQQSGQGRQGQQSGQRQQDQSGQGQQP GQRQPGYYSTSPQQLGQGQPRYYPTSPQQPGQEQ QPRQLQQPEQGQQGQQPEQGQQGQQQRQGEQGQ QPGQGQQGQQPGQGQPGYYPTSPQQSGQGQPGY YPTSPQQSGQLQQPAQGQQPGQEQQGQQPGQGQ QPGQGQPGYYPTSPQQSGQEQQLEQWQQSGQGQ PGHYPTSPLQPGQGQPGYYPTSPQQIGQGQQPGQL QQPTQGQQGQQPGQGQQGQQPGEGQQGQQPGQ GQQPGQGQPGYYPTSLQQSGQGQQPGQWQQPGQ GQPGYYPTSSLQPEQGQQGYYPTSQQQPGQGPQP GQWQQSGQGQQGYYPTSPQQSGQGQQPGQWLQ PGQWLQSGYYLTSPQQLGQGQQPRQWLQPRQGQ QGYYPTSPQQSGQGQQLGQGQQGYYPTSPQQSG QGQQGYDSPYHVSAEHQAASLKVAKAQQLAAQL PAMCRLEGGDALLASQ	815
LMW glutenin	Q8W3V5	MKTFLVFALIAVVATSAIAQMETSCISGLERPWQQ QPLPPQQSFSQQPPFSQQQQQPLPQQPSFSQQQPPF SQQQPILSQQPPFSQQQQPVLPQQSPFSQQQQLVLP PQQQQQQLVQQQIPIVQPSVLQQLNPCKVFLQQQ CSPVAMPQRLARSQMWQQSSCHVMQQQCCQQL	303

		QQIPEQSRYEAIRAIIYSIILQEQQQGFVQPQQQQPQ QSGQGVSQSQQQSQQQLGQCSFQQPQQQLGQQP QQQQQQVLQGTFLQPHQIAHLEAVTSIALRTLPTM CSVNVPLYSATTSVPFGVGTGVGAY	
alpha-gliadin	A0A0E3Z527	MKTFLILALLAIVATTATIAVRVPVPQLQPQNPSQ QQPQEQVPLMQQQQQFPGQQEQFPPQQPYPHQQP FPSQQPYPQPQPFPPQLPYPQTQPFPPQQPYPQPQP QYPQPQQPISQQQAQQQQQQQQILQQILQQQLIPC RDVVLQQHNIAHASSQVLQQSTYQLVQQLCCQQL WQIPEQSRCQAIHNVVHAIILHQQQQQQQQQQQQ PLSQVSFQQPQQQYPSGQGSFQPSQQNPQAQGSV QPQQLPQFEEIRNLALETLPAMCNVYIPPYCTIAPV GIFGTN	284
gamma gliadin	P21292	MKTLLILTILAMATTIATANMQVDPSGQVQWPQQ QPFPQPQQPFCQQPQRTIPQPHQTFHHQPQQTFPQP QQTYPHQPQQQFPQTQQPQQPFPQPQQTFPQQPQL PFPQQPQQPFPQPQQPQQPFPQSQQPQQPFPQPQQ QFPQPQQPQQSFPQQQQPAIQSFLQQQMNPCKNFL LQQCNHVSLVSSLVSIILPRSDCQVMQQQCCQQLA QIPQQLQCAAIHSVAHSIIMQQEQQQGVPILRPLFQ LAQGLGIIQPQQPAQLEGIRSLVLKTLPTMCNVYV PPDCSTINVPYANIDAGIGGQ	302
omega gliadin	Q6PNA3	MKPHHDGYKYTCSIIVTFHYPNFKHQDQKHQFQE SIKHKSKMKTFIIFVLLSMPMSIVIAARHLNPSDQE LQSPQQQFLEKTIISAATISTSTIFTTTTISHTPTIFPP STTTTISPTPTTNPPTTTMTIPLATPTTTTTFSPAPTT ISLATTTTISLAPTTNSPITTTTIPAATPETTTTIPPAT RTNNYASTATTISLLTATTTPPATPTTILSATTTTIS PAPTIISPATRTNNSLATPTTIPPATATTIPPATRTNN SPATATTIPPAPQQRFPHTRQKFPRNPNNHSLCSTH HFPAQQPFPQQPGQIIPQQPQQPLPLQPQQPFPWQP EQRSSQQPQQPFSLQPQQPFS	354

Highlighted in : predicted opioid peptide sequences

4.4.2. Selection of opioid peptides using BIOPEP database and PeptideRanker

BIOPEP is a database of biologically active peptide sequences and a tool for evaluation of proteins as the precursor of bioactive peptides. The database was used to compare the presence of structural motifs comprising of di or tripeptides in identified peptides in comparison to known opioid peptides (**Table 4.2**). The peptides were classified according to different search terms and for every sequence within the class, EC_{50} values are reported. YYP (part of YYPG) structural motif is present in eight opioid peptides with EC_{50} as low as 60 µM (gluten A5 exorphin) (**Table 4.2**). Similarly, five known opioid peptides contain structural motif IP (part of YIPP), whereas Casoxin C, containing YIP (part of YIPP) is an antagonist. YVP is present in only one known opioid peptide. YP (part of YPG, YPQ, YPH, and YPN) was present in 92 out of 156 (nearly 60%) opioid peptides present. It is also realised that YP can be present at amino or carboxyl terminals or in the middle of peptide sequences and may be followed either by aromatic or non-aromatic amino acids. Majority of opioid peptides have aromatic amino acid tryptophan (W), phenylalanine (F) or tyrosine (as in YPYY) and had EC_{50} value as low as 0.01 µM (YPFGFR, YPFGFRG) and 0.02 µM (YPFGFS, YPFGFK, YPFGFG, YPFGFGG, YPFGFKG and YPFGFSG) making these very effective opioids. Non-aromatic amino acids are also present as isoleucine (I) in YPISL, leucine (L) in YPLG and YPLSL, serine (S) in YPSYGLN, YPSF and YPS, valine (V) in YPVSL, alanine (A) in YPASL, threonine (T) in YPTSL and YPTS (**Table 4.2**). EC_{50} values of YPISL, YPVSL and YPASL were 13.50 µM, 200 µM and 200 µM, respectively. This implies that peptides having YP structural motif followed by non-aromatic

amino acids as predicted in **Table 4.1** (YPG, YPQ, YPH, YPT, YPS and YPN) may have opioid activity. With limited information available of EC_{50} value of the sequences (**Table 4.2**), it is also evident that within the search term, the opioid activity is dependent on chain length of the peptide sequence. For example, high EC_{50} values of sequences YYPT (1000 µM) and YYP (800 µM) are suggestive of their weak or negligible opioid activity. Similarly, it can also be stated that casein are superior to gluten as a protein source for the opioid peptides as the EC_{50} values of peptides containing YP motif of casomorphin are significantly lower.

Table 4.2: Opioid peptides as predicted by BIOPEP database

Search term – YYP by sequence				
	Name	**Sequence**	**Molecular mass**	**EC_{50} (μM)**
1	gluten A5 exorphin	GYYPT	599.61	60
2	gluten A4 exorphin	GYYP	498.50	70
3		YYPT	542.56	800
4		YYP	441.45	1000
5		RYYP	597.64	190
6		WYYP	627.67	n.d.
7		SYYP	528.53	200
8		GYYPTS	686.69	72
Search term – IP by sequence				
1		FGGFTGRIPKLWDNQ	1735.95	n.d.
2		YPFVEPIP	961.10	n.d.
3		YPFPGPIP	887.02	n.d.
4	casoxin C *	YIPIQYVLSR	1251.46	50
5	β-casomorphin-11 (60-70)	YPFPGPIPNSL	1201.37	10
Search term – YVP by sequence				
1	fragment of human αs1-casein	YVPFP	621.71	n.d.
Search term - YP by sequence				
1	VV-hemorphin-7	VVYPWTQRF	1194.60	34.3
2	VV-hemorphin-5	VVYPWTQ	891.43	78.2

3	oryzatensin*	GYPMYPLPR	1092.52	n.d.
4		YPFT	526.23	n.d.
5		LVVYPWTQR	1160.62	n.d.
6		YPFVEP	750.34	n.d.
7		YPFV	524.25	n.d.
8		YPFVEPIP	960.48	n.d.
9		YPFVE	653.29	n.d.
10	opioid peptide	YPFP	522.23	n.d.
11	opioid peptide	YPFPGPIP	886.44	n.d.
12	opioid peptide	YPF	425.18	n.d.
13	opioid fragment of dermorphin	YAFGYPS	803.33	n.d.
14	opioid fragment of β-lipotropin β-neoendorphin	YGGFLRKYP	1099.56	n.d.
15	opioid fragment of β-lipotropin β/α-neoendorphin	YGGFLRKYPK	1227.66	n.d.
16	LVV-hemorphin-7	LVVYPWTQRF	1307.68	29.1
17	LVV-hemorphin-5	LVVYPWTQ	1004.51	80.5
18	hemorphin-8	YPWTQRFF	1143.53	4.6
19	hemorphin-7	YPWTQRF	996.46	2.9
20	hemorphin-6	YPWTQR	849.40	4.3
21	hemorphin-5	YPWTQ	693.29	46.3
22	hemorphin-4	YPWT	565.24	45.2

23	gluten C exorphin	YPISL	591.31	13.5
24	gluten A5 exorphin	GYYPT	599.24	60
25	gluten A4 exorphin	GYYP	498.19	70
26	gliadin 2 exorphin	YPLG	448.22	n.d.
27	casoxin from bovine kappa-casein fr: 35-41	YPSYGLN	812.35	n.d.
28	casoxin (fr.33-38 of bovine kappa-casein) *	SRYPSY	771.34	n.d.
29	Casoxin	YPYY	604.24	n.d.
30	β-casomorphin	YPSF	512.21	n.d.
31	β-casomorphin-11 (60-70)	YPFPGPIPNSL	1200.60	10
32	β-casomorphin-7 (60-66)	YPFPGPI	789.39	14
33	β-casomorphin-5 (60-64)	YPFPG	579.25	1.1
34		YPFGFF~	775.35	0.06
35		YPFGFE~	757.33	0.12
36		YPFGFW~	814.36	0.09
37		YPFGFCQ~	756.34	0.08
38		YPFGFD~	743.31	0.07
39		YPFGFV~	727.35	0.05
40		YPFGFS~	715.32	0.02
41		YPFGFL~	741.37	0.05
42		YPFGFT~	729.33	0.04
43		YPFGFI~	741.37	0.04

44		YPFGFY~	791.35	0.04
45		YPFGFH~	765.34	0.04
46		YPFGFM~	759.32	0.03
47		YPFGFQ~	756.34	0.03
48		YPFGFP~	725.34	0.03
49		YPFGFA~	699.32	0.03
50		YPFGFK~	756.38	0.02
51		YPFGFG~	685.30	0.02
52		YPFGFR~	784.38	0.01
53		YPFGFN~	742.33	0.03
54		YPFGFGG	743.31	0.02
55		YPFGFNG	800.33	0.03
56		YPFGFAG	757.33	0.03
57		YPFGFQG	814.35	0.03
58		YPFGFKG	814.38	0.02
59		YPFGFMG	817.33	0.03
60		YPFGFRG	842.39	0.01
61		YPFGFSG	773.32	0.02
62		YPFGFDG	801.31	0.07
63		YPFGFEG	815.33	0.12
64		YPFGFPG	783.34	0.03
65		YPFGFCQG	814.35	0.08
66		YPFGFFG	833.36	0.06
67		YPFGFVG	785.36	0.05
68		YPFGFLG	799.37	0.05

69		YPFGFTG	787.34	0.04
70		YPFGFIG	799.37	0.04
71		YPFGFYG	849.35	0.04
72		YPFGFHG	823.35	0.04
73		YPFGFWG	872.37	0.09
74		YYPT	542.22	800
75		YYP	441.17	1000
76		YPVSL	577.29	200
77		YPLSL	591.31	200
78		YPASL	549.26	n.d.
79		YPTSL	579.27	n.d.
80		YPFSL	625.29	70
81		RYYP	597.27	190
82		YPWSL	664.31	70
83		WYYP	627.25	n.d.
84		SYYP	528.21	200
85		GYYPTS	686.27	72
86		YPFW~	610.27	n.d.
87		YPFWG	668.28	n.d.
88		YPFF~	571.26	n.d.
89		YPFFG	629.27	n.d.
90	soymorphin-5	YPFVV	623.31	6
91	soymorphin-6	YPFVVN	737.36	9.2
92	soymorphin-7	YPFVVNA	808.39	13

* Opioid antagonist, n.d. = not defined

PeptideRanker is a web based application that predicts probability of the peptide to be bioactive (Mooney, Haslam et al. 2013). In this study, PeptideRanker was used to rank bioactivity of peptides as identified from **Table 4.1** and compared to known gluten exorphins (opioid peptides from wheat) and presented in **Table 4.3.** Peptide Ranking of known gluten exorphins varied from 0.55 to 0.96 (**Table 4.3**) and exorphins A4, B4 and B5 show higher likelihood of bioactivity based on high ranking than exorphins A5 and C. The ranking for the unknown 11 peptides as identified from **Table 4.1** varied between 0.38 - 0.83. Only 3 out of 11 peptides, YPG, YYPG and YIPP were ranked > 0.77. These 3 peptides were used for confirmation of opioid activity.

Table 4.3: Ranking of the predicted peptide sequences obtained by PeptideRanker (bioware.ucd.in) as compared to known exorphins from gluten

Known peptides	**Peptide ranking**
GYYPT (exorphin A5)	0.58
GYYP (exorphin A4)	0.8
YGGWL (exorphin B5)	0.96
YGGW (exorphin B4)	0.96
YPISL (exorphin C)	0.55
Predicted sequences from Table 4.1	
YPG	0.83
YYPG	0.78
YIPP	0.77
YVPP	0.52
YPH	0.59
YPISP	0.52
YPTSP	0.41
YPQ	0.47
YPS	0.44
YPN	0.55
YPT	0.38

4.4.3. Assessment of wheat protein derived peptides for opioid activity

Based on the *in silico* analysis, peptides YPG, YYPG and YIPP are opioid and should bind to opioid receptors to exert bioactivity. The activity of these peptides was confirmed by using cyclic AMP assay, which is based on inhibition of the adenylate cyclase enzyme. The decreasing concentration of cAMP within the cells is taken to be an indication of a positive test and used in determination of activity expressed as EC_{50} values. All three peptides - YPG, YYPG and YIPP inhibited production of cAMP in the presence of 10 µM forskolin in cells expressing µ and κ opioid receptors (**Figure 4.1** and **Figure 4.2**). Decrease in concentration of cAMP is graphed against concentration of peptides using GraphPad Prism 7 software and EC_{50} of the peptides were calculated from a sigmoid response curve. Calculated EC_{50} values of all tested peptides was greater than 1.0 mM for both µ and κ receptors which indicated that a high dose of these peptides is required for them to exert opioid activity. For µ opioid receptor, EC_{50} values of YPG, YYPG and YIPP were 5.3 mM, 1.5 mM and 2.9 mM respectively, while for κ opioid receptor, EC_{50} values were 1.8 mM, 1.2 mM and 3.2 mM, respectively. For both receptors, YYPG had the lowest EC_{50} value, and is more potent opioid peptide than either YPG or YIPP which can be due to presence of 2 Y residues in the peptide. Also, these peptides have higher affinity to κ opioid receptors than for µ opioid receptors. As shown in **Table 4.2**, YYP has EC_{50} of 1 mM, and presence of glycine (G) at the amino terminal end in GYYP (gluten exorphin A4) decreased its EC_{50} value to 70 µM making it more effective peptide than YYP. Presence of non-aromatic amino acid (Threonine, T) at the carboxyl terminal (YYPT) also decreased its EC_{50} value to 800 µM but presence of G at the carboxyl terminal (YYPG) did

not decrease EC_{50} value. Despite of these peptides binding to opioid receptors (μ and κ), they are not as effective (higher EC_{50} values) in their native form and therefore need modification to improve their binding and agonistic activities. For example, Torino, Mollica et al. (2010) reported improvement in opioid activity though modification of native endomorphine-2. Further research needs to be conducted to find if these peptides can be adsorbed intact or pass the blood brain barrier (BBB) to make them effective for clinical application. Small size of predicted peptide assures that these peptides can pass the GI tract (De Noni, FitzGerald et al. 2009). Use of D-amino acids, β-amino acids, various types of synthetic residues and backbone cyclization can improve stability against enzymatic hydrolysis (Mollica, Pinnen et al. 2013) and therefore modify their fate in human system. However, even if these peptides may not be able to pass BBB or absorbed, they can also stimulate the brain by brain-gut axis (Stefanucci, Mollica et al. 2016). They are still worth further investigation for further pharmaceutical development because of their high selectivity and low toxicity (Mollica, Pinnen et al. 2013, Garg, Nurgali et al. 2016, Stefanucci, Mollica et al. 2016). These food-derived opioid peptides can also be introduced carefully into the market as diet supplements, food additives, or as constituents of functional foods and nutraceuticals (Stefanucci, Mollica et al. 2018).

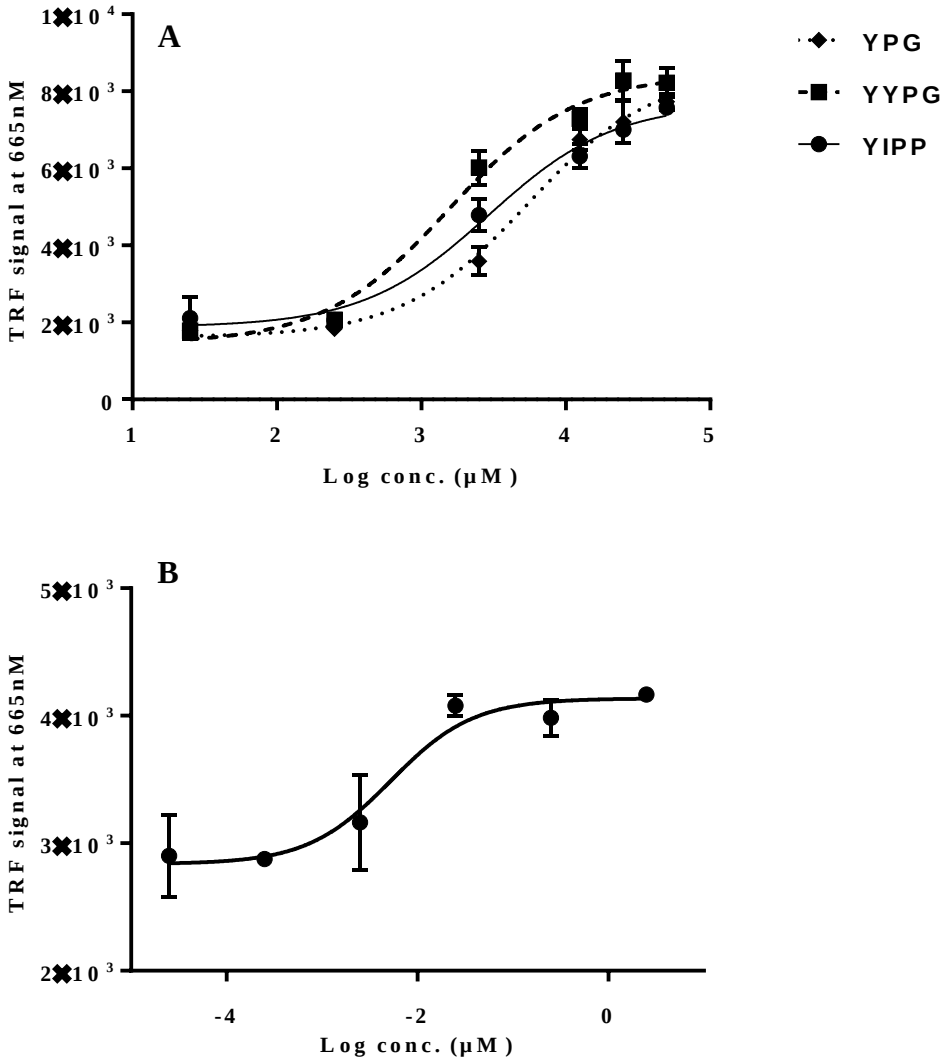


Figure 4.1: Dose response curves of three peptides (A) and DAMGO (B) against µ-opioid receptors based on inhibition of adenylate cyclase activity as depicted by TRF signal measured at 665 nm.

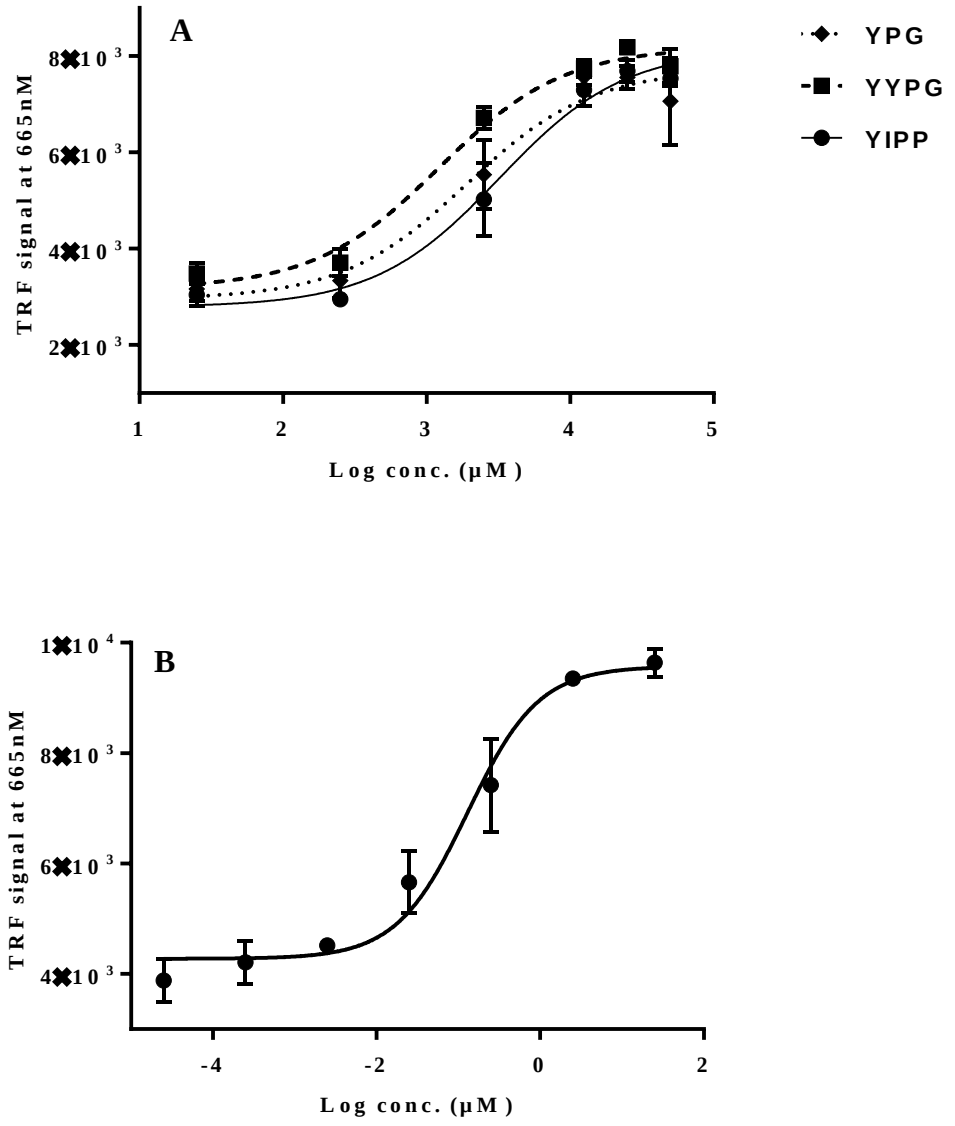


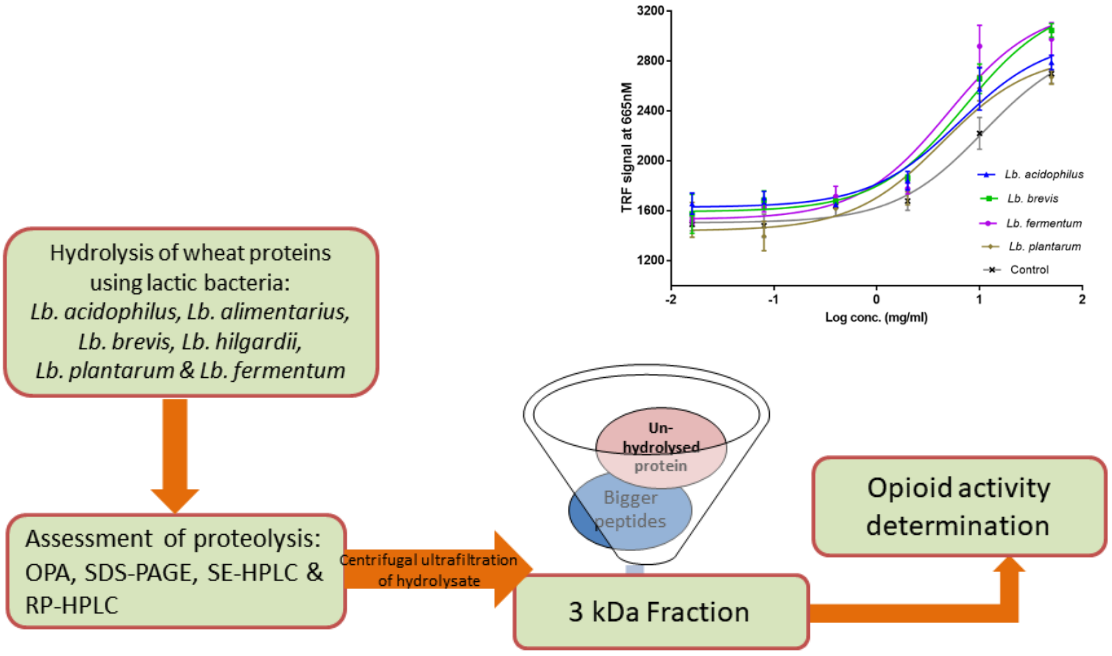
Figure 4.2: Dose response curves of three peptides (A) and dynorphin A (B) against κ-opioid receptors based on inhibition of adenylate cyclase activity as depicted by TRF signal measured at 665 nm.

4.5. Conclusion

Bioinformatics approach was used for the identification of opioid peptides from wheat gluten proteins as a cost-effective approach for selection and comparison of proteins for production of opioid peptides. Structural motifs, as particular amino acids or their combinations responsible for opioid bioactivity were identified. Using tyrosine and proline residues in the peptide sequences as predictors of opioid peptides, HMW glutenin was found to be the best source of opioid peptides. It was predicted that eleven peptides from wheat gluten could have opioid activity, out of which, YPG, YYPG and YIPP were selected as these showed higher ranking of 0.83, 0.78 and 0.77, respectively. The opioid activity of these predicted peptides were determined using cAMP assay in cell lines expressing opioid receptors. Based on the lowest EC_{50} value, YYPG is found to be more potent opioid peptide than YPG or YIPP. The study confirmed that gluten proteins are potential protein source for production of opioid peptides. In the following chapters, lactic acid fermentation and the use of commercial proteolytic enzymes for hydrolysis of wheat proteins for production of opioid peptides were evaluated.

Chapter 5. Proteolytic and opioid activities of wheat flour fermented by selected lactic acid bacteria

The material presented in this chapter has been submitted to 'International Journal of Food Science and Technology' for publication.



5.1. Summary

In the previous chapter, the presence of tyrosine and proline in peptides from wheat gluten protein was confirmed as key determinants for opioid activity. In this chapter, wheat flour was fermented using lactic acid bacteria: *Lb. acidophilus, Lb. alimentarius, Lb. brevis, Lb. fermentum, Lb. hilgardii* and *Lb. plantarum* at 37 °C for 24 hours with a view to produce opioid peptides. Proteolysis was measured by change in free amino group and SDS-PAGE. The peptide profile of the water/salt soluble extract was obtained using RP-HPLC and SE-HPLC. The opioid activity of the 3 kDa fractions was measured by adenylate cyclase assay. The relative concentration of bands in SDS-PAGE and peptide profile of fractions obtained by *Lb. acidophilus, Lb. brevis, Lb. fermentum* and *Lb. plantarum* fermentation showed significant proteolysis. The opioid activity as expressed by EC_{50} values of *Lb. acidophilus, Lb. brevis, Lb. fermentum* and *Lb. plantarum* fractions were 6.3, 7.2, 4.9 and 4.3 mg/mL, respectively. *Lb. plantarum* is the most effective bacteria to produce opioid peptides from wheat proteins.

5.2. Introduction

There has been considerable increase in interest in bioactive peptides derived from food proteins. These bioactive peptides are encrypted in protein sequence and must be released by proteolysis by using commercial enzymes (Kong, Zhou et al. 2008, Wu, Zhang et al. 2016), gastrointestinal digestive enzymes (Stuknytė, Maggioni et al. 2015) in vivo, by fermentation using microorganisms containing proteolytic enzymes (Muguerza, Ramos et al. 2006, Rizzello, Nionelli et al. 2012) and by using proteolytic enzymes that are activated during germination of grains (Mamilla and Mishra 2017). These peptides can have significantly increased opioid, mineral binding, immune-modulatory, anti-microbial, anti-oxidative, anti-thrombotic, hypo-cholesterolemic and anti-hypertensive activities (Udenigwe and Aluko 2012, Agyei, Potumarthi et al. 2015, Garg, Nurgali et al. 2016) compared to native proteins from which these are sourced from. Unlike proteins, most of the bioactive peptides are known to have low molecular weight and are non-immunogenic (Wang, Mejia et al. 2005) and preferred for food and pharmaceutical applications (Agyei, Potumarthi et al. 2015).

Bioactive opioid peptides derived from food proteins have been considered as future dietary supplement for their health benefits (Stefanucci, Mollica et al. 2018). A number of animal and plant food proteins show presence of opioid peptides within. Several opioid peptides, such as casomorphins, exorphins, soymorphin and rubiscolin have been identified in β-casein (milk), gluten (wheat), β-conglycinin (soybean) and rubisco (spinach) proteins, respectively, and have been reviewed in chapter 2. Out of these, the most studied milk derived opioid peptides are β-casomorphins -5 and -7 and they have EC_{50} values of 6.5 and 57 μM,

respectively, determined using guinea pig ileum assay. Plant derived opioid peptides, the gluten exorphin B5, rubiscolin-6 have EC_{50} of 0.017 µM and 24.4 µM in mouse *vas deferens*, respectively, and soymorphin-5 has EC_{50} of 32.0 µM determined using guinea pig ileum assay as reviewed by Garg, Nurgali et al. (2016).

Many fermented dairy products are known to contain bioactive opioid peptides. These peptides are present in kefir (Ebner, Arslan et al. 2015) and cheese (Muehlenkamp and Warthesen 1996, Jarmołowska, Kostyra et al. 1999, Sienkiewicz-Szłapka, Jarmołowska et al. 2009). Similarly, sourdough fermentation of wheat proteins releases number of bioactive peptides from hydrolysis of albumin, globulin and gliadin (Di Cagno, De Angelis et al. 2002, Zotta, Piraino et al. 2006, Gänzle, Loponen et al. 2008). Another plant based fermented food, kimchi shows the presence of bioactive cyclic dipeptide having antimicrobial activity, but they are suggested to be originating from lactic acid bacteria (Liu, Kim et al. 2017) rather than from vegetable proteins. The lactic acid bacteria present in sourdough contain cell envelop associated serine proteases (degrade proteins to peptides) and a range of intracellular peptidases (endopeptidases and exopeptidases) (Gänzle, Loponen et al. 2008, Chavan and Chavan 2011) that are responsible for hydrolysing proteins to peptides. These peptides produced during sourdough fermentation have shown anti-cancer (Rizzello, Nionelli et al. 2012), anti-hypertensive (Rizzello, Cassone et al. 2008), anti-oxidant (Coda, Rizzello et al. 2012), anti-bacterial (Coda, Rizzello et al. 2008, Garofalo, Zannini et al. 2012) activities. However, there is no information available on the use of lactic acid bacterial fermentation of wheat proteins to produce peptides having opioid activity.

The opioid peptides are categorized into 2 groups: one group contains peptides which must have tyrosine-proline at amino termini followed by either aromatic or non-aromatic amino acid while the other group does not have proline at second position but they must have aromatic amino acid (Yoshikawa 2013). So, the presence of tyrosine, proline and aromatic amino acids can be used as key determinant of opioid activity of a peptide (**Chapter-2**). Arguably, these amino acids must be present in a significant proportion in the food protein structure to be a suitable protein source for production of opioid peptides. Comparing the amino acid composition of albumin and globulin with gluten proteins, the albumin and globulin contains 3.4 % and 2.3 % tyrosine, 5 % and 3.2% phenylalanine (aromatic amino acid), 6.23 % and 3.2% proline, respectively, whereas, gliadin and glutenin proteins contain 2.6 and 3.6 % tyrosine, 5.6 and 4.8 % phenylalanine and 13.1 and 10% proline, respectively (Mohsen, Yaseen et al. 2010). This suggests that albumin and globulin hydrolysis during sourdough fermentation may release opioid peptides by lactic acid bacteria (Di Cagno, De Angelis et al. 2002). To the best of our knowledge none of the studies have investigated the release of opioid peptides from wheat proteins using lactic acid bacteria.

This study aimed to assess the proteolysis of wheat flour proteins by selected lactic acid bacteria (LAB) with a view to release opioid peptides. Hydrolysis pattern was determined by measuring change in free amino group and peptide profile was obtained using SDS-PAGE, RP-HPLC and SE-HPLC. The hydrolysates were fractionated by 3 kDa cut off ultrafiltration membrane and the opioid activity was determined by using adenylate cyclase assay in cells expressing opioid receptors.

5.3. Materials and methods

Tris buffer and hydrochloric acid, triflouroacetic acid and acetonitrile were from Merck (Australia). o-phthalaldehyde (OPA), methionine, 3- Isobutyl-1-methylxanthine (IBMX), trypsin-EDTA, forskolin and [D-Ala^2, NMePhe^4, Gly^5-ol]-enkephalin (DAMGO), tween-20 and HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) and all other chemicals were from Sigma Aldrich (Australia). Mini-PROTEAN TGX gel (12%), 10X Tris/Glycine/SDS buffer and 2X Laemmli sample buffer were obtained from Bio-Rad (Melbourne, Australia). Cell culture media, Dulbecco's Modified Eagle's Medium (DMEM) (Catalogue number- 12430054), fetal bovine serum (FBS), antibiotic–antimycotic (100X) and hygromycin-B and phosphate buffer saline (PBS) – pH 7.2, were acquired from Life Technologies (Carlsbad, California, US). Lance cAMP detection kit (AD0263), and bovine serum albumin (BSA) stabiliser and opti-plate were purchased from Perkin Elmer Life Sciences (Cambridge, MA).

5.3.1. Lactic acid cultures and inoculum preparation

Six strains of LAB commonly identified in sourdough (*Lactobacillus acidophilus, Lactobacillus alimentarius, Lactobacillus brevis, Lactobacillus fermentum, Lactobacillus hilgardii and Lactobacillus plantarum)* were used in this study. *Lactobacillus acidophilus,* were acquired from Victoria University culture collection, *Lactobacillus alimentarius (*ATCC 29643), *Lactobacillus brevis* (ATCC 13648), *Lactobacillus hilgardii* (ATCC 8290) and *Lactobacillus plantarum* (ATCC 8014) were acquired from ATCC culture collection and *Lactobacillus fermentum* (LMG 26741) and *Lb. plantarum* (LMG 6907) were acquired from BCCM. de Man,

Rogosa, and Sharpe (MRS) broth was obtained from Oxoid (Basingstoke, Hampshire, England).

All cultures were stored at -80° C in sterile MRS broth containing 0.4 volumes of glycerol as cryo-protectant. These LAB were routinely propagated for 48 h at 37°C in MRS broth. The bacterial cells were allowed to grow overnight to have actively dividing cells in exponential phase, harvested by centrifugation (3274 g for 10 min at 4°C), washed twice in 50 mM phosphate buffer (pH 7.0) and resuspended in tap water before inoculating the flour slurry at a concentration of 10^9 cfu/mL. Cell enumeration was carried out by serial dilution and plating onto MRS agar (agar at 1.2%) at 37°C for 48 hours.

5.3.2. Lactic acid fermentation

Wheat flour was acquired from Allied Mills (Australia). The composition of the flour as supplied by the company was 14% moisture, 69.4% carbohydrate, 12.5%, protein, 1.2% fat and 0.55 % ash. The wheat flour slurry was prepared by mixing 120 g flour and 280 g of tap water with dough yield (DY; dough weight * 100/flour weight) of 330 (DY330) (Coda, Rizzello et al. 2012). The fermentation was carried out under stirring conditions (120 rpm) at 37°C for 24 h after inoculation by selected LABs. Control doughs (DY 330) was also prepared without any bacterial inoculum and incubated under the same conditions for comparison. Water/salt-soluble extracts (WSE) containing albumins/globulins was prepared as described by Coda, Rizzello et al. (2012). Briefly, 10 mL of fermented slurry was mixed with 10 mL of Tris-HCI buffer (50 mM, pH 8.8) for 30 min with vortexing in between to extract hydrolysed products, followed by centrifugation (20,000 g, 20

min at 4°C). The supernatant was collected and filtered through 0.45 µm filter and stored at -20°C used for further analysis.

5.3.3. Measurement of proteolysis by OPA

The concentration of peptides in the WSE was determined by the OPA method (Church, Swaisgood et al. 1983). The OPA reagent was prepared by mixing 25 mL of sodium phosphate buffer (50 mM; pH 8.3), 2.5 mL of sodium dodecyl sulphate (20%, w/v), 40 mg of OPA (dissolved in 1mL of methanol), 100 µL of β-mercaptoethanol and diluting to 50 mL with water. In a test tube, 300 µL of WSE was mixed with 3 mL of freshly prepared OPA reagent and mixed gently for 5 s. The absorbance was measured after two min at 340 nm using a Nova Spec-II spectrophotometer (Pharmacia, England, UK). Methionine was used as standard (0.01 – 1.00 mM) and buffer was used as a blank.

5.3.4. SDS-PAGE of the fermented hydrolysate

SDS-PAGE was carried out under reducing conditions to study protein hydrolysis. 10 µL of the fermented samples were diluted with 2X Laemmli sample buffer containing β-mercaptoethanol followed by heating at 95°C for 5 min. Samples were then loaded on the gel (20 µL) and ran at 200 V for 45 min. The gels were stained using 0.15 % Coomassie Brilliant Blue R250 dye in 72% isopropanol and 3% acetic acid for 1 h followed by overnight de-staining (10% isopropanol, 10% acetic acid) on a shaker. The gel images were captured using a Chemidoc imager (Chemidoc MP; Bio-Rad Laboratories) and relative concentration of protein corresponding to different molecular weight was analysed using Image Lab version-6 (Bio-Rad, Australia).

5.3.5. RP-HPLC of the fermented hydrolysate

The WSE of fermented samples were profiled onto C-18 monomeric column (5 mm, 300A˚, 250mm*4.6mm; Grace Vydac, Hesperia CA, USA) attached to Prominence-i, LC-2030C (Shimadzu, Kyoto, Japan) comprising an auto-sampler, solvent delivery system, UV-Vis detector, interfaced with a PC with a software package (Lab solutions) for system control and data acquisition. Solvent A and Solvent B consisted of 0.1 % TFA in water and acetonitrile, respectively. Ten µL of the sample was eluted with solvent flowing at 0.75 mL/min in a linear gradient from 95 % to 5 % solvent A in solvent B over 90 min and peaks were detected at 214 nm.

5.3.6. SE-HPLC of the fermented hydrolysate

SE-HPLC was used for determination of molecular weight distribution of the hydrolysates samples after fermentation. The WSE were profiled onto Yarra SEC 3000 column (Phenomenex, Torrance, United States) attached to Prominence-i, LC-2030C (same as above). Ten µL of the samples were eluted with 50 mM sodium phosphate buffer (pH 6.8) containing 300 mM NaCl at flow rate of 0.4 mL/min over 20 min and peaks were detected at 214 nm using UV/Vis detector. Albumin (67 kDa), β-lacto globulin (18.4 kDa), RNAse (13.7 kDa) and vitamin B_{12} (1.3 kDa) were used to prepare a standard curve by plotting the molecular weights and the corresponding retention times determined by running these proteins under the same conditions of chromatography. This standard curve was used to determine the molecular weights of the proteins/ peptides present in samples.

5.3.7. Membrane fractionation of hydrolysates

The hydrolysates were fractionated by using centrifugal ultrafiltration membrane (Z740200, Sigma Aldrich, Australia) with 3 kDa cut off. The hydrolysates were centrifuged at 3200 X g for 120 minutes at 4°C to collect filtrates. The collected fractions were lyophilised and subsequently used for determination of opioid activity.

5.3.8. Determination of opioid activity of the fractions (<3 kDa)

5.3.8.1. Cell Lines and culture conditions

FlpIn CHO (Chinese hamster ovary) cells stably transfected with pOG44 vector encoding Flp recombinase and pDEST vector encoding human μ receptors were kind gift from Dr. Meritxell Canals, Monash Institute of Pharmaceutical Sciences, Melbourne, Australia. The cells were transfected using polyethylenimine as transfection reagent and hygromycin-B (200 μg/mL) was used as selection agent (Burford, Livingston et al. 2015). Cells were grown in a humidified incubator containing 5% CO_2 maintained at 37°C in DMEM containing 10% FBS, 1% antibiotic-antimycotic and 200 μg/mL hygromycin-B (Burford, Livingston et al. 2015).

5.3.8.2. Adenylate cyclase assay

Opioid activity of the fractions was determined on the basis of inhibition of adenylate cyclase enzyme, which decreases cAMP within the cell as indicated by an increase in TRF signal and hence positive opioid activity. The method has been optimised for receptor expression by Burford, Livingston et al. (2015) and was adopted. Briefly, CHO cells transfected with μ receptors were grown and stimulated

at different concentrations (0.00064 to 50 mg/mL) of fractions for 30 mins. After incubation, stimulation buffer (containing peptides from fractions) was removed and cells were lysed using lysis buffer (0.3% tween-20, 5 mM HEPES and 0.1% BSA). Change in concentration of cAMP in the lysate was determined using Lance cAMP detection kit (Perkin Elmer, Melbourne) following instructions by the supplier of the kit. Briefly, 10 µL of lysate and 10 µL of Alexa flour-647 anti-cAMP antibody was mixed followed by addition of 20 µL of detection mix (prepared according to kit instructions). Time-resolved fluorescence (TRF) with excitation at 337 nm and emission read at 665 nm was measured using an Envision plate reader (PerkinElmer, Melbourne). DAMGO was used as positive control. Nonlinear logistic regression analysis was used to fit the dose response data using Graph Pad Prism, version 7 (San Diego, California, US). The same software was used for determining EC_{50} values.

5.4. Results and discussion

5.4.1. LAB growth, change in pH and proteolysis

Bacterial growth (as measured by colony forming unit (cfu)), pH and proteolysis (as measured by change in free amino group) after lactic acid fermentation of wheat flour slurry are presented in Table 5.1. For the same conditions of fermentation, bacteria grew differently as suggested by differences in the $\log_{10}$ (cfu/mL) and pH of the fermented slurry. The growth was minimum for *Lb. hilgardii* (log cfu/ml-6.31) and maximum for *Lb. acidophilus* (log cfu/ml- 8.88). As LAB grew, the drop in pH was maximum for *Lb. acidophilus* (3.49) and minimum for *Lb. hilgardii* (4.86) (Table 5.1). The WSE of fermented slurry also

showed an increase in free amino content as compared to control suggesting proteolysis. The increase in free amino group content ranged from 0.065 (*Lb. hilgardii)* to 0.203 mM equivalent of methionine (*Lb. acidophilus*). Results indicate that for the same fermentation conditions, the proteolysis follow the pattern *Lb. acidophilus* > *Lb. brevis* > *Lb. fermentum* > *Lb. plantarum* > *Lb. alimentarius* > *Lb. hilgardii*. A statistical correlation analysis showed that proteolytic activity of these bacteria is positively correlated to growth (R^2= 0.848, $P < 0.005$) and negatively correlated to pH (R^2 = -0.897, $P < 0.005$). Proteolysis therefore appears to be growth dependent and suggests that higher growth would have also increased proteolysis.

Table 5.1: Growth (cfu/mL), pH and the proteolytic activity as measured by increase in free amino content of fermented wheat flour

Microorganisms used	Log_{10} cfu/mL	pH	Increase in free amino group (mM equivalent of methionine)
Lb. acidophilus	8.88^{a}	3.49 ± 0.01^{d}	0.203 ± 0.00^{a}
Lb. alimentarius	7.52^{b}	4.33 ± 0.06^{b}	0.130 ± 0.00^{bc}
Lb. brevis	8.60^{ab}	3.74 ± 0.01^{c}	0.187 ± 0.00^{ab}
Lb. fermentum	8.81^{a}	3.5 ± 0.02^{d}	0.175 ± 0.01^{ab}
Lb. hilgardii	6.31^{b}	4.86 ± 0.08^{a}	0.065 ± 0.02^{c}
Lb. plantarum	8.62^{ab}	3.62 ± 0.02^{cd}	0.148 ± 0.02^{ab}

For each bacterium used for fermentation, three samples were prepared. Values are expressed as mean ± standard deviation for triplicate. Within a column, different lowercase letters (a–d) indicate a significant difference ($P < 0.05$) based on the Tukey's test.

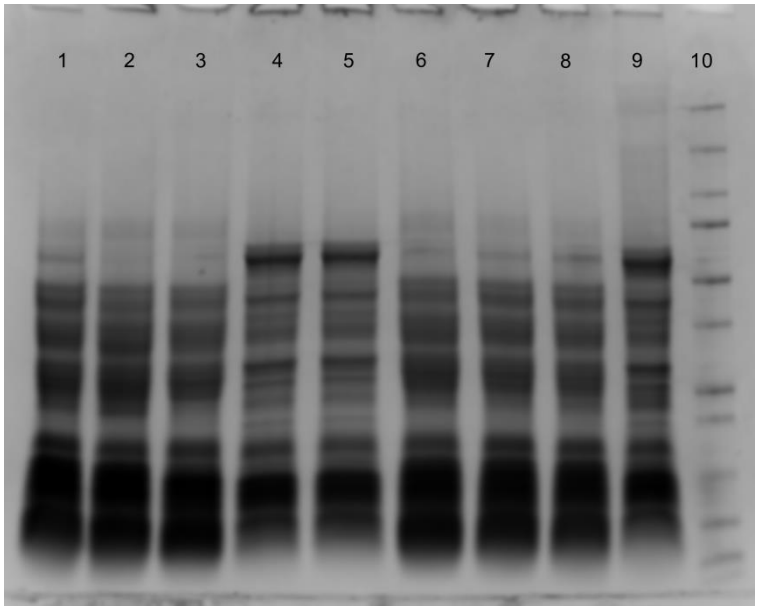
5.4.2. SDS-PAGE pattern of Water/salt soluble extract obtained after fermentation

The changes in the WSE of fermented slurry were analysed by SDS PAGE. This is also presented as **Figure 5.1**. The WSE of the control sample shows band between 10-70 kDa, with some prominent bands at 60, 30 and 25 kDa (Zotta, Piraino et al. 2006, Gobbetti and Gänzle 2013). These bands are also present intact after fermentation with *Lb. alimentarius* and *Lb. hilgardii,* which indicates that these bacteria did not hydrolyse the proteins under selected conditions. However, reduction in the prominent bands intensity and appearance of more bands in the low molecular weight regions confirm increased proteolytic activity of *Lb. acidophilus, Lb. brevis, Lb. fermentum and Lb. plantarum*. A smear can also be seen at the end of lane in those hydrolysates indicating proteolysis.

The quantitative analysis of the protein bands shows more significant differences between the control and fermented samples as shown in Table 5.2. The table shows that the relative concentration of protein between 55-60 kDa is 9.7 % in control and it is not significantly different in fermented hydrolysates of *Lb. alimentarius* and *Lb. hilgardii,* however, a decrease in relative concentration (0.6 – 1.9 %) compared to fermented hydrolysate of *Lb. acidophilus, Lb. brevis, Lb. fermentum and Lb. plantarum*. Samples fermented by these four bacteria also show increase in relative concentration of protein band at 42 - 49 kDa to nearly 9.5 to 12.0 % which is relatively low in control, *Lb. alimentarius* and *Lb. hilgardii* samples. The relative content of band at 38-40 kDa is 2.2% for control which reduced to 0.9 % in *Lb. alimentarius* and *Lb. hilgardii* and an increase of relative content of band at 28-30 kDa. Higher relative content of band maxima can be seen

between 34-36 kDa, 24-26 kDa and 14-15 kDa in *Lb. acidophilus, Lb. brevis, Lb. fermentum* and *Lb. plantarum.* In control, *Lb. alimentarius* and *Lb. hilgardii,* the maxima for smear is present between 9-10 kDa, which shifted to lower molecular weight 7-8 kDa in *Lb. acidophilus, Lb. brevis, Lb. fermentum* and *Lb. plantarum* indicating proteolysis by these bacteria. These data support previous observations of growth and degree of proteolysis measured by OPA.

(a)



(b)

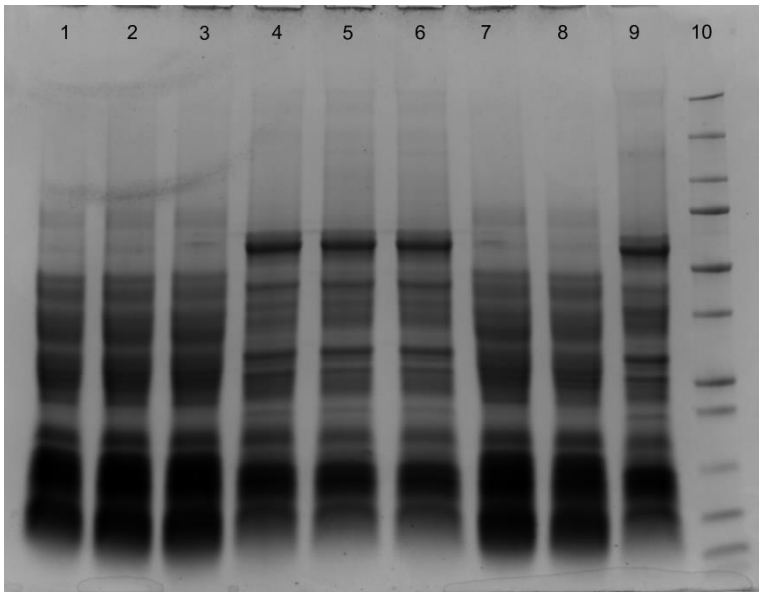


Figure 5.1: SDS-PAGE profile of fermented wheat flour hydrolysate produced by selected lactic acid bacteria: Lb. acidophilus (a, lane 1-3), Lb. alimentarius (a, lane 4-5), Lb. brevis (a, lane- 6-8), Lb. fermentum (b, lane 1-3), Lb. hilgardii (b, lane 4-6), Lb. plantarum (b, lane7-8) as compared to control (a and b, lane 9) and protein ladder (a and b, lane 10)

Table 5.2: Change in relative concentration of protein bands on fermentation by selected lactic acid bacteria as observed by SDS- PAGE

Molecular mass	**>60 kDa**	**55-60 kDa**	**42-49 kDa**	**38-40 kDa**	**34-36 kDa**	**28-30 kDa**	**24-26 kDa**	**17-18 kDa**	**14-15 kDa**	**12-13 kDa**	**9-10 kDa**	**7-8 kDa**
Lb. acidophilus	0.9 ± 0.6^{b}	1.0 ± 1.1^{b}	10.5 ± 0.5^{ab}	N.A.	8.7 ± 1.6^{ab}	N.A.	19.8 ± 0.6^{a}	8.4 ± 1.3^{ab}	18.9 ± 0.5^{a}	N.A.	N.A.	31.8 ± 1^{a}
Lb. alimentarius	N.A.	9.0 ± 0.5^{a}	2.0 ± 0.4^{c}	0.9 ± 0.3^{b}	2.3 ± 0.2^{c}	4.6 ± 0.5^{a}	4.1 ± 0.2^{b}	10.0 ± 0.2^{a}	N.A.	36.7 ± 0.9^{a}	30.2 ± 1.7^{a}	N.A.
Lb. brevis	N.A.	1.9 ± 0.9^{b}	9.5 ± 1.6^{b}	N.A.	7.2 ± 1.1^{b}	N.A.	19.7 ± 2.2^{a}	8.0 ± 1.0^{b}	18.7 ± 2.2^{a}	N.A.	N.A.	35.8 ± 7.8^{a}
Lb. fermentum	2.2 ± 0.4^{a}	0.6 ± 0.2^{b}	12.1 ± 1.5^{a}	N.A.	9.9 ± 1.6^{a}	N.A.	18.2 ± 0.1^{a}	6.5 ± 0.4^{c}	16.5 ± 1.3^{a}	N.A.	N.A.	34.5 ± 2.2^{a}
Lb. hilgardii	N.A.	9.7 ± 0.6^{a}	2.9 ± 0.3^{c}	0.9 ± 0.3^{ab}	2.1 ± 0.4^{c}	4.0 ± 0.2^{ab}	6.1 ± 0.2^{b}	8.4 ± 0.2^{ab}	N.A.	35.8 ± 1.9^{a}	29.2 ± 2.7^{a}	N.A.
Lb. plantarum	1.8 ± 0.7^{ab}	0.8 ± 0.2^{b}	9.6 ± 1.0^{ab}	N.A.	8.6 ± 1.2^{ab}	N.A.	19.7 ± 1.8^{a}	6.9 ± 0.8^{bc}	18.3 ± 0.9^{a}	N.A.	N.A.	34.5 ± 0.7^{a}
Control	N.A.	9.6 ± 2.3^{a}	2.6 ± 0.8^{c}	2.2 ± 1.0^{a}	2.2 ± 0.6^{c}	3.5 ± 0.8^{c}	4.6 ± 1.1^{b}	7.8 ± 0.3^{bc}	N.A.	33.5 ± 0.9^{b}	33.8 ± 6.6^{a}	N.A.

For each bacterium used for fermentation, three samples were prepared. Values are expressed as mean ± standard deviation for triplicate. Within a column, different lowercase letters (a–c) indicate a significant difference ($P < 0.05$) based on the Tukey's test.

5.4.3. Peptide profile by RP-HPLC and SE-HPLC

Figure 5.2 shows the differences in the RP-HPLC chromatograms of WSE of fermented wheat flour slurry as compared to control. The WSE of control shows major protein peaks at 23.8, 29.0, 29.9, 31.9, 33.3, 33.7, 36.5, 37.4 mins and small peaks at 13.4, 15.8, 34.4, 41.5, 43.9 and 45.7 mins. The chromatograms of fermented samples of *Lb. alimentarius* and *Lb. hilgardii* do not show much differences in the distribution of peaks as compared to control. In chromatogram of control sample, peaks present at 33.3 and 33.7 mins are nearly of same height while in hydrolysates produced by *Lb. alimentarius* and *Lb. hilgardii*, the relative intensity of peak at 33.7 min decreased as compared to 33.3 min. Besides, relative concentration of peak at 16.7, 24.8, 29.8 mins decreased while relative concentration of peak at 36.5 min increased. However, the chromatograms of fermented samples of *Lb. acidophilus*, *Lb. fermentum*, *Lb. brevis* and *Lb. plantarum* are significantly different from that of control. After fermentation with these bacteria, peaks appeared at 17.7, 18.2, 18.8, 19.3, 22.2, 22.8 and 28 mins which are not present in control, indicating proteolysis. There is a significant decrease in the relative concentration of peak at 29.8 min and increase in relative concentration of peaks at 31.2, 31.9, 36.2, 37.4, 37.7 and 39.7 mins. The control, *Lb. alimentarius* and *Lb. hilgardii* show peaks at 41.5, 43.9 and 45.7 mins representing intact proteins present in the sample but are absent in fermented WSE prepared by *Lb. acidophilus*, *Lb. fermentum*, *Lb. brevis* and *Lb. plantarum* indicating proteolysis.

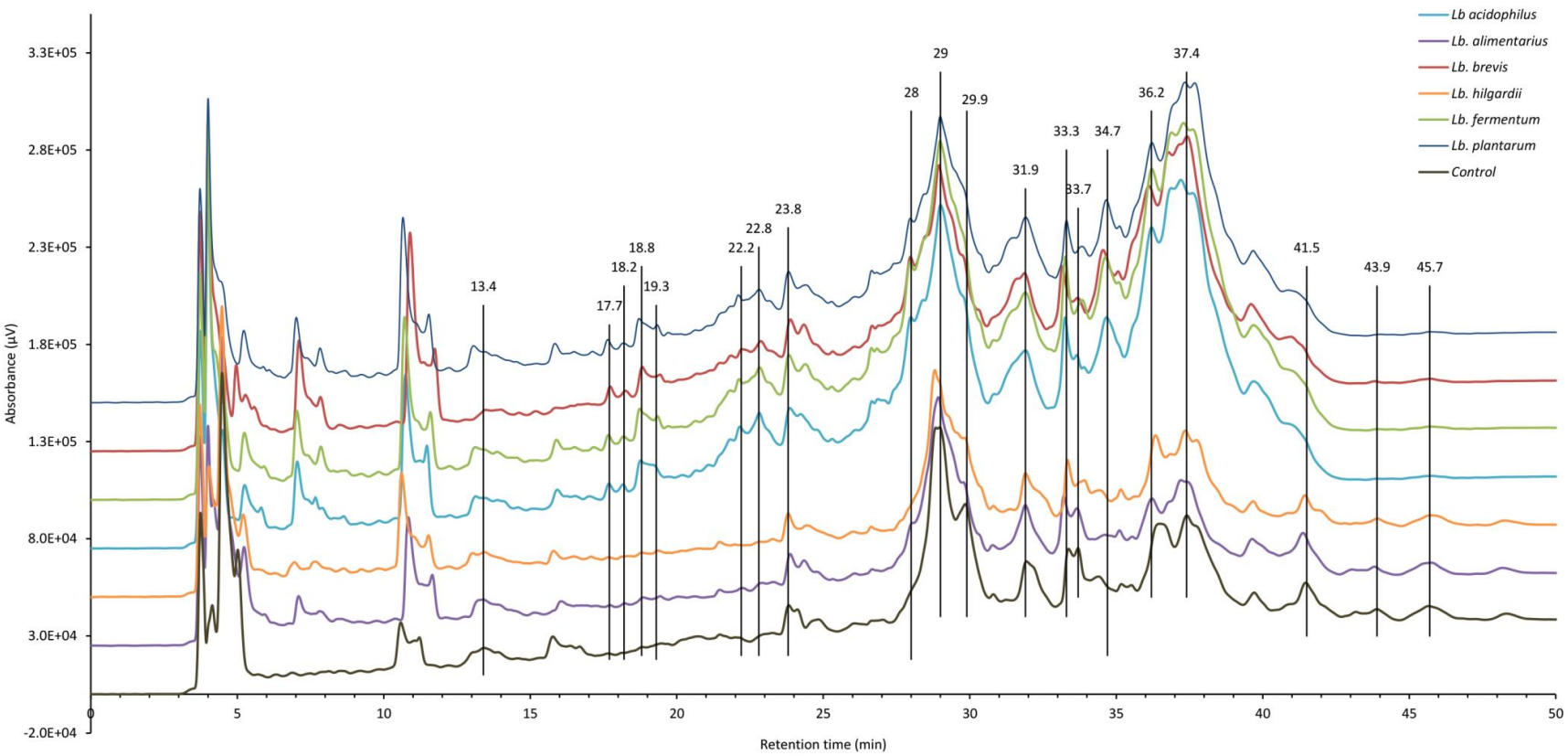


Figure 5.2: RP-HPLC chromatogram fermented wheat flour extracts

produced by selected lactic acid bacteria as compared to unfermented flour slurry used as control

Figure 5.3 shows SE-HPLC chromatograms of standard proteins. The peak maxima for albumin, β-lactoglobulin, RNAse and VitB_{12} are present at 8.2 min, 8.9, 9.5 and 10.8 minutes and was used to compare the size of proteins and peptides present in the hydrolysate.

Figure 5.4 shows SE-HPLC chromatograms of WSE of fermented wheat slurry prepared by different bacteria as compared to unfermented control. The control and the fermented samples did not show any peak at lower retention time indicating absence of gluten in the samples. The chromatogram of unfermented control is different from fermented samples by *Lb. acidophilus*, *Lb. fermentum*, *Lb. brevis* and *Lb. plantarum* showing proteolysis as a result of fermentation. The control shows protein peaks at 23 kDa, 13.0 kDa, 6.6 kDa, 3.3 kDa and 0.25 kDa, respectively, determined by comparison of retention times of the peaks with a standard curve. The chromatogram of *Lb. alimentarius* and *Lb. hilgardii* fermented hydrolysates are similar to control. However, the chromatograms of fermented hydrolysates of *Lb. acidophilus*, *Lb. fermentum*, *Lb. brevis* and *Lb. plantarum* are significantly different from control as there is an increase in intensity of proteins at 23 kDa, 13.0 kDa and 6.6 kDa and 3.3 kDa. The protein peak at 13.0 kDa in control shifted to 9.5 kDa, indicating hydrolysis to comparatively smaller peptides in fermented samples.

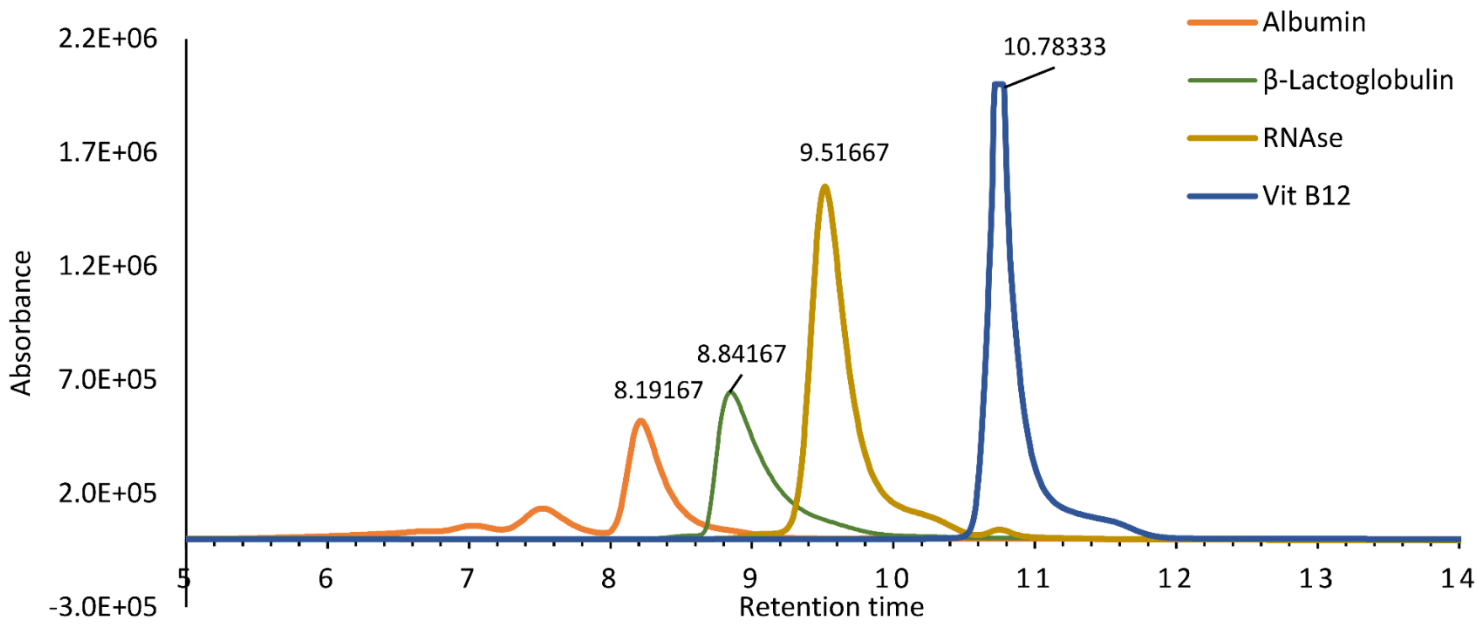


Figure 5.3: SE-HPLC chromatogram of standard proteins: albumin (67 kDa), β-lactoglobulin (18.4 kDa), RNAse (13.7 kDa) and Vit B_{12} (1.3 kDa)

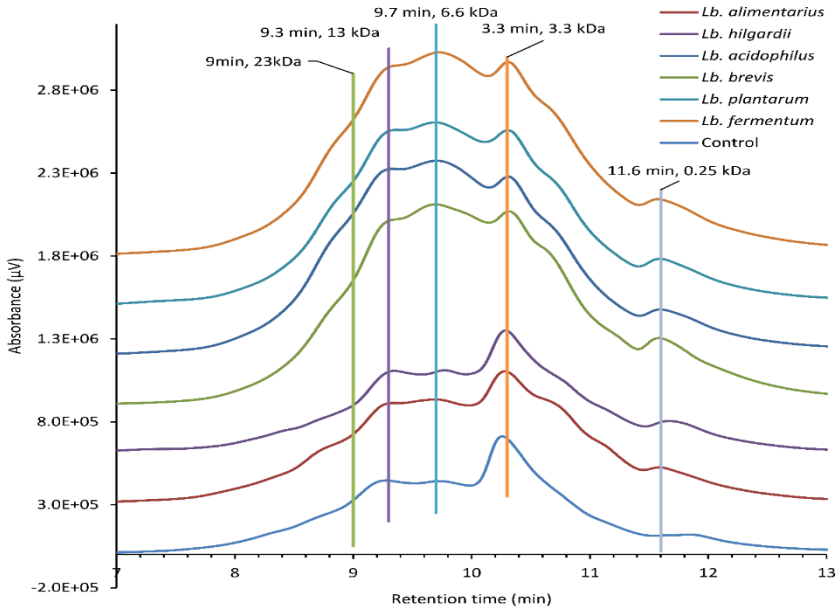


Figure 5.4: SE-HPLC chromatogram of extract of fermented wheat flour produced by selected lactic acid bacteria as compared to unfermented control

The quantitative change in concentration of different peaks relative to total protein was analysed and presented in Table 5.3. As evident from the chromatograms, there *w*ere no significant differences of relative protein concentration between control and fermented hydrolysate of *Lb. alimentarius* and *Lb. hilgardii* which indicates that proteins were not hydrolysed by these bacteria. The relative concentration of peaks in fermented hydrolysate of *Lb. acidophilus, Lb. brevis, Lb. fermentum* and *Lb. plantarum* hydrolysates was significantly higher at 23kDa, 13.0 kDa and 6.6 kDa, indicating increased hydrolysis and solubility of protein on fermentation by theses LABs. The peaks at 3.3 kDa in these fermented samples were significantly lower than the control. The data suggests that fermentation by these bacteria caused hydrolysis of the proteins to peptides up to 6.6 kDa. This supports the pattern obtained with SDS-PAGE.

Table 5.3: Relative concentration of different protein / peptide peaks obtained using SE-HPLC after fermentation of wheat flour by selected lactic acid bacteria as compared to unfermented control

Retention time (min)	**23 kDa**	**13.0 kDa**	**6.6 kDa**	**3.3 kDa**	**0.25 kDa**
Lb. acidophilus	0.151 ± 0.001^{ab}	0.183 ± 0.001^{a}	0.198 ± 0.000^{a}	0.353 ± 0.002^{d}	0.107 ± 0.001^{c}
Lb. alimentarius	0.155 ± 0.005^{a}	0.142 ± 0.003^{d}	0.154 ± 0.005^{b}	0.401 ± 0.000^{b}	0.124 ± 0.005^{ab}
Lb. brevis	0.124 ± 0.002^{c}	0.166 ± 0.000^{b}	0.189 ± 0.003^{a}	0.377 ± 0.001^{c}	0.137 ± 0.004^{a}
Lb. hilgardii	0.146 ± 0.002^{ab}	0.136 ± 0.004^{d}	0.155 ± 0.001^{b}	0.419 ± 0.003^{a}	0.137 ± 0.001^{a}
Lb. plantarum	0.140 ± 0.001^{b}	0.180 ± 0.000^{a}	0.199 ± 0.000^{a}	0.367 ± 0.001^{c}	0.108 ± 0.001^{c}
Lb. fermentum	0.138 ± 0.001^{bc}	0.172 ± 0.002^{ab}	0.194 ± 0.002^{a}	0.368 ± 0.001^{c}	0.119 ± 0.003^{bc}
Control	0.159 ± 0.004^{a}	0.154 ± 0.000^{c}	0.159 ± 0.001^{b}	0.409 ± 0.004^{ab}	0.115 ± 0.001^{bc}

Values are expressed as mean ± standard deviation for triplicate and lowercase (a-d) superscript within a column represents the values which are statistical different using tukey's test ($P < 0.05$) 23 kDa, 13.0 kDa, 6.6 kDa, 3.3 kDa and 0.25 kDa

5.4.4. Opioid activity of fractions

The opioid activity was tested using cAMP assay, which is based on the inhibition of the adenylate cyclase enzyme as measured by a decrease in concentration of cAMP (increase in TRF signal) in the cells expressing opioid receptors. The opioid activity of fermented sample fractions (<3kDa) as determined by inhibition of the adenylate cyclase enzyme is presented as dose response curves in **Figure 5.5**, which shows TRF signals plotted as function of hydrolysate concentration. The fractions (<3kDa) of fermented samples *Lb. acidophilus, Lb. brevis, Lb. fermentum and Lb. plantarum* inhibited production of cAMP in the presence of 10 µM forskolin in cells expressing µ opioid receptors and gave sigmoid dose response curve. **Figure 5.5** clearly shows higher opioid activities of fermented samples when compared to unfermented control as evidenced by higher TRF responses for the concentration range used in the study. The slope of control curve changes at higher concentrations as compared to fermented samples. This clearly indicates that fermented fractions have increased opioid activity due to presence of opioid peptides released during fermentation. A comparison of opioid activity of fermented sample fractions is more vivid when a comparison of EC_{50} of the fractions as calculated from dose response curve is made. The EC_{50} of the fractions were 6.3, 7.2, 4.9 and 4.3 mg/mL for *Lb. acidophilus, Lb. brevis, Lb. fermentum and Lb. plantarum* (Table 5.4). These values confirm that from the selected bacteria use for fermentation *Lb. fermentum* and *Lb. plantarum* outperform the others in releasing opioid peptides from wheat proteins.

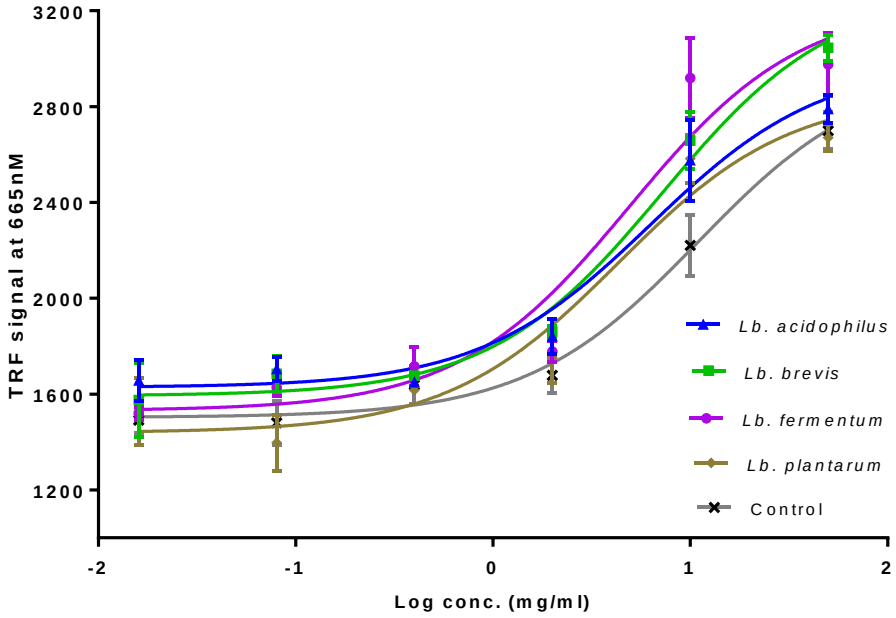


Figure 5.5: Dose response curve of fermented hydrolysate fractions (<3 kDa) for opioid activity as measured by TRF signal

Table 5.4: EC_{50} values of fermented hydrolysate fraction (<3 kDa fraction) as obtained by change in TRF signal dose response curve

Microorganisms used	**EC_{50} (mg/mL)**
Lb. acidophilus	6.3
Lb. brevis	7.2
Lb. fermentum	4.9
Lb. plantarum	4.3
Control	10.9

The data from proteolysis, RP- HPLC, SE- HPLC and SDS-PAGE clearly shows that out of the six LAB used for fermentation, *Lb. acidophilus, Lb. brevis, Lb. fermentum and Lb. plantarum* grew more and hydrolysed proteins and have superior proteolytic enzymes system to release peptides. *Lb. alimentarius* and *Lb. hilgardii* grew slower and pattern of HPLC and SDS-PAGE were similar to control samples indicating very limited proteolysis by these bacteria. It has been known that proteolysis during sourdough fermentation is due to activation of native cereal proteases (also called primary proteolysis) and proteolytic system of LAB (secondary proteolysis) (Gänzle 2014). Cereal proteases are mainly aspartic proteinases (Bleukx and Delcour 2000) and carboxypeptidases (Drzymała, Prabucka et al. 2012) which causes protein hydrolysis leading to production of different sized peptides. The proteolytic system of LAB consists of extracellular cell envelop proteinases (CEPs) mainly serine proteases, transporters proteins and intracellular enzymes present within bacterial system. CEPs cleave the proteins into peptides ranging from 4 to 30 amino acids, which are released in extracellular media. The peptides so produced are transported into bacteria by oligopeptide permease, ion linked transporter (for di and tri- peptides) and ATP-binding cassette transporter (for 2-9 amino acid long peptides) and further hydrolysed into smaller peptides and amino acids by action of intracellular enzymes. These enzymes are endopeptidases (PepO, PepF, PepE and PepG), aminopeptidases (PepN, PepC, PepS, PepA and PepI), tri-peptidases (PepT), dipeptidases (PepD and PepV) and proline-specific peptidases (PepQ, PepI, PepR, PepX and PepP) (Gänzle, Loponen et al. 2008, Raveschot, Cudennec et al. 2018). Different lactobacillus species possess different proteolytic enzymes. *Lb. plantarum* possess PrtR (a CEPs)

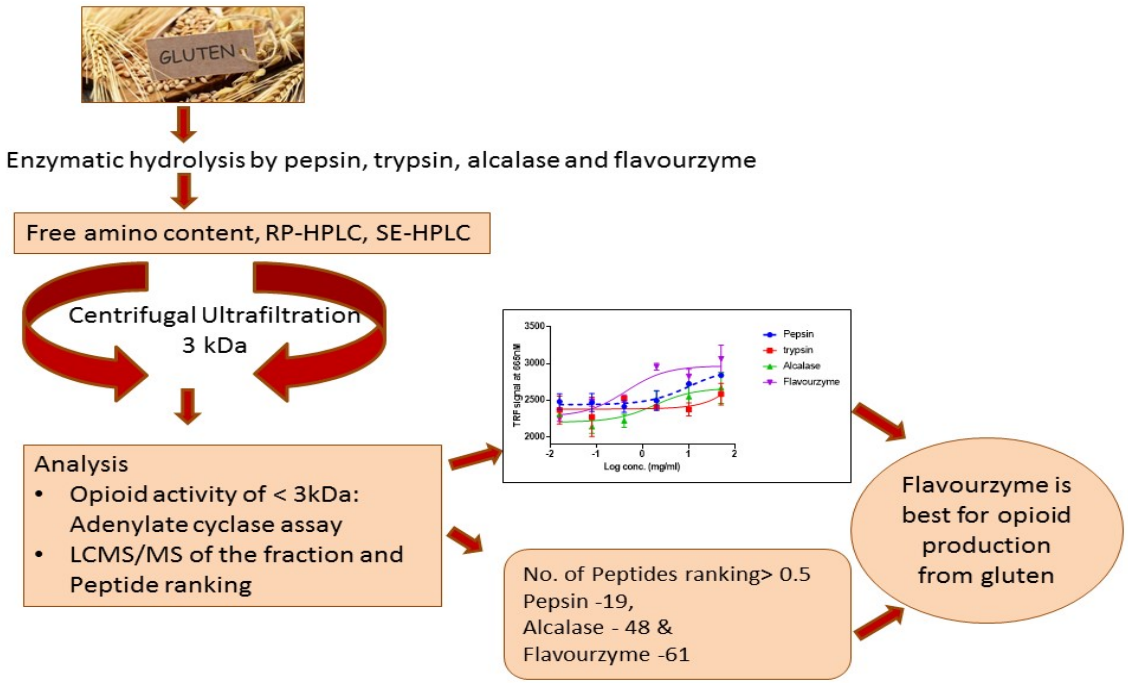
(Vukotić, Strahinić et al. 2016, Chen, Huang et al. 2018), leucyl aminopeptidase (Roudj, Belkheir et al. 2009) and high Pep X activity and intermediate PepQ, PepN, and PepO activities (Hu, Stromeck et al. 2011). *Lb. acidophilus* possess prolyl endopeptidases (Brzozowski and Lewandowska 2014), aminopeptidase and dipeptidase (Shihata and Shah 2000). *Lb. brevis* contains aminopeptidase, di-peptidase, tri-peptidase, imino-peptidase (Gobbetti, Smacchi et al. 1996) and leucyl peptidase (Roudj, Belkheir et al. 2009). The different enzymes present in different lactobacillus species drives their ability to hydrolyse food proteins for the production of bioactive peptides with different functionalities and opioid activity as has been observed in the present study. Low PepO and high PepN activities of starter cultures are known to contribute to increased release of antihypertensive tripeptides LQP and LLP during sourdough fermentation (Hu, Stromeck et al. 2011). In this study, acidification of the fermented slurry due to increased growth of *Lb. acidophilus, Lb. brevis, Lb. fermentum* and *Lb. plantarum* appeared to have contributed to activation of cereal proteases and solubilisation of gluten. It can be explained on the basis that proteolysis observed (during 24 hours) is mainly restricted to cereal proteases with relatively smaller contribution to proteolysis by bacterial proteolytic system. This aspect needs further investigation. Absence of amino acid peak in SE-HPLC chromatogram also indicates that endopeptidases (which mainly hydrolyse the bigger peptides into smaller peptides and amino acids) were not activated under tested conditions. Opioid activity of the fermented sample (<3 kDa) fractions, as expressed in terms of EC_{50} values, decreased by factor of 0.58, 0.66, 0.48 and 0.39 times for *Lb. acidophilus, Lb. brevis, Lb. fermentum and Lb. plantarum*, respectively, after fermentation.

5.5. Conclusion

The lactic acid bacteria that produced reasonable opioid activity under the specified fermentation conditions are *Lb. fermentum* and *Lb. plantarum.* There is an evidence of positive relationship between the growth of bacteria and proteolysis in all of the lactic acid bacteria studied. The scope of this study can be enhanced by including fermentation conditions, like temperature, agitation, size of inoculum and pH in order to identify optimal fermentation conditions required for maximum production of opioid peptides. In the present study, it is not possible to distinguish clearly whether the proteolysis and the resultant opioid activity were due to proteolytic enzymes from wheat or bacterial proteases. Considering this, wheat proteins were hydrolysed using pure enzymes, and opioid activity of the hydrolysate was investigated and the results are presented in the next chapter. Proteolysis using pure enzymes offer advantage as enzymes are specific and hydrolysis can be controlled all for the release of opioid peptides.

Chapter 6. Opioid activity of enzymatic hydrolysate of wheat gluten

The material presented in this chapter has been submitted to 'Journal of Functional foods' for publication.



6.1. Summary

The opioid activity of the fermented wheat flour samples by lactic acid bacteria was low due to low proteolytic activity of selected bacteria, so, selected commercially available enzymes were used for the proteolysis of gluten in this chapter. Wheat gluten was hydrolysed using pepsin, trypsin, alcalase and flavourzyme and hydrolysis was measured by change in free amino group. The hydrolysate was ultrafiltered through 3 kDa membrane and peptide profile was obtained using RP-HPLC and SE-HPLC. The opioid activity was measured by adenylate cyclase assay and peptides were identified using LC-MS/MS and sequenced using MASCOT followed by selecting peptides with the highest peptide ranking. Among the tested enzymes, Flavourzyme produced maximum hydrolysis. The opioid activity as EC_{50} values of pepsin, alcalase and flavourzyme hydrolysates were 9.9, 2.3 and 0.46 mg/mL, respectively. The number of peptides showing > 0.5 ranking were 19, 48 and 61 for pepsin, alcalase and flavourzyme fractions. The majority of the opioid peptides originated from gliadin fraction. Peptides, PQQPFPL, QQPPFW and QPFPQPQPFP were identified as most potent with ranking ≥ 0.9 and therefore show promise to be further tested for their opioid activity.

6.2. Introduction

Protein hydrolysis has been widely used for the improvement of functional and nutritional properties of food proteins. These hydrolysates find applications as nutritional, pharmaceutical and functional ingredients in cosmetics and personal care products and for flavour and colour improvement in foods. The reviews published in the literature proves significant interest in the use of food protein as a source to produce bioactive peptides, which have anti-oxidant, anti-cancerous, anti-microbial, anti-hypertensive, opioid and immune-stimulating properties (Korhonen and Pihlanto 2003, Gobbetti, Minervini et al. 2004, Pepe, Tenore et al. 2013, Garg, Nurgali et al. 2016). Typically, these bioactive peptides have low molecular weight (Barbana and Boye 2010, Choonpicharn, Tateing et al. 2016) and are sourced from food proteins either by commercial enzymes (Pihlanto-Leppälä, Koskinen et al. 2000, Kong, Zhou et al. 2008), gastrointestinal digestive enzymes *in vivo* (De Noni 2008, De Noni and Cattaneo 2010, Stuknytė, Maggioni et al. 2015, Ul Haq, Kapila et al. 2015), or during food processing (Rizzello, Nionelli et al. 2012, Mamilla and Mishra 2017).

Enzymatic hydrolysis is the most preferred hydrolysis method for production of bioactive peptides as enzymes are specific and, therefore, the degree of hydrolysis can be controlled to produce peptides with specific bioactivity from different plants (Yang, Marczak et al. 2004, Kong, Zhou et al. 2008, Barbana and Boye 2010, Ribeiro, Leclercq et al. 2017) and animal proteins (Pihlanto-Leppälä, Koskinen et al. 2000, De Noni 2008, Ul Haq, Kapila et al. 2015). Wheat gluten (WG) is a widely available by-product of the starch industry and can serve as a cheap source of protein for the production of bioactive peptides. Gluten is a mixture

of two distinct proteins, glutenins ((high molecular weight (HMW) and low molecular weight (LMW)) and gliadins (alpha, γ and omega) and contribute to different functionalities due to their structural differences.

Opioid peptides, β-casomorphins, exorphins soymorphins and rubiscolin obtained from food proteins are from milk, wheat gluten, soybean and spinach proteins, respectively. The opioid bioactivity has been reported to vary not only due to difference in the composition of hydrolysates containing peptides but also different assays used for determination of opioid activities. EC_{50} value as measured by a guinea-pig ileum assay was 6.5 and 57 μM for β-casomorphins-7 and -5, respectively (Brantl, Teschemacher et al. 1981). The EC_{50} value of soymorphin -5, -6 and -7 on guinea pig ileum were 6.0, 9.2 and 13 μM (Ohinata, Agui et al. 2007) and rubiscolin - 5 and -6 on mouse *vas deferens* were 51 and 24.4 μM, respectively (Yang, Yunden et al. 2001), thus implying that β-casomorphins-5 and soymorphin-5 are more potent food derived opioid peptides. Rubiscolins and soymorphins were synthesized on the basis of presence of tyrosine-proline structural motifs in the proteins. However, β-casomorphins have been released from β-casein after pepsin hydrolysis of milk (Schmelzer, Schöps et al. 2007) or simulated gastrointestinal digestion of milk products (De Noni and Cattaneo 2010). These opioid peptides were also detected in Greek yogurt (Papadimitriou, Vafopoulou-Mastrojiannaki et al. 2007), kefir (Ebner, Arslan et al. 2015) and different cheese varieties (Sienkiewicz-Szłapka, Jarmołowska et al. 2009). The hydrolysis of gluten by pepsin showed opioid activity (Zioudrou, Streaty et al. 1979) due to the presence of exorphin A5, A4, B5, B4 and C (Fukudome and Yoshikawa 1993) in the hydrolysates. Opioid activity is also noted in pepsin–trypsin (Huebner, Lieberman

et al. 1984) and pepsin-pancreatic (Fukudome, Jinsmaa et al. 1997) digests of gluten. Kong *et al.* (2008) demonstrated enhanced opioid activity of gluten hydrolysates produced by alcalase, pepsin and combination of pepsin and pancreatin. The alcalase hydrolysate showed stronger inhibitory effects on the contraction of guinea-pig ileum with EC_{50} of 1.21 mg protein/mL, however, peptides responsible for opioid activity were not identified. Combination of flavourzyme and alcalase to hydrolyse gluten showed no improvement in opioid activity (Kong, Zhou et al. 2008).

In this study, the ability of different enzymes (pepsin, trypsin, alcalase and flavourzyme) to hydrolyse opioid peptides from WG was assessed. The peptide profile of the hydrolysates was determined using RP-HPLC and SE-HPLC and compared to less than 3 kDa fractions. The opioid activities of the fractions (<3 kDa) were measured by adenylate cyclase assay and constituent peptides were identified by LC-MS/MS followed by sequence analysis using Mascot. The identified peptides in different hydrolysates were ranked using PeptideRanker for identification of the most likely opioid sequences within the hydrolysate.

6.3. Materials and methods

6.3.1. Materials

Wheat flour (protein content 12.5%) was obtained from Allied Mills, Australia. o-phthalaldehyde (OPA) and methionine were from Sigma Aldrich (Steinheim, Germany). Enzymes pepsin (P77160), trypsin (T1426) and alcalase (30079) were from Sigma Aldrich while flavourzyme was from Novozyme (Denmark). Cell culture media, Dulbecco's Modified Eagle's Medium (DMEM)

containing 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonicacid (HEPES), fetal bovine serum (FBS), antibiotic-antimycotic-100X (15240062) and hygromycin-B and phosphate buffer saline (PBS, pH 7.2), were from Life Technologies (Carlsbad, CA). Lance cAMP detection reagents, and bovine serum albumin (BSA) stabilizer and optiplate were purchased from PerkinElmer Life Sciences (Cambridge, MA). 3-Isobutyl-1-methylxanthine (IBMX), trypsin-EDTA, forskolin, [D-Ala^2, N-MePhe^4,Gly^5-ol]-enkephalin (DAMGO), dynorphin A, HCl, NaOH and all other chemicals were purchased from Sigma Aldrich (Australia) unless otherwise stated.

6.3.1.1. Cell lines

FlpIn Chinese hamster ovary (CHO) cells stably transfected with pOG44 vector encoding Flp recombinase and pDEST vector encoding human μ receptor were kindly gifted by Dr Meritxell Canals, Monash Institute of Pharmaceutical Sciences, Melbourne, Australia. The cells were transfected using polyethylenimine as a transfection reagent and hygromycin-B (200 μg/mL) was used as a selection agent (Burford, Livingston et al. 2015).

6.3.2. Preparation of gluten

Gluten was extracted from wheat flour by hand-washing using AACC method 38-10 (Approved methods of the American association of cereal chemists, 2000). The extracted gluten was freeze dried using Alpha 1–4 LSC freeze dryer (Martin Christ, Germany) and powdered using a coffee grinder. The gluten powder was passed through a 212 μm sieve before using and had protein concentration of

75% (correction factor N * 5.7) as measured by using Kjeldahl method (Qiu, Sun et al. 2014).

6.3.3. Preparation of hydrolysates

Gluten suspension (50 mg/mL) was prepared in Milli-Q water and pH of the suspension was adjusted to optimum pH and temperature as specified in Table 6.1 for each enzymes used. Hydrolysis was carried out using enzyme: substrate ratio of 1:100 (Kong, Zhou et al. 2008) for 2 hours at optimum pH under pH stat conditions. Hydrolysates were kept in boiling water bath for 10 min to inactivate the enzyme, cooled to room temperature, and centrifuged at 3274 g for 20 min. The supernatants were filtered through 0.45 µM PTFE membranes and freeze dried using Alpha 1–4 LSC Christ freeze dryer (Martin Christ, Germany). Dried hydrolysates were stored at -20 °C until used for further analysis.

Table 6.1: Experimental conditions used for hydrolysis of wheat gluten by different enzymes

Enzymes	**Source of enzyme**	**Activity**	**Temperature**	**pH**	**Time**
Pepsin (P77160)	Porcine stomach mucosa	543 U/mg	37°C	2	2 hrs
Alcalase (30079)	*Bacillus licheniformis*	5900 U/mg	60°C	8	2 hrs
Trypsin (T1426)	Porcine pancreas	1300 U/mg	37°C	8	2 hrs
Flavourzyme	*Aspergillus oryzae*	500 LAPU/g	50°C	6	2 hrs

6.3.4. Determination of free amino group concentration using OPA reagent.

Freeze dried samples (10.0 mg) were suspended in 1 mL of 50 mM sodium phosphate buffer (pH 8.3) vortexed and centrifuged at 3274 g for 15 min at 4 °C and supernatant so obtained was passed through 0.45 µm PTFE syringe filter and stored at -20 °C until assayed. The OPA reagent was prepared fresh by combining 25 mL of sodium phosphate buffer (50 mM; pH 8.3), 2.5 mL of sodium dodecyl sulphate (20%, w/v), 40 mg of OPA (dissolved in 1 mL of methanol), and 100 µL of β-mercaptoethanol and diluting to 50 mL with water. Samples (50 µL) were added to a test tube containing 3 mL of the OPA reagent and mixed gently for 5 sec. The absorbance was measured at 340 nm after two min using a Nova Spec-II spectrophotometer (Pharmacia, England, UK). Buffer was used as a blank and concentration of free amino group was determined using methionine as a standard (Church, Swaisgood et al. 1983).

6.3.5. RP-HPLC of the hydrolysate

The peptides present in the control and hydrolysed gluten were profiled on a Prominence-i, LC-2030C (Shimadzu, Kyoto, Japan) liquid chromatography equipped with C-18 monomeric column (5mm, 300A˚, 250 mm*4.6 mm; Grace Vydac, Hesperia CA, USA). Samples were prepared by dissolving 10.0mg of freeze-dried hydrolysates in 1.0 mL of MQ water. Ten µL of solution was eluted by a linear gradient from 95% to 5% solvent A (0.1% trifluoroacetic acid (TFA) in deionised water) in solvent B (0.1% TFA in acetonitrile) over 90 min (Donkor, Henriksson et al. 2007). Separations were conducted at room temperature at a flow rate of 0.75 mL/min. The eluted peptides were detected at 214 nm using UV/Vis

detector. All samples and solvents were filtered through a 0.45 mm membrane filter (Schleicher & Schuell GmbH, Germany).

6.3.6. Molecular weight distribution using SE HPLC

SE-HPLC was conducted on Prominence-i, LC-2030C (Shimadzu, Kyoto, Japan) with automatic injection. Ten µL of sample was loaded onto a Yarra SEC 3000 column (Phenomenex, Torrance, United States). Samples were eluted with 50 mM sodium phosphate buffer (pH 6.8) containing 300 mM NaCl at flow rate of 0.4 mL/min and detected at 214 nm using UV/Vis detector.

6.3.7. Fractionation of hydrolysate to <3 kDa

The hydrolysates obtained using pepsin, trypsin, alcalase and flavourzyme were passed through an ultra-filtration membrane with molecular weight cut-off of 3 kDa to obtain fractions (<3 kDa). These fractions were lyophilised and subsequently used for opioid activity determination. RP-HPLC and SE-HPLC analysis of the fractions was conducted to compare the differences in peptide profiles of whole hydrolysate and fractions (<3 kDa).

6.3.8. Opioid activity measurement using adenylate cyclase activity

Opioid activity of the hydrolysates was determined on the basis of inhibition of adenylate cyclase using transfected cell lines expressing µ opioid receptors. Cells were grown and maintained in DMEM media containing 10% FBS, 1% antibiotic-antimycotic and 200 µg/mL hygromycin-B in a humidified incubator containing 5% CO_2 at 37°C (Burford, Livingston et al. 2015). Confluent cells (90%) were harvested and resuspended at 2 x 10^6 cells/mL in media and 100 µL of cell

suspension was transferred into sterile 96 well plates and incubated at 37°C and 5% CO_2 overnight. The culture media in all the wells were replaced with stimulation buffer (PBS, 50 mM IBMX and BSA stabiliser) and incubated for 30 min. Cells were treated with different concentrations of hydrolysate in the presence of forskolin (10 µM) for 30 min. The stimulation buffer was removed and 50 µL of 100% ethanol (ice cold) was added to each well. Ethanol was allowed to be evaporated followed by addition of 75 µL of lysis buffer (0.3% tween-20, 5 mM HEPES and 0.1% BSA). Change in concentration of cAMP in the lysate was estimated using Lance cAMP detection kit (Perkin Elmer, Melbourne) using kit instructions. Time-resolved fluorescence (TRF) was detected using an Envision plate reader (PerkinElmer, Melbourne) with excitation at 337 nm and emission read at 615 nm and 665 nm. Data were analysed and EC_{50} values determined using nonlinear regression analysis to fit a logistic equation using Graph Pad Prism, version 7 (Graph Pad San Diego, CA). DAMGO was used as a positive control.

6.3.9. LC-MS analysis

For LC-MS analysis, samples were dissolved in 2% acetonitrile containing 0.1% TFA and analysed using an Ultimate 3000 nano HPLC (ThermoFisher scientific) through MicroTOFq quadrupole TOF (Bruker Daltonics) mass spectrometer. The samples were separated over a 30 min gradient on a pepmap 100, 75 µm id, 100Å pore size, reversed phase nano column with 95% buffer A (0.1% formic acid) to 70% B (80% acetonitrile and 0.1% formic acid), at a flow rate of 300 nL/min. The eluent was nebulised and ionised using the Bruker nano ESI source with a capillary voltage of 4000 V. Peptides are selected for MS/MS analysis in auto MSn mode based on peak intensity selecting 4 precursor ions and active

exclusion released after 2 min. Prior to analysis, the qTOF mass spectrometer was calibrated using tune mix. Data from LC/MS/MS run were exported in Mascot generic file format (*.mgf) and searched against wheat database downloaded from UniProt using the MASCOT search engine (version 2.4). The search parameters used were: missed cleavages, 1; peptide mass tolerance, ± 20 ppm; peptide fragment tolerance, ± 0.04 Da; peptide charge, 2+, 3+ and 4+; fixed modifications, carbamidomethyl; variable modification, methionine oxidation.

6.3.10. Bioactivity prediction using Peptide ranker

The ranking of peptides was based on the fact that different functional classes of bioactive peptides share structural features. The ranking of the peptides (<3 kDa) produced by selected enzymes was predicted by PeptideRanker (http://bioware.ucd.ie/~compass/biowareweb/Serverpages/peptideranker.php). The peptides with a score of > 0.5 were considered bioactive in the present study (Qiao, Tu et al. 2018).

6.4. Results and discussion

6.4.1. Gluten hydrolysis and peptide profile on RP-HPLC and SE-HPLC

The pattern of gluten hydrolysis using selected proteases (pepsin, trypsin, alcalase and flavourzyme) is shown in terms of change in the concentration of free amino groups (Table 6.2). Based on these data, maximum and minimum hydrolysis was achieved using flavourzyme (0.777 mM equivalent of methionine) and trypsin (0.055 mM equivalent of methionine), respectively. It can be explained on the basis of specificity of the enzymes used. Pepsin cleaves specifically after

Phenylalanine (F) (Narita, Oda et al. 2002) and Leucine (L) (Keil 1992) whereas trypsin splits peptide bonds adjacent to Arginine (R) or Lysine (K) (Olsen, Ong et al. 2004) residues. Thus, both these enzymes will lead to protein hydrolysis only at their specific sites. On the other hand, flavourzyme and alcalase are a mixture of proteases sourced from *Aspergillus oryzae* and *Bacillus licheniformis*, respectively. Alcalase contains endopeptidases glutamyl endopeptidase and subtilisin. However, flavourzyme, contains 3 endopeptidases, 2 aminopeptidases (specific for leucine), 2 dipeptidyl peptidases (specific for proline) (Merz, Eisele et al. 2015) and the presence of all these enzymes in flavourzyme increases the hydrolysis of gluten to shorter peptides and amino acids, thus, increasing the free amino content compared to pepsin, trypsin and alcalase (**Table 6.2).**

Table 6.2: Gluten hydrolysis by different enzymes as measured by free amino acid content using o-phthalaldehyde (OPA) method

Enzymes	**Increase in conc. of free amino group (mM equivalent of methionine)**
Pepsin (P77160)	0.284±0.004 [b]
Alcalase (30079)	0.233±0.020 [b]
Trypsin (T1426)	0.055±0.002 [c]
Flavourzyme	0.777±0.069 [a]

Values in column are expressed as mean ± standard deviation. Within a column, different lowercase letters (a–c) indicate a significant difference ($P < 0.05$) based on the Tukey's test.

RP-HPLC chromatogram of hydrolysates produced by pepsin, trypsin, alcalase and flavourzyme compared to the corresponding <3 kDa fractions are shown in **Figure 6.1**. The chromatogram of pepsin hydrolysate shows peaks between 10–34 min. In trypsin and alcalase hydrolysates the majority of the peaks are present between 10-39 mins. The chromatogram of flavourzyme hydrolysate is very different from that of the other enzymatic hydrolysates as all peaks are present between 6-28 min with prominent peaks appearing at 6.5, 8.9 and 12.7 min. The peaks present in the pepsin and trypsin hydrolysate RP-chromatograms showed significant difference between the chromatogram before and after passing through 3 kDa membrane due to gluten being hydrolysed to smaller proteins and not peptides. Comparison of RP-HPLC chromatograms of whole hydrolysate and after passing through 3 kDa ultrafiltration membrane shows that peptides produced by pepsin and trypsin hydrolysates were mainly >3 kDa as the majority of the peaks are absent in less than 3 kDa fraction. The chromatogram of flavourzyme hydrolysate did not show much difference before and after passing through 3 kDa ultrafiltration membrane which indicates that majority of peptides produced by flavourzyme were <3 kDa which further supports increased hydrolysis to smaller molecular weight peptides due to the presence of exopeptidases in flavourzyme.

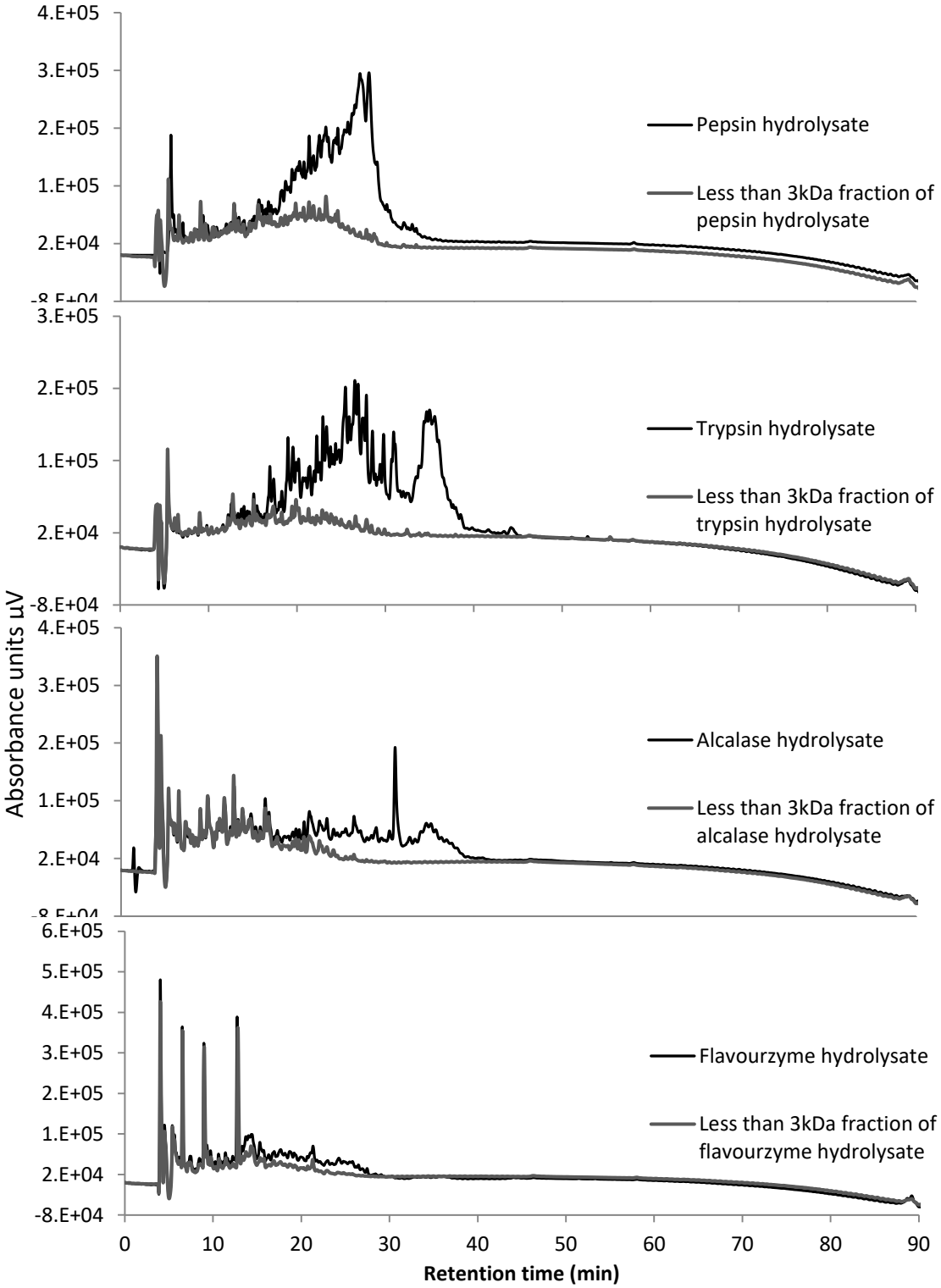


Figure 6.1: RP-HPLC chromatogram of the enzymatic hydrolysates as compared to their <3 kDa fraction

The separation mechanism in RP-HPLC column is based on hydrophobic interactions of peptides with non-polar stationary phase and can be explained on the basis of hydrophobicity of the proteins and peptides in the hydrolysate. Different researchers have different opinion on hydrophobicity of hydrolysates. Some researchers believe that enzymatic hydrolysis increases hydrophobicity (Moure, Domínguez et al. 2005, Wang, Zhao et al. 2007), whilst others suggest it decreases hydrophobicity (De La Barca, Ruiz-Salazar et al. 2000). The early chromatogram consists of highly hydrophilic molecules and probably includes soluble components other than peptides. In the RP chromatogram, the peaks eluted early are hydrophilic protein or peptides compared to later peaks at higher retention time which are hydrophobic protein/peptides. Legay et al (1997) determined that peptides eluting around 30% acetonitrile predominantly contained glutamic acid, proline and phenylalanine, whereas those being eluted at 45% contained lysine after enzymatic hydrolysis of gliadin (Legay, Popineau et al. 1997). We assigned the peptides eluted at 15 to 30% acetonitrile concentration as hydrophilic and the peptides eluted at higher acetonitrile concentration as hydrophobic and relative content of hydrophilic peptides is represented in Figure 6.2. In hydrolysates, the relative content of hydrophilic peptides was maximum for flavourzyme. Following hydrolysates being passed through ultrafiltration membrane (3 kDa), the relative content of hydrophilic peptides increased in all hydrolysate and followed the order: flavourzyme > alcalase > pepsin > trypsin. A positive correlation between hydrophobicity and bioactivity has been established in previous studies (Pihlanto-Leppälä, Koskinen et al. 2000, Gobbetti, Minervini et al. 2004).

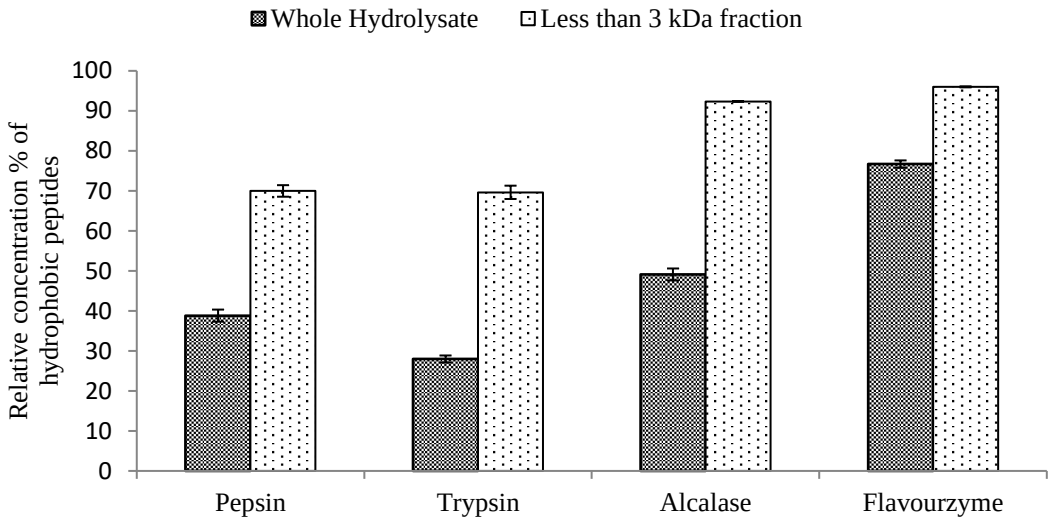


Figure 6.2: Comparison of concentration of hydrophilic peptides (% of the total) present in hydrolysates and <3 kDa fractions based on chromatographic pattern

The SE-HPLC chromatograms of the control gluten (a), hydrolysates produced by selected enzyme (b) and less than 3 kDa fraction (c) and shows differences in the size of protein or peptides as further comparison (**Figure 6.3**). Generally, higher molecular weight proteins have lower retention time as compared to low molecular weight protein, peptides or amino acids. The hydrolysates produced by pepsin, trypsin, alcalase and flavourzyme elute at 8.2 min, 7.2 min, 8.0 min and 8.6 min, respectively. This indicates that from the four enzymes used for hydrolysis, trypsin hydrolysate contains relatively larger molecular weight protein and peptides than those produced by flavourzyme. This observation corroborates the results of the extent of hydrolysis reported in Table 6.2. The ability of investigated enzymes to hydrolyse gluten to smaller peptides follows the order: flavourzyme > pepsin > alcalase > trypsin. Extended time taken for elution (11.6 - 12.4 min) in the case of flavourzyme hydrolysate suggests production of hydrolysate containing amino acids and smaller peptides due to its exopeptidase activity. In hydrolysate fraction (<3 kDa), the peaks start eluting at 9.6 min, which confirms that fraction is now free of proteins and larger peptides and only contain low molecular weight peptides. The data show that flavourzyme is best for the hydrolysis of gluten as compared to pepsin, trypsin and alcalase. This is particularly significant as most of the food derived bioactive peptides are known to be of a low molecular weight (Kong, Zhou et al. 2008, Choonpicharn, Tateing et al. 2016) including ones that have opioid activity. Hence, the opioid activity assay was carried out only on <3 kDa fractions of hydrolysate.

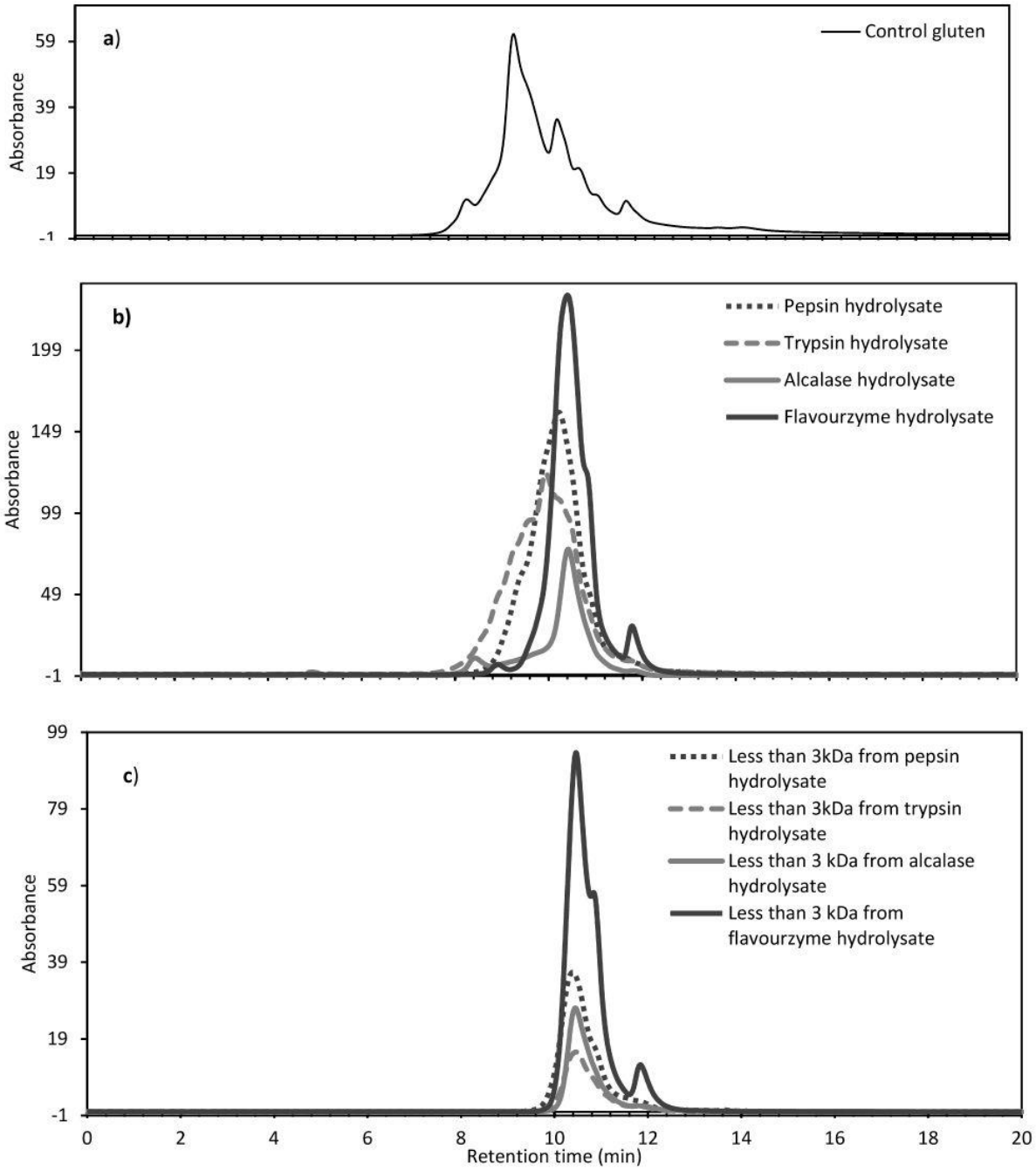


Figure 6.3: SE-HPLC chromatogram of control gluten (a), enzymatic hydrolysate (b) and fraction (<3 kDa)

6.4.2. Opioid activity of <3 kDa fraction of the hydrolysate

The opioid activity was tested using cAMP assay, which is based on the inhibition of the adenylate cyclase enzyme as measured by a decrease in concentration of cAMP (increase in TRF signal) in the cells expressing opioid receptors (Zioudrou, Streaty et al. 1979). The increase in TRF signal (which expresses the decrease in concentration of cAMP) is graphed against log concentration of hydrolysate. It is noted that the fractions (<3 kDa) produced by pepsin, alcalase and flavourzyme inhibited the production of cAMP in the presence of 10 µM forskolin in cells expressing µ opioid receptors. These dose-response curves show differences in the opioid activity of fractions. The EC_{50} of the hydrolysates calculated from a sigmoid response curve (**Figure 6.4**) were 9.9 mg/mL, 2.3 mg/mL and 0.46 mg/mL for pepsin, alcalase and flavourzyme hydrolysates, respectively. Trypsin hydrolysate dose-response was flat indicating no change in adenylate cyclase activity irrespective of the concentration. The dose-response curve did not fit the sigmoid and R^2 value for the curve fit was extremely low (0.27). This is due to an inability of trypsin to hydrolyse gluten to produce smaller bioactive peptides. This confirms that from all the hydrolysates, the flavourzyme fraction showed maximum opioid activity.

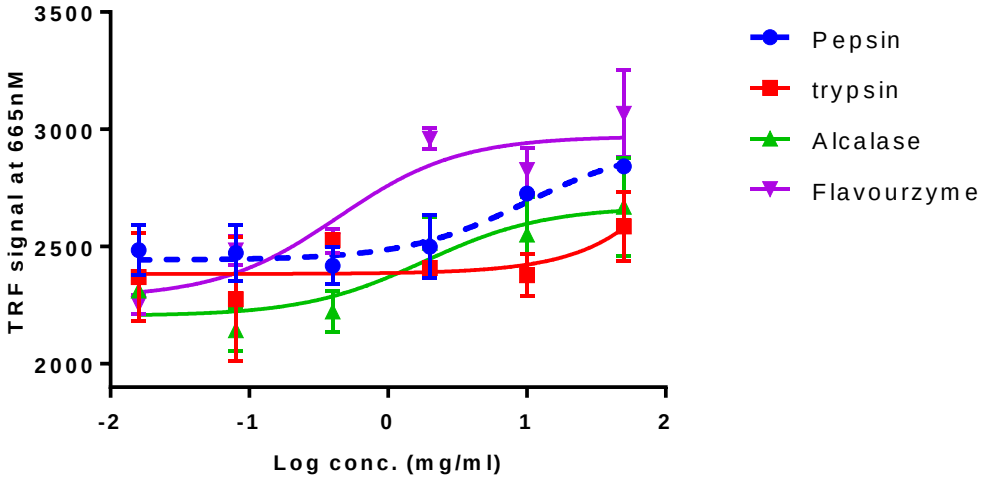


Figure 6.4: Opioid activity of gluten hydrolysate fractions (<3 kDa) as produced by different proteolytic enzymes

6.4.3. Peptide identification and ranking

The hydrolysate is generally fractionated into different fractions and the bioactivity of each of these fractions are tested. The bioactivity results from additive and/or synergistic effects of various components present in each fraction. Fractionation is followed by identification of the peptide sequence in the most bioactive fraction using LC-MS/MS, however, some bioactive peptides present in the other fractions but not showing overall bioactivity could be missing. In this study, the pepsin, alcalase and flavourzyme hydrolysates were found to be effective in releasing number of peptides that contribute to opioid activities (**Table 6.2, Figure 6.1**) as demonstrated by lower EC_{50} value (**Figure 6.4**); it is essential to identify the peptides present in the hydrolysate. The molecular weights of the peptides present in the hydrolysates were identified using LC-MS/MS and the sequence was analysed using MASCOT. Identifications were considered significant when the overall protein score was higher than 50 (Ribeiro, Leclercq et al. 2017). The letter U in **Table 6.3** represents peptides that are unique to specific protein family. The MASCOT of <3 kDa fraction identified 88 peptides each from pepsin and alcalase hydrolysates and 152 peptides from flavourzyme hydrolysate. This observation confirmed that flavourzyme was the most effective proteolytic enzyme for hydrolysis of gluten to peptides.

The identified peptide sequences were ranked for bioactivity by using peptide ranker. The rank of these peptides ranged 0.1 to 0.97. **Table 6.3** shows sequence of peptides identified from pepsin, alcalase and flavourzyme hydrolysates (<3 kDa) in the order of their decreasing bioactivity ranking as predicted by peptide

ranker (Mooney, Haslam et al. 2012, Qiao, Tu et al. 2018). This analysis shows that in pepsin hydrolysate, 11 peptides were released from HMW glutenin and 6 of them showed higher ranking. Out of 17 peptides from LMW glutenin, only 1 of the peptides showed ranking > 0.5 and the rest were from gliadin (α, β and γ). In alcalase hydrolysate, all identified peptides were from gliadin (α, β and γ) and none of them was from glutenin. This indicates that alcalase could not hydrolyse glutenin. In flavourzyme hydrolysate 33 and 10 peptides were identified to be from LMW and HMW glutenin, respectively. Nearly 45 % (15 peptides) of peptides released from LMW glutenin showed ranking >0.5 as compared to only 20 % (2 peptides) peptides released from HMW glutenin. Interestingly, most of the identified peptides from HMW glutenin in flavourzyme hydrolysate are bigger, thus indicating that the enzyme was not efficient in hydrolysing glutenin. Based on these observations, it can also be concluded that glutenins are not hydrolysed easily by alcalase and flavourzyme. Peptides from pepsin hydrolysate showed higher ranking and were attributed to HMW glutenin. This may be due to the use of optimal pH for pepsin activity being in the acidic range **(Table 6.1**) which favours increased solubilisation of glutenin and thereby enhancing proteolysis.

In pepsin hydrolysate, the peptide PQQPFPL was ranked > 0.9 and FPQQPSF, ILRPLF, QQRIFWGIPAL, RIFWGIPALL, AGRLPWSTGL, QQLAGRLPWSTGL, RIFWGIPAL peptides were ranked from 0.8 to 0.9. Peptide ranking from 0.5 to 0.8 was noted for 11 peptides. Most of these peptides contain phenylalanine and leucine at amino terminal end as expected (Keil 1992) except AGRLPWSTGLQ, ILRPLFQ, RPLFQLAQGLG and QPFPQSKQPQQPFPQPQ. In alcalase hydrolysate, QPYPQPQPF, WSIIWPQ, PQQPFPQL, QPYPQPQPFP,

LPYPQPQPF and YIPPY showed peptides ranking between 0.8-0.9. Peptide ranking from 0.5 to 0.8 was shown by 42 peptides. In flavourzyme hydrolysate, peptides QQPPFW and QPFPQPQPFP had peptide ranking > 0.9, while FPQQPSF, QPFPQP, PQQPFPQL, QPQQPFP, FPQQPQQPFP, QPILPQLPF, QPQQLFP, QPQQPF and QPFPQPQLPYP showed ranking between 0.8 - 0.9. Peptide ranking between 0.7 - 0.8 was shown by 13 peptide sequences, whilst 20 and 18 peptides showed ranking between 0.6 - 0.7 and 0.5 - 0.6, respectively. In general, the number of amino acids in the peptides in the hydrolysates varied between 5 to 19 amino acids, except peptides ASPQQPGQGQQPGKWQEPGQGQQW and QPGQGQQSGQGQQSGQGHQPGQGQQSGQEKQG present in flavourzyme hydrolysate and RPSQQNPQAQGSVQPQQLPQF in pepsin hydrolysate. Interestingly, most of the identified peptides showing higher ranking (> 0.7) contained proline, an aromatic amino acid (Y, W or F) and glutamine except ILRPLF, RIFWGIPALL, AGRLPWSTGL, RIFWGIPAL, PVGIF in pepsin hydrolysate, and YIPPY in alcalase hydrolysate, thus indicating that presence of proline, aromatic amino acid and glutamine is mainly responsible for bioactivity of a peptide. Tyrosine and proline are considered as key amino acids for opioid activity (Yoshikawa 2013, Garg, Apostolopoulos et al. 2018). There is sufficient evidence that hydrolysates produced by using the selected proteolytic enzymes contain low molecular weight peptides with proline and aromatic amino acids as key amino acids for peptides to be bioactive.

Table 6.3: Sequences of peptides obtained in <3 kDa fractions of hydrolysates prepared by pepsin (a), alcalase (b) and flavourzyme (c) listed in order of their descending peptide ranking

a) Pepsin hydrolysate

	Observed Mass	Mr (expt)	Mr (calc)	ppm	Score	Expect	Unique	Peptide	Peptide ranking	Source
1	826.5	825.4	825.4	6.51	31	6.7	U	PQQPFPL	0.92	γ gliadin
2	850.4	849.4	849.4	7.49	21	1.20E+02	U	FPQQPSF	0.88	LMW glutenin
3	758.5	757.5	757.5	8.18	26	10	U	ILRPLF	0.87	γ gliadin
4	664.9	1327.8	1327.7	10.3	45	0.34	U	QQRIFWGIPAL	0.87	HMW glutenin
5	593.4	1184.7	1184.7	0.98	34	2.4	U	RIFWGIPALL	0.84	HMW glutenin
6	529.3	1056.6	1056.6	5.7	54	0.044		AGRLPWSTGL	0.84	HMW glutenin
7	536.8	1071.6	1071.6	7.71	38	1.5	U	RIFWGIPAL	0.81	HMW glutenin
8	713.9	1425.8	1425.8	2.46	40	1.3		QQLAGRLPWSTGL	0.82	HMW glutenin
9	532.3	531.3	531.3	10.9	16	2.30E+02		PVGIF	0.79	α/β gliadin
10	1195.7	1194.6	1194.6	2.7	44	0.51		QLQPFPQPQL	0.74	α/β gliadin
11	593.3	1184.6	1184.6	8.65	40	1.2		AGRLPWSTGLQ	0.69	HMW glutenin
12	500.3	998.6	998.6	10.9	27	5.6	U	ILRPLFQL	0.65	γ gliadin
13	705.5	704.5	704.5	3.75	16	25	U	HILLPL	0.61	γ gliadin

14	868.5	867.5	867.5	1.8	20	38	U	HILLPLY	0.58	γ gliadin
15	443.8	885.6	885.5	10	33	3.1	U	ILRPLFQ	0.58	γ gliadin
16	1182.1	2362.2	2362.2	5.76	29	29		RPSQQNPQAQGSVQPQQLPQF	0.55	α/β gliadin
17	600.3	1198.7	1198.7	0.93	32	5.7	U	RPLFQLAQGLG	0.51	γ gliadin
18	602.3	1202.7	1202.7	6.38	48	0.21		FLQPHQIAHL	0.5	γ gliadin
19	670.0	2007.0	2006.0	515	17	3.80E+02	U	QPFPQSKQPQQPFPQPQ	0.5	γ gliadin
20	962.5	1923.0	1923.0	4.2	82	0.00014		VSFQQPLQQYPLGQGSF	0.48	α/β gliadin
21	742.4	1482.8	1482.7	3.66	60	0.016	U	NIQVDPSGQVQWL	0.47	γ gliadin
22	913.4	1824.9	1824.8	19.9	14	7.40E+02	U	VSFQQPQQQYPSGQGF	0.46	α/β gliadin
23	909.9	1817.9	1817.9	6.94	32	13	U	SQQQQQQLFPQQPSF	0.45	LMW glutenin
24	811.4	1620.8	1620.8	12.4	65	0.0058	U	PQQPPFSQQQQPVL	0.44	LMW glutenin
25	818.4	1634.9	1634.8	6.67	29	25	U	QQPPFSQQQPILPQ	0.44	LMW glutenin
26	797.4	796.4	796.4	-2.24	18	94		AQIPQQL	0.42	γ gliadin
27	672.4	1342.7	1342.7	4.21	80	0.00013	U	RAPFASIVAGIGGQ	0.39	γ gliadin
28	817.5	816.5	816.5	-10.01	19	180	U	IFLPLSQ	0.37	γ gliadin
29	776.4	1550.8	1550.8	17.5	87	0.000026		AQGTFLQPHQIAQL	0.37	LMW glutenin
30	885.1	2652.3	2652.3	5.85	19	320	U	VSFQPSQLNPQAQGSVQPQQLPQF	0.35	α/β gliadin
31	603.8	1205.7	1205.7	4.06	42	0.78		AIHSVVHSIIM	0.35	γ gliadin

32	781.4	1560.9	1560.9	1.75	51	0.091	U	AQGLGIIQPQQPAQL	0.34	γ gliadin
33	1144.9	3431.7	3431.7	-7.9	5	11000		QPQPFPSQQPYLQLQPFPQPQPFPPQLPY	0.33	α/β gliadin
34	1012.5	3034.5	3034.5	0.5	36	6.6	U	LGQQQPFPPQQPYPQPQPFPSQQPYL	0.32	α/β gliadin
35	994.5	993.5	993.5	10.6	54	0.06	U	HSVAHSIIM	0.32	γ gliadin
36	541.3	1080.6	1080.6	12.9	32	7.3		FLQPHQIAQ	0.3	LMW glutenin
37	833.0	1663.9	1663.9	5.97	96	3.2E-06		LAQGTFLQPHQIAQL	0.3	LMW glutenin
38	699.5	698.5	698.4	15.2	21	28		IRNLAL	0.3	α/β gliadin
39	771.5	770.5	770.5	3.61	30	3.5	U	AIRSLVL	0.28	γ gliadin
40	838.0	1674.0	1673.9	3.42	72	0.0006	U	LAQGLGIIQPQQPAQL	0.28	γ gliadin
41	955.8	2864.5	2864.5	5.92	27	45	U	VRVPVPQLQPQNPSQQQPQEQVPLM	0.28	α/β gliadin
42	524.3	1046.6	1046.6	23.6	42	0.42		LQPHQIAQL	0.28	LMW glutenin
43	1185.6	3553.8	3552.7	293	2	18000	U	PQQPFPQTQQPQQPFPQQPQQPFPQTQQPQ	0.27	γ gliadin
44	934.5	933.5	933.5	6.89	20	99	U	YLTSPLQL	0.27	HMW glutenin
45	1148.5	1147.5	1146.5	879	31	8.6		GQQPGQGQQGY	0.27	HMW glutenin
46	724.9	1447.8	1447.8	5.23	61	0.0044	U	VQQQIPVVQPSIL	0.27	LMW glutenin
47	1269.1	2536.3	2536.2	3.98	37	5	U	SQQQPPFWQQQPPFSQQQPIL	0.27	LMW glutenin
48	1072.5	4286.1	4286.1	15.3	19	430	U	PQQPYPQQPQQPFPQTQQPQQLFPQSQQPQQQFSQP	0.26	γ gliadin
49	656.8	1311.6	1311.6	8.81	67	0.0033	U	PQQPPFSQQQQ	0.26	LMW glutenin

50	987.9	2960.6	2960.6	6.38	61	0.013		VRVPVPQLQPQNPSQQQPQEQVPLVQ	0.25	α/β gliadin
51	1030.6	3088.7	3088.6	5.36	46	0.53		VRVPVPQLQPQNPSQQQPQEQVPLVQQ	0.25	α/β gliadin
52	874.0	3491.8	3491.8	4.56	37	4.7		VRVPVPQLQPQNPSQQQPQEQVPLVQQQQF	0.25	α/β gliadin
53	511.8	1021.6	1021.5	14.3	73	0.00064		HSVVHSIIM	0.25	γ gliadin
54	454.3	906.6	906.6	10.3	31	0.82		VRVPVPQL	0.24	α/β gliadin
55	648.4	1294.7	1294.7	6.46	49	0.12		TFLQPHQIAQL	0.23	LMW glutenin
56	719.9	1437.8	1437.7	11.3	69	0.0019		AQGTFLQPHQIAQ	0.23	LMW glutenin
57	802.9	1603.9	1603.9	9.36	64	0.005		VQGQGIIQPQQPAQL	0.23	γ gliadin
58	803.9	3211.6	3211.6	14.9	24	120	U	QPFLQPQQPFPQQPQQPFPQTQQPQQP	0.23	γ gliadin
59	747.9	1493.8	1493.8	5.09	62	0.0067	U	VPLSQQQQVGQGIL	0.22	γ gliadin
60	1074.6	1073.6	1073.6	5.26	30	18		VSFQQPLQQ	0.22	α/β gliadin
61	700.5	699.5	699.5	13.4	21	23		IRSLVL	0.22	γ gliadin
62	899.5	898.5	898.5	9.54	43	0.31		AEIRNLAL	0.22	α/β gliadin
63	588.8	1175.6	1175.6	8.15	49	0.2		LQPHQIAQLE	0.21	LMW glutenin
64	968.5	1935.1	1935.1	8.03	78	0.0002	U	HILLPLYQQQQVGQGTL	0.21	γ gliadin
65	859.5	1717.0	1717.0	1.47	44	0.52		LVQGQGIIQPQQPAQL	0.2	γ gliadin
66	779.4	2335.2	2335.2	-1.92	35	7.3	U	QQHNIVHGKSQVLQQSTYQL	0.2	α/β gliadin
67	905.2	2712.4	2712.4	22.9	49	0.28	U	IILHQQQQQQQQQQQQQQPLS	0.2	α/β gliadin

68	716.9	1431.7	1431.7	19.4	65	0.0062		QQHNIAHASSQVL	0.19	α/β gliadin
69	1106.6	2211.2	2211.2	4.42	16	470	U	IVVPPKGGSFYPGETTPPQQL	0.17	HMW glutenin
70	828.5	827.5	827.5	2.66	27	13		EIRNLAL	0.17	α/β gliadin
71	1149.1	2296.2	2296.2	7.16	52	0.14		VRVPVPQLQPQNPSQQQPQE	0.17	α/β gliadin
72	900.6	899.5	899.5	5.87	34	2.6	U	EAIRSLVL	0.17	γ gliadin
73	799.5	798.5	798.5	1.14	35	0.65	U	VIRSLVL	0.15	γ gliadin
74	634.9	1267.8	1267.8	8.68	49	0.048	U	KVAKAQQLAAQL	0.15	HMW glutenin
75	884.7	3534.7	3534.7	4.48	17	610	U	FSQPQQQFPQPQQPQQSFPQQQPPFIQPSL	0.14	γ gliadin
76	826.4	1650.8	1650.8	6.95	22	110		VSFQQPQQQYPSSQ	0.13	α/β gliadin
77	939.0	1875.9	1875.9	9.13	37	3.9		QQHNIAHASSQVLQQST	0.13	α/β gliadin
78	957.5	956.5	956.5	13.8	40	1		EEIRNLAL	0.12	α/β gliadin
79	1002.5	3004.5	3004.5	19	54	0.095	U	IILHQQHHHHQQQQQQQQQQPLSQ	0.12	α/β gliadin
80	769.9	3075.6	3075.5	10.9	34	11	U	AIILHQQHHHHQQQQQQQQQQPLSQ	0.12	α/β gliadin
81	891.5	890.5	890.5	10.9	53	0.047		HSVVHSII	0.11	γ gliadin
82	1177.6	1176.6	1176.6	23.3	84	0.000063	U	HVSVEHQAASL	0.11	HMW glutenin
83	642.8	1283.7	1283.7	8.97	27	29		YRTTTSVPFGVG	0.1	LMW glutenin
84	835.4	1668.9	1668.8	9	85	0.000058		YRTTTSVPFGVGTGVGA	0.09	LMW glutenin
85	917.0	1831.9	1831.9	5.95	83	0.000097		YRTTTSVPFGVGTGVGAY	0.09	LMW glutenin

86	610.8	1219.6	1219.6	6.81	38	2.5		QQHNIAHASSQ	0.08	α/β gliadin
87	670.0	2007.0	2007.0	7.83	43	1.1	U	AIILHHHQQQQQQPSSQ	0.07	α/β gliadin
88	665.3	664.3	664.3	9.21	1	8700		HSVVHS	0.07	γ gliadin

b) Alcalase hydrolysate

	Observed	Mr (expt)	Mr (calc)	ppm	Score	Expect	Unique	Peptide	Peptide ranking	Source of peptides
1	1101.5	1100.5	1100.5	12.1	37	2.5		QPYPQPQPF	0.87	α/β gliadin
2	929.5	928.5	928.5	-1.71	32	7.7	U	WSIIWPQ	0.86	γ gliadin
3	954.5	953.5	953.5	0.75	22	68	U	PQQPFPQL	0.84	γ gliadin
4	1198.6	1197.6	1197.6	19.5	16	350		QPYPQPQPFP	0.83	α/β gliadin
5	1086.6	1085.6	1085.6	4.1	45	0.45		LPYPQPQPF	0.81	α/β gliadin
6	652.3	651.3	651.3	2.84	21	87		YIPPY	0.8	α/β gliadin
7	744.4	743.4	743.4	3.59	20	95		PQQQPF	0.79	α/β gliadin
8	874.0	1745.9	1745.9	2.07	22	130	U	PQPQPFLPQLPYPQP	0.77	α/β gliadin
9	900.5	899.5	899.5	6.48	23	60	U	PQQQRPF	0.77	γ gliadin
10	768.9	1535.8	1535.8	-2.49	39	2.2		PQPQLPYPQPQPF	0.77	α/β gliadin
11	1180.7	1179.6	1178.6	883	14	390	U	PQQPQLPFPQ	0.77	γ gliadin
12	607.8	1213.6	1213.6	3.37	54	0.054		QPFPQPQLPY	0.76	α/β gliadin
13	955.0	1908.0	1908.0	3.6	50	0.23	U	QPFPQPQLPYPQPQPF	0.75	α/β gliadin
14	962.5	1922.9	1922.9	-0.25	66	0.0052		QQPFPPQQPYPQPQPF	0.75	α/β gliadin
15	841.4	840.4	840.4	5.64	40	1		QPFPQPQ	0.74	γ gliadin
16	735.4	1468.7	1468.7	-0.63	48	0.28	U	QQQPFPQPQQPF	0.74	γ gliadin

17	671.3	1340.7	1340.7	0.15	53	0.07	U	FPQPQQPQQPF	0.71	γ gliadin
18	664.4	1326.7	1326.7	1.02	49	0.18		LQPFPQPQLPY	0.7	α/β gliadin
19	1011.5	2021.0	2021.0	-1.77	58	0.035	U	LQPFPQPQLPYPQPQPF	0.69	α/β gliadin
20	938.0	1874.0	1874.0	-2.36	25	59	U	PQPQPFLPQLPYPQPQ	0.69	α/β gliadin
21	743.4	742.4	742.4	4.52	26	22	U	SIIWPQ	0.69	γ gliadin
22	728.4	1454.8	1454.8	-2.45	55	0.046		QLQPFPQPQLPY	0.68	α/β gliadin
23	942.0	1881.9	1881.9	-1.22	62	0.012		QFPPQQPYPQPQPFPS	0.65	α/β gliadin
24	664.9	1327.7	1327.7	-2.68	47	0.27		QLPQFAEIRNL	0.64	α/β gliadin
25	969.5	968.5	968.5	-1.24	21	94		QQPFPQPQ	0.64	γ gliadin
26	1054.5	2107.0	2107.0	2.89	88	0.000037		QQPFPPQQPYPQPQPFPS	0.63	α/β gliadin
27	776.9	1551.8	1551.8	-2.87	53	0.093		LQPFPQPQLPYPQ	0.63	α/β gliadin
28	1116.1	2230.1	2230.1	-0.73	60	0.023	U	QPFPQPQPFPPQLPYPQPQ	0.63	α/β gliadin
29	1017.0	2032.0	2032.0	0	29	27	U	QQPQLPFPQQPQQPFPQ	0.62	γ gliadin
30	693.4	1384.7	1384.7	-10.85	46	0.36	U	QLPQFQEIRNL	0.62	α/β gliadin
31	745.4	744.4	744.4	-0.89	24	46		QPQLPY	0.59	α/β gliadin
32	946.5	1890.9	1890.0	509	16	490		PQPQLPYPQPQLPYPQ	0.59	α/β gliadin
33	1073.0	2144.0	2144.0	-2.36	31	17	U	QQPQQPFPQPQQPQQPFP	0.59	γ gliadin
34	1180.6	1179.6	1178.6	891	15	420		PQQPYPQPQP	0.58	α/β gliadin

35	1172.6	2343.2	2343.2	-0.21	64	0.0096	U	LQPFPQPQPFPPQLPYPQPQ	0.58	α/β gliadin
36	1142.6	1141.6	1140.6	879	26	29	U	PQQPYPQQR	0.56	α/β gliadin
37	954.5	953.5	953.5	5.56	31	8.6		LQPFPQPQ	0.56	α/β gliadin
38	801.8	2402.3	2402.3	1.19	52	0.15	U	LQPFPQPQLPYPQPQPFRPQ	0.56	α/β gliadin
39	1024.5	2047.0	2047.0	2.37	27	47	U	QPQQPFPQPQQQFPQPQ	0.54	γ gliadin
40	1194.6	1193.6	1193.6	0.1	36	4.5		QQPFPQPQQP	0.54	γ gliadin
41	889.5	1776.9	1776.9	-2.79	78	0.00035		LQPFPQPQLPYPQPQ	0.54	α/β gliadin
42	920.0	1838.0	1838.0	-2.12	58	0.029		VQPQQLPQFEEIRNL	0.54	α/β gliadin
43	693.9	1385.7	1385.7	6.51	67	0.0034		QLPQFEEIRNL	0.52	α/β gliadin
44	992.0	1982.0	1982.0	-1.31	76	0.00046		GSVQPQQLPQFEEIRNL	0.51	α/β gliadin
45	738.7	2213.1	2212.1	436	27	46		PQPQLPYPQPQLPYPQPQP	0.5	α/β gliadin
46	858.5	857.5	857.5	-6.45	22	69	U	LQPQLPY	0.5	α/β gliadin
47	842.4	841.4	841.4	2.74	29	12		LPYPQPQ	0.5	α/β gliadin
48	924.5	1847.0	1847.0	-1.53	80	0.00013	U	GIIQPQQPAQLEGIRSL	0.5	γ gliadin
49	753.4	1504.8	1504.8	-8.09	70	0.0014	U	QEQQQGVPILRPL	0.49	γ gliadin
50	1082.5	1081.5	1081.5	2.32	56	0.038		QPYPQPQPQ	0.49	α/β gliadin
51	797.5	796.5	796.5	-4.11	24	6	U	VSIILPR	0.48	γ gliadin
52	1097.5	1096.5	1096.5	-1.66	26	31		PQQQFPQPQ	0.48	γ gliadin

53	911.5	2731.4	2731.4	1.48	33	12	U	QPFPQPQLPYPQPQLPYPQPQPF	0.47	α/β gliadin
54	1024.1	2046.1	2046.1	-2.71	30	14	U	GQGIIQPQQPAQLEAIRSL	0.47	γ gliadin
55	1038.1	2074.1	2074.2	-3.27	23	73	U	GQGIIQPQQPAQLEVIRSL	0.47	γ gliadin
56	945.5	1889.1	1889.1	-3.42	73	0.00052	U	GIIQPQQPAQLEVIRSL	0.46	γ gliadin
57	968.5	1934.9	1934.9	4.69	41	1.5		QPQQPYPQQPQQPFPQ	0.46	γ gliadin
58	551.3	1100.6	1100.6	1.64	57	0.029		QLPQFAEIR	0.45	α/β gliadin
59	629.8	1257.7	1257.7	-1.22	34	6.8		LPQFEEIRNL	0.45	α/β gliadin
60	931.5	1861.0	1861.0	-5.65	76	0.00034	U	GIIQPQQPAQLEAIRSL	0.43	γ gliadin
61	660.3	659.3	659.3	-4.94	9	1600		QTLPAM	0.39	α/β gliadin
62	649.4	648.3	648.3	-2.34	14	460		TTIAPF	0.37	α/β gliadin
63	838.4	837.4	837.4	-6.39	23	56		QPQQLPQ	0.37	α/β gliadin
64	775.4	1548.8	1548.8	-2.27	61	0.014	U	QQVPQPQQPQQPF	0.37	γ gliadin
65	732.4	1462.7	1462.7	11.7	54	0.077		RPQQPYPQPQPQ	0.35	α/β gliadin
66	779.4	1556.7	1556.7	-0.73	25	47		QPYPQPQPQYSQP	0.35	α/β gliadin
67	580.3	1158.6	1158.6	1.14	58	0.025		QLPQFEEIR	0.34	α/β gliadin
68	938.5	937.5	937.5	0.99	18	210		QPQEQVPL	0.33	α/β gliadin
69	516.3	1030.6	1030.5	5.94	55	0.049		LPQFEEIR	0.33	α/β gliadin
70	1198.6	1197.6	1197.6	2.03	29	16		TQQPQQPFPQ	0.32	γ gliadin

71	832.4	831.4	830.4	1224	21	120	U	QPLSQVC	0.31	alpha/bet
72	764.4	3053.5	3053.5	3.16	19	320	U	QPFPQPQQPQQPFPQSQQPQQPFPQP	0.3	γ gliadin
73	982.5	981.5	981.5	7.89	33	6.8		QTFPHQPQ	0.28	γ gliadin
74	980.0	1958.0	1958.0	2.07	28	37		LQPQNPSQQQPQEQVPL	0.27	α/β gliadin
75	460.7	919.5	919.5	1.96	34	6.9		FEEIRNL	0.25	α/β gliadin
76	1048.5	1047.5	1046.5	956	18	260		YSQPQQPIS	0.25	α/β gliadin
77	454.3	906.6	906.6	0.43	42	0.11		VRVPVPQL	0.24	α/β gliadin
78	764.5	763.5	763.5	-0.11	22	5.1		VVHAIIL	0.23	α/β gliadin
79	840.4	839.4	839.5	-14.03	34	3.6		GQGIIQPQ	0.23	γ gliadin
80	824.4	823.4	823.4	1.29	15	380	U	QPQQQVP	0.22	γ gliadin
81	640.3	1278.6	1278.6	2.82	23	75		QPQNPSQQQPQ	0.22	α/β gliadin
82	697.7	2090.0	2090.0	-11.53	17	410		PQPQYPQPQQPISQQQAQ	0.21	α/β gliadin
83	1175.6	1174.6	1174.6	2.05	26	38		YSQPQQPISQ	0.19	α/β gliadin
84	696.9	1391.7	1391.7	7.28	36	4.5		LQPQNPSQQQPQ	0.17	α/β gliadin
85	695.4	694.4	694.4	-0.65	21	22		RVPVPQ	0.17	α/β gliadin
86	1039.5	1038.5	1038.5	-4.94	17	250		LQPQNPSQQ	0.16	α/β gliadin
87	794.5	793.5	793.5	10	33	0.86		VRVPVPQ	0.12	α/β gliadin
88	729.4	728.4	728.4	-3.95	20	84		RDVVLQ	0.08	α/β gliadin

c) Flavourzyme hydrolysate

	Observed Mass	Mr (expt)	Mr(calc)	ppm	Score	Expect	Unique	Peptide	Peptide ranking	Source of peptide
1	802.4	801.4	801.4	-0.87	19	120	U	QQPPFW	0.97	LMW glutenin
2	1182.6	1181.6	1181.6	0.59	21	110		QPFPQPQPFP	0.9	α/β gliadin
3	850.4	849.4	849.4	1.6	19	180	U	FPQQPSF	0.88	LMW glutenin
4	713.4	712.4	712.4	-1.2	23	41		QPFPQP	0.87	α/β gliadin
5	954.5	953.5	953.5	-6.72	29	15	U	PQQPFPQL	0.84	γ gliadin
6	841.4	840.4	840.4	5.62	34	3.6		QPQQPFP	0.83	γ gliadin
7	607.3	1212.6	1212.6	3.62	37	2.9		FPQQPQQPFP	0.82	γ gliadin
8	1052.6	1051.6	1051.6	-9.77	30	6.9	U	QPILPQLPF	0.82	LMW glutenin
9	857.4	856.4	856.4	-6.22	25	30	U	QPQQLFP	0.82	γ gliadin
10	744.4	743.4	743.4	1.96	13	450		QPQQPF	0.81	γ gliadin
11	656.3	1310.7	1310.7	2.15	50	0.15		QPFPQPQLPYP	0.8	α/β gliadin
12	841.4	840.4	840.4	7.11	23	46		QQPFPQP	0.78	γ gliadin
13	876.9	1751.8	1751.8	-0.14	39	2.4	U	WPQQQPFPQPQQPF	0.79	γ gliadin
14	816.5	815.4	815.4	38.7	10	1500	U	LSQQPPF	0.78	γ gliadin
15	874.0	1745.9	1745.9	-2.65	27	37	U	PQPQPFLPQLPYPQP	0.77	α/β gliadin

16	1214.6	1213.6	1213.6	5.24	-47	0.28		QPFPQPQLPY	0.76	α/β gliadin
17	1164.6	1163.6	1163.6	-3.39	24	51	U	QPILPQQPPF	0.76	LMW glutenin
18	878.1	2631.2	2631.2	2.78	12	1200		ASPQQPGQGQQPGKWQEPGQGQQW	0.74	HMW glutenin
19	587.8	1173.6	1173.6	2.45	30	15	U	QPVPQPHQPF	0.73	γ gliadin
20	1066.5	1065.5	1065.5	2.96	47	0.3		QPQQPFPQP	0.73	γ gliadin
21	703.3	702.3	702.3	1.48	20	91		PQQPSF	0.72	LMW glutenin
22	729.4	728.4	728.3	3.5	42	0.54		QPYPQP	0.72	α/β gliadin
23	985.5	984.5	984.5	-7.23	36	3.1		QPQQLPQF	0.72	α/β gliadin
24	671.3	1340.7	1340.7	2.25	37	3.3		FPQQPQQPFPQ	0.72	γ gliadin
25	601.3	600.3	600.3	4.99	19	88		SPVAMP	0.69	LMW glutenin
26	484.3	966.5	966.5	1.45	49	0.1		IPGLERPW	0.69	LMW glutenin
27	913.5	912.5	912.5	2.42	35	3	U	LPQQPPFS	0.68	LMW glutenin
28	1052.0	2102.1	2102.1	0.3	65	0.0074		QPFPQPQPFPPQLPYPQP	0.68	α/β gliadin
29	1179.6	1178.6	1178.6	2.72	21	110	U	QPQLPFPQQP	0.68	γ gliadin
30	702.9	1403.7	1403.7	-4.35	17	370	U	PQQPQLPFPQQP	0.68	γ gliadin
31	985.5	984.5	984.5	-3.67	34	4.5	U	QPQQLFPQ	0.67	γ gliadin
32	712.4	2134.1	2134.1	-2.68	48	0.33		QPFPQPQLPYPQPQLPYP	0.67	α/β gliadin
33	896.4	1790.9	1790.9	2.27	82	0.00012	U	QPQQPFPQQPQQPFP	0.66	γ gliadin

34	905.5	904.5	904.5	-3.44	37	1.3	U	QPILPQLP	0.63	LMW glutenin
35	1116.1	2230.1	2230.1	0.69	71	0.0019	U	QPFPQPQPFPPQLPYPQPQ	0.63	α/β gliadin
36	1071.5	1070.5	1069.5	939	20	130		QPQQPFPQT	0.63	γ gliadin
37	1194.6	1193.6	1193.6	5.02	52	0.095		QPQQPFPQPQ	0.62	γ gliadin
38	443.235	884.4554	884.4538	1.78	52	0.063		SPVAMPQR	0.62	LMW glutenin
39	626.3	1250.7	1250.7	-2.4	34	5.3	U	QPILPQQPPFS	0.62	LMW glutenin
40	690.4	1378.7	1378.7	-7.83	54	0.067	U	QQPILPQQPPFS	0.6	LMW glutenin
41	803.4	1604.8	1604.8	0.95	45	0.6	U	WPQQQPFPQPQQP	0.61	γ gliadin
42	960.5	1918.9	1918.9	0.51	85	0.00007	U	QQPQQPFPQQPQQPFP	0.61	γ gliadin
43	954.5	953.5	953.5	2.62	31	8.9		QPYPQPQP	0.61	α/β gliadin
44	611.8	1221.6	1221.6	6.28	42	1.1		QPQPFRPQQP	0.6	α/β gliadin
45	745.4	744.4	744.4	-3.34	24	54		QPQLPY	0.59	α/β gliadin
46	1147.1	2292.1	2292.1	-0.24	12	1300		GQQQPFPPQQPYPQPQPFPS	0.59	α/β gliadin
47	960.5	1918.9	1918.9	0.62	82	0.00013	U	QPQQPFPQQPQQPFPQ	0.59	γ gliadin
48	567.4	566.3	566.3	-0.03	10	150		LPQIP	0.59	LMW glutenin
49	729.4	728.4	728.4	3.14	23	38		SPVAMPQ	0.55	LMW glutenin
50	860.4	2578.3	2577.3	400	20	240	U	PQQQPFPQPQQPFCQQPQRTI	0.58	γ gliadin
51	1140.6	1139.6	1139.6	-3.6	27	23		QPVLPQQSPF	0.55	γ gliadin

52	1024.5	2047.0	2047.0	-1.44	53	0.1	U	QQPQQPFPQQPQQPFPQ	0.55	γ gliadin
53	808.6	3230.4	3230.4	-5.57	-10	1000	U	QPGQGQQSGQGQQSGQGHQPGQGQQSGQEKQG	0.54	HMW-glutenin
54	1103.5	2205.1	2205.1	0.46	30	23	U	FPGQQQPFPPQQPYPQPQP	0.53	α/β gliadin
55	726.4	1450.7	1450.7	2.19	31	13		FPQPQQTFPHQP	0.52	γ gliadin
56	876.4	1750.9	1750.9	-6.03	47	0.41	U	QPFPQPQLPYSQPQP	0.52	α/β gliadin
57	889.5	888.5	887.5	1137	21	140		PQQQFLQ	0.52	γ gliadin
58	783.9	1565.8	1565.8	0.44	18	300	U	WPQQQPVPQPHQP	0.51	γ gliadin
59	646.8	1291.7	1291.7	1.07	47	0.35		QPQLPYPQPQP	0.5	α/β gliadin
60	1058.5	2115.1	2115.1	-3.85	56	0.059		QPQLPYPQPQLPYPQPQP	0.5	α/β gliadin
61	738.7	2213.1	2212.1	436	27	41		PQPQLPYPQPQLPYPQPQP	0.5	α/β gliadin
62	690.4	1378.7	1378.7	-8.83	43	0.88	U	QPILPQQPPFSQ	0.5	LMW glutenin
63	840.6292	3358.488	3358.502	-4.21	37	2.2	U	QQPGQGQQSGQGQQSGQGHQPGQGQQSGQEKQG	0.49	HMW glutenin
64	877.4313	3505.696	3505.721	-7	13	1500	U	QPFPQPQLPYSQPQPFRPQQPYPQPQPQY	0.49	α/β gliadin
65	856.4206	1710.827	1709.816	591	36	4.8	U	QPQQPFPQQPQQPY	0.48	γ gliadin
66	675.8513	1349.688	1349.684	2.96	32	11	U	QPRQPFPQQPQ	0.48	γ gliadin
67	677.3446	1352.675	1352.673	1.67	35	5	U	QPPFSQQPPISQ	0.47	LMW glutenin
68	904.1183	2709.333	2709.326	2.6	12	1600	U	QQPQQPFPQPQQQLPQPQQPQQS	0.47	γ gliadin
69	774.3838	1546.753	1546.753	0.15	78	0.00029		QPQQPFPQQPQQP	0.47	γ gliadin

70	943.969	1885.923	1884.948	517	19	250	U	PQPQQPQQPFLQPQQP	0.46	γ gliadin
71	862.446	2584.316	2584.311	1.78	47	0.5	U	QPFPQPQLPYPQPQLPYPQPQP	0.46	α/β gliadin
72	929.4651	2785.373	2785.373	0.34	30	27	U	QPRQPFPQQPQQPYPQQPQQPFP	0.46	γ gliadin
73	1137.061	2272.107	2272.103	1.97	49	0.27	U	QPQQPFPQPQQQFPQPQQP	0.46	γ gliadin
74	710.3574	1418.7	1418.694	4.28	74	0.0008		PQQPFPQQPQQP	0.45	γ gliadin
75	1075.028	2148.041	2148.039	0.86	28	37		QQPQQPFPQTQQPQQPFP	0.44	γ gliadin
76	1073.029	2144.043	2144.044	-0.3	39	2.8	U	PQQPFPQPQQQFPQPQQP	0.44	γ gliadin
77	668.351	1334.687	1334.673	10.7	54	0.075		RPQQPYPQPQP	0.44	α/β gliadin
78	767.3838	1532.753	1532.751	1.24	17	340		VPPKGGSFYPGETTP	0.44	HMW glutenin
79	1141.53	1140.523	1140.52	2.53	24	52		DPSGQVQWPQ	0.44	γ gliadin
80	910.4899	909.4827	908.4716	1113	26	26		QPQQPAQL	0.43	γ gliadin
81	1027.536	1026.529	1026.525	3.89	24	44	U	QPVPQPHQP	0.43	γ gliadin
82	980.5042	2938.491	2938.502	-3.7	36	6.2	U	QPQLPYPQPQLPYPQPQLPYPQPQP	0.42	α/β gliadin
83	791.4284	790.4212	790.4225	-1.69	20	130		SVNVPLY	0.41	γ gliadin
84	585.3039	1168.593	1168.588	4.85	38	2.1	U	LPQQPPFSQQ	0.41	LMW glutenin
85	754.4004	1506.786	1506.783	2.13	55	0.05	U	QPILPQQPPFSQQ	0.41	LMW glutenin
86	818.4277	1634.841	1634.842	-0.46	87	0.000041	U	QQPILPQQPPFSQQ	0.4	LMW glutenin
87	937.1179	2808.332	2808.33	0.81	22	150		GQQQPFPPQQPYPQPQPFPSQQPY	0.4	α/β gliadin

88	1136.91	3407.709	3407.734	-7.32	46	0.68	U	QPFPQPQLPYPQPQLPYPQPQLPYPQPQP	0.39	α/β gliadin
89	775.391	1548.767	1548.769	-0.74	34	6.9	U	QQVPQPQQPQQPF	0.37	γ gliadin
90	391.2339	780.4533	780.4494	5.1	42	0.26		IPGLERP	0.37	LMW glutenin
91	908.4809	907.4737	907.4763	-2.95	29	15	U	QPLPPQQT	0.37	LMW glutenin
92	675.4163	674.409	674.4115	-3.78	7	1300	U	IPVIHP	0.37	LMW glutenin
93	803.4754	802.4681	802.4701	-2.52	16	260	U	QIPVIHP	0.37	LMW glutenin
94	822.3962	1642.778	1642.774	2.34	76	0.00044	U	PPFSQQQQPPFSQQ	0.37	LMW glutenin
95	923.7872	2768.34	2768.396	-20.39	13	1100	U	QQPYPQPQPFPSQQPYLQLQPFL	0.36	α/β gliadin
96	1014.483	2026.952	2026.95	0.96	42	1.1	U	QQQQPPFSQQQQPPFSQ	0.36	LMW glutenin
97	886.4249	1770.835	1770.833	1.54	55	0.058	U	QPPFSQQQQPPFSQQ	0.35	LMW glutenin
98	732.3757	1462.737	1462.732	3.49	43	0.96		RPQQPYPQPQPQ	0.35	α/β gliadin
99	813.907	1625.799	1625.795	2.72	43	1		RPQQPYPQPQPQY	0.34	α/β gliadin
100	839.4228	1676.831	1676.827	2.33	35	6	U	QQQVPQPQQPQQPF	0.34	γ gliadin
101	598.7779	1195.541	1195.537	3.56	23	68		QPGQGQQGHYP	0.34	HMW glutenin
102	938.4869	937.4797	937.4869	-7.72	25	40		QPQEQVPL	0.33	α/β gliadin
103	861.9302	1721.846	1721.808	21.8	67	0.0041		SPQQPGQGQQLGQGQQG	0.33	HMW glutenin
104	951.4721	950.4648	949.4617	1056	22	110		QPQQPQQP	0.33	γ gliadin
105	696.8838	1391.753	1391.752	0.68	42	0.94	U	QEQQQGVPILRP	0.32	γ gliadin

106	712.3964	711.3892	711.3915	-3.36	24	25		GQGIIQP	0.32	γ gliadin
107	733.3932	732.386	732.3807	7.23	31	13		PFGVGTGV	0.32	LMW glutenin
108	909.7944	2726.361	2726.32	15.1	41	1.9	U	QPQQPFPQQPQQPFPQTQQPQQP	0.32	γ gliadin
109	950.452	1898.89	1898.891	-0.85	43	0.84	U	QQPPFSQQQQPPFSQQ	0.32	LMW glutenin
110	980.4787	1958.943	1958.939	2.12	32	13		FPHQPQQQFPQPQQPQ	0.32	γ gliadin
111	566.365	565.3577	565.3588	-1.91	6	99		AIILH	0.31	α/β gliadin
112	684.4031	683.3958	683.3966	-1.19	23	26		IPGLER	0.31	LMW glutenin
113	859.4346	2575.282	2575.282	0.03	33	13	U	QQQPPFSQQQPILPQQPPFSQQ	0.31	LMW glutenin
114	699.3368	1396.659	1396.652	4.74	29	20	U	YPHQPQQQFPQ	0.31	γ gliadin
115	1056.021	2110.027	2110.012	7.03	23	110		QPYPQPQPQYSQPQQPIS	0.31	α/β gliadin
116	612.2925	1222.57	1222.573	-2.19	39	1.8	U	QPQQTYPHQP	0.31	γ gliadin
117	681.3936	680.3863	680.3857	0.8	11	360		QPVLPQ	0.31	γ gliadin
118	867.1073	2598.3	2598.262	14.8	28	38	U	PQQPFPQQPQQPFPQTQQPQQP	0.31	γ gliadin
119	909.109	2724.305	2723.382	339	12	1500	U	PQQPFPQLQQPQQPFPQPQQQLP	0.3	γ gliadin
120	861.4344	2581.281	2580.236	405	17	560	U	QQPPFSQQQQQPLPQQPSFSQQ	0.3	γ gliadin
121	769.3842	2305.131	2305.149	-8	10	2300	U	PQQPYPQQQPQYLQPQQPI	0.3	α/β gliadin
122	923.4596	1844.905	1844.902	1.65	46	0.45		QPQNPSQQQPQEQVPL	0.29	α/β gliadin
123	952.4807	2854.42	2854.419	0.36	28	38	U	PQQPQQPFLQPQQPFPQQPQQPFP	0.29	γ gliadin

124	1033.843	3098.507	3098.5	2.31	30	24	U	QQPQQPFPQTQQPQQPFPQQPQQPFP	0.29	γ gliadin
125	909.7947	2726.362	2726.32	15.5	49	0.31	U	PQQPQQPFPQTQQPQQPFPQQPQ	0.28	γ gliadin
126	662.8065	1323.598	1323.596	2.09	45	0.43		QQPGQGQQGHYP	0.28	HMW glutenin
127	976.1599	2925.458	2925.42	12.9	-15	940	U	PFPQPQQPQQPFPQSQQPQQPFPQP	0.28	γ gliadin
128	732.3721	2925.459	2925.42	13.5	24	110	U	PFPQPQQPQQPFPQSQQPQQPFPQP	0.28	γ gliadin
129	1076.527	3226.559	3226.559	0.18	48	0.41	U	QQPQQPFPQQPQQPFPQTQQPQQPFPQ	0.28	γ gliadin
130	831.9183	3323.644	3323.611	9.83	35	8.5	U	QPQQPFPQQPQQPFPQTQQPQQPFPQQP	0.28	γ gliadin
131	1028.863	3083.567	3083.525	13.6	22	170	U	PQQPFPQTQQPQQPFPQLQQPQQPFP	0.27	γ gliadin
132	871.4375	3481.721	3481.728	-2.02	17	560	U	PQQPFPQPQQQLPQPQQPQQSFPQQQRPF	0.27	γ gliadin
133	986.1768	2955.509	2955.467	14.2	16	620	U	PQQPFPQPQQTFPQQPQLPFPQQPQ	0.27	γ gliadin
134	1038.761	4151.015	4150.009	243	28	51	U	PQQPFPQQPQQPFPQTQQPQQPFPQQPQQPFPQTQ	0.27	γ gliadin
135	817.4028	2449.187	2449.186	0.47	18	330	U	FPGQQQPFPPQQPYPQPQPFP	0.27	α/β gliadin
136	805.8707	1609.727	1609.723	2.23	45	0.44		SQQQPGQGQQGHYPA	0.27	HMW glutenin
137	721.353	720.3457	720.3443	1.99	11	1100		YPLGQGS	0.26	α/β gliadin
138	964.137	2889.389	2889.388	0.61	28	38	U	FPGQQQPFPPQQPYPQPQPFPSQQP	0.25	α/β gliadin
139	1018.493	3052.456	3052.451	1.74	30	24	U	FPGQQQPFPPQQPYPQPQPFPSQQPY	0.25	α/β gliadin
140	997.2979	4981.453	4981.469	-3.13	32	21	U	QQPFPQQPQQPFPQTQQPQQPFPQLQQPQQPFPQP QQQLPQP	0.25	γ gliadin

141	713.6755	2138.005	2138	2.13	17	390	U	QPGQGQPGQRQQPGQGQHPE	0.25	HMW glutenin
142	1011.517	3031.528	3031.515	4.36	39	3.5	U	QQPFPQPQQPQQSFPQQQPSLIQQSL	0.25	γ gliadin
143	991.493	1980.971	1980.007	487	25	73	U	SQQQQLVLPQQPPFSQQ	0.25	LMW glutenin
144	981.234	3920.907	3920.902	1.15	51	0.21	U	QPQQPFPQQPQQPFPQTQQPQQPFPQQPQQPFP	0.23	γ gliadin
145	578.3679	577.3606	577.3588	3.18	31	1.4	U	IPVIH	0.23	LMW glutenin
146	640.3051	1278.596	1278.595	0.34	50	0.14		QPQNPSQQQPQ	0.22	α/β gliadin
147	1017.005	4063.989	4063.906	20.4	12	2100	U	PQQQFPQPQQPQQSFPQQQQPAIQSFLQQQMNPC	0.2	γ gliadin
148	1102.52	1101.513	1101.509	3.5	19	150		YPTSPQQPGQ	0.19	HMW glutenin
149	1053.528	1052.521	1052.525	-3.9	17	310		QPQQPISQQ	0.18	α/β gliadin
150	866.9228	1731.831	1731.818	7.7	59	0.024		QPQNPSQQQPQEQVP	0.16	α/β gliadin
151	555.3502	554.3429	554.3428	0.22	29	2		IPVVQ	0.1	LMW glutenin

U stands for unique peptides

Mr stands for molecular mass

6.5. Conclusion

There are significant differences in the proteolysis of gluten by the investigated enzymes. Flavourzyme was the best of all in terms of both producing highest proteolysis and yield of opioid peptides. Hydrolysis of gluten by pepsin, alcalase and flavourzyme released peptides with opioid activity in the order: flavourzyme > alcalase > pepsin. This suggests that proteolysis by flavourzyme should be investigated in future at conditions other than those presented in this study to identify optimal reaction conditions for maximal yield of opioid peptides. The results of this study also suggest that wheat gliadin can be easily hydrolysed using enzymes and peptides produced from gliadin were of higher bioactivity ranking. The higher opioid activity, as evident from lowest EC_{50} values of the flavourzyme hydrolysate, appears to be due to the presence of a large number of peptides having higher ranking. Peptides, PQQPFPL from pepsin hydrolysate and, QQPPFW and QPFPQPQPFP from flavourzyme hydrolysate, should be further explored for determination of opioid activity as their peptide ranking was greater than or equal to 0.9. Enzymatic hydrolysis of wheat gluten showed both increased proteolysis and peptides having increased opioid activity compared to fermentation approach (**Chapter 4**). It was noticed throughout the experimentation that glutenin was not completely dispersed and this may have affected the proteolysis and yield of opioid peptides. Gluten is a complex protein and is known to have poor dispersion in water affecting its functionality. Dispersion of gluten and how it is affected by varying pH and heat treatment was investigated and the results are reported in the next chapter.

Chapter 7. Effect of heating and acidic pH on characteristics of wheat gluten suspension

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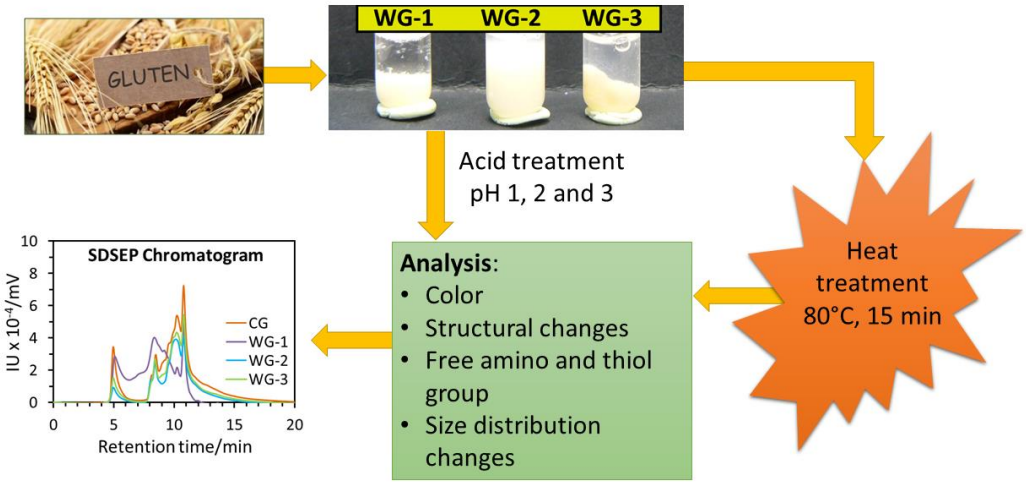

7.1. Summary

In the previous chapter, it was realized that accessibility of glutenin by proteolytic enzymes may have limited the proteolysis and the yield of opioid peptides. Considering this fact, this chapter investigates the effects of different pH treatments with and without heating on the characteristics of wheat gluten suspension. At pH 1, maximum changes in colour were observed with a concurrent 65% decrease in free-thiol content compared to the control gluten. The SDS-Extractability of protein (SDS-EP) chromatogram eluted at lower retention time and the presence of bands at the top lane even during reducing conditions in SDS-PAGE gel suggested complex formation involving bonds other than disulphides. An increase in the free-amino group content and the presence of an additional peak at a higher retention time in the SDS-EP chromatogram was suggestive of hydrolysis. At pH 2 and 3, similar decreases in SDS-EPs and free-thiol content indicated formation of complexes. When heated, the free-thiol content of the dispersions increased compared to the non-heated dispersions indicating disruption of disulphide bonds with changes in gluten structure and size distribution.

7.2. Introduction

Wheat gluten (WG) is a heterogeneous mixture of gliadin and glutenin protein subunits which is produced as a by-product of the wheat starch industry. It is widely used in a range of food (bakery products) as well as non-food applications which includes production of bioplastics (Langstraat, Jansens et al. 2015) through to animal feeds and adhesives (Day, Augustin et al. 2006). Gluten can be hydrolysed to produce bioactive peptides with anti-oxidant, anti-microbial, anti-cancer, opioid or anti-hypertensive activities (Korhonen and Pihlanto 2003, Shahidi and Zhong 2008, Zhu, Su et al. 2011, Janković, Barać et al. 2015, Garg, Nurgali et al. 2016, Wu, Zhang et al. 2016). Enzymatic hydrolysis is the preferred method for the production of bioactive peptides and the extent of hydrolysis depends on the number, nature of amino acids and their sequence. The conformational differences associated with gluten proteins, availability of cleavage sites (Liao, Wang et al. 2012), product inhibition of enzymes, enzymatic specificity and autopeptidolysis of enzyme (Giesler, Linke et al. 2014) reduce hydrolysis of gluten and consequent redution in the yield of peptides.

The inherent complexity and poor water dispersion of WG limits its use for various applications. Poor dispersion results from the presence of high concentrations of proline, leucine, and glutamine and low concentrations of lysine, arginine, glutamic acid and aspartic acid (Yildiz 2009). Heat treatment can change physico-chemical and viscoelastic properties of gluten (Falcão-Rodrigues, Moldão-Martins et al. 2005, Lagrain, Thewissen et al. 2008) via cleavage of intra-molecular disulphide (S-S) bonds and formation of inter-molecular S-S bonds (Lagrain, Thewissen et al. 2008). Cross-linking start with glutenins, and at higher

temperatures, gliadin also gets incorporated into the network (Singh and MacRitchie 2004), which decreases protein extractability due to the formation of aggregates. Improvement in the yield of peptides of >10 kDa were observed when thermally treated gluten was hydrolysed by papain (Wang, Wei et al. 2009). Functional properties of gluten normally improve in both acidic and alkaline pH range away from isoelectric point of gluten. At alkaline pH, the formation of lanthionine (Jansens, Lagrain et al. 2013) and lysinoalanine (Rombouts, Lagrain et al. 2010) leads to more aggregates due to crosslinking. On the other hand, the ability of gluten to bind with other polymers is favoured under acidic pH as a result of reduced cross-linking (Langstraat, Jansens et al. 2015) paving way for application of modified gluten in bioplactics. Reddy and Yang (2007) reported development of gluten based fibre with improved mechanical properties similar to that of wool. Other non-food applications of modified gluten are as medical bandages (Quimby Jr, Birdsall et al. 1994) and biodegradable substitute for polylactic acid fibres (Li, Reddy et al. 2008).

Hydrothermal treatment of gluten in acidic solutions improves functional properties of gluten including solubility, emulsifying and foaming properties (Liao, Zhao et al. 2010). The extent of these changes depends on the balance between intra-molecular electrostatic repulsion and the non-covalent and S-S bond formation (Liao, Liu et al. 2011). Consequently, more amino acids are exposed for binding to enzymes and increasing susceptibility to enzymatic hydrolysis (Liao, Wang et al. 2012) required for increasing the yield of bioactive peptides. When used as pre-treatment, increased hydrolysis of gluten by pancreatin increased number of peptides with MWs below 3000 Da (Cui, Hu et al. 2013). Similarly, thermal

pretreatment improved the yield of peptides with higher antioxidant activity using gluten as a substrate and alcalase as proteolytic enzyme (Elmalimadi, Jovanović et al. 2017). Hydrothermal treatment of gluten in acidic solution improves molecular flexibility (Liao, Han et al. 2016). Use of high temperature and acidic concentration hydrolyse peptide bonds, thus impairing the protein quality, however, moderate acid concentration improves emulsifying and foaming properties (Liao, Han et al. 2016). Knowledge of enhancing physicochemical, structural and functional properties of wheat gluten as affected by various treatments is necessary for extending the uses of gluten, which is cheap and readily available natural protein.

Hence, this study was carried out to investigate the changes in WG in hydrochloric acid at different pH with and without heating in order to optimise the treatment conditions for improved dispersibility. The changes in the structure of the protein, size distribution, free-thiol content, and amino content were determined to evaluate protein aggregation and hydrolysis.

7.3. Materials and methods

7.3.1. Materials

Wheat flour with a protein content of 12.5% was obtained from Allied Mills (Australia). Hydrochloric acid (HCl) was purchased from Merck (Melbourne, Australia); mini-PROTEAN TGX gel (12%), 10X Tris/Glycine/SDS buffer and 2X Laemmli sample buffer were obtained from Bio-Rad (Melbourne, Australia); o-phthalaldehyde (OPA), methionine, 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB), glutathione and all other chemicals were from Sigma Aldrich (Steinheim, Germany).

7.3.2. Preparation of gluten and heat treatment

Gluten was extracted from the wheat flour by hand-washing using AACC method 38-10, 2000 followed by freeze drying and grinding. The powder was passed through a 212 µm sieve and its protein content was 75% (conversion factor N × 5.7) as measured by Kjeldahl (Qiu, Sun et al. 2014). Gluten powder (400 mg) was dispersed in 5 mL aqueous HCl solution at pH 1, 2 and 3 in glass tubes to prepare 8% gluten dispersions which were sealed and shaken thoroughly to obtain homogenous dispersions named WG-1, WG-2, WG-3, respectively. Another set of samples at pH 1, 2 and 3 were heated in an oil bath set at 80ºC for 15 min and were designated WG-1a, WG-2a and WG-3a, respectively. Each sample was then freeze-dried using an Alpha 1–4 LSC freeze dryer (Martin Christ, Germany) before further analysis. All samples were prepared and analysed in triplicate.

7.3.3. Colour measurement

A portable Konika Minolta (Tokyo, Japan) Chroma Meter CR-300 with illuminant D65 and a 2° standard observer was used to measure the colour. A measuring head area of 8 mm diameter was used with diffuse illumination and 0º viewing angle, and a white chromameter standard tile (L = 97.47, a = 0.13, b = 1.83) was used for calibration. Samples were placed on the white standard plate to measure the CIE $L^*a^*b^*$ colour values which were taken in triplicate.

7.3.4. SEM of treated gluten

Images were obtained using a JOEL NeoScope SEM (JCM-5000, Frenchs Forest, NSW, Australia). Freeze-dried samples were mounted on an aluminium sample holder and coated with gold for 6 min using JOEL NeoCoater

(MP19020NCTR). The images were acquired at a scale of 10 µm under high vacuum using an accelerating voltage of 10 kV.

7.3.5. FTIR analysis of treated gluten

The FTIR absorbance spectra were acquired using Perkin Elmer Frontier™ FTIR spectrophotometer (Waltham, USA) in attenuated total reflectance (ATR) mode using a diamond ATR crystal. Background spectra were recorded at the beginning of the measurements with a blank diamond ATR cell. For each sample, 16 scans were collected at a resolution of 4 cm^{-1}. For determination of the quantitative changes in secondary structure of the proteins, deconvolution of spectrum was performed using Origin Pro 8.5 software. The spectra were analysed by taking second derivatives and applying the "fit peak function", an option available within peak analyser feature of the software, in the amide I region (1700-1600 cm^{-1}). Assignment of bands in the de-convoluted spectra was as follows: the bands located at 1611-1600 cm^{-1} were assigned to hydrated extended chains, 1640-1619 cm^{-1} and 1690-1670 cm^{-1} to β-sheets, 1670-1660 cm^{-1} to β-turns, 1660-1650 cm^{-1} to α-helix, and 1645 cm^{-1} to random coil (Ukai, Matsumura et al. 2008, Wang, Luo et al. 2016). The secondary structures were determined from the relative areas of the individual assigned bands according to Ukai et al. (2008). The results are expressed as means of three independent replicates and the statistical significance of difference was determined at 5%. Tukey's test was used for statistical comparison of means using Minitab, version 7.

7.3.6. SE-HPLC analysis of treated gluten

The SE-HPLC analysis was performed using a Prominence-i, LC-2030C instrument (Shimadzu, Kyoto, Japan). Samples were prepared by dissolving 1 mg of sample in 1 mL of 50 mM sodium phosphate buffer (pH 6.8) containing 2% SDS (extractability buffer) for 60 min followed by centrifugation (20 min at 10,000 g) (Langstraat *et al*., 2015). The supernatant (10 µL) was loaded onto a Yarra SEC 3000 column (Phenomenex, Torrance, United States) and eluted with 50 mM sodium phosphate buffer (pH 6.8) containing 300 mM NaCl at flow rate of 0.4 mL/min with detection set at 214 nm. The elution profiles were divided into higher and lower MW protein fractions that were identified as SDS-extractable glutenin and SDS-extractable gliadin, respectively (Jansens, Lagrain et al. 2013).

7.3.7. SDS-PAGE analysis of treated gluten

Samples were dissolved in the SDS buffer (10 µg/µL) and mixed with a volume of 2× sample buffer containing β-mercaptoethanol followed by heating at 95°C for 5 min for the reducing SDS-PAGE analysis. For the non-reducing SDS-PAGE analysis, all the conditions were the same except that the sample buffer did not contain β-mercaptoethanol and it was not heated. Samples were then loaded on the gel (20 µL) and electrophoresis was conducted at 200 V for 45 min. The gels were stained using 0.15% Coomassie Brilliant Blue R250 dye in 72% isopropanol and 3% acetic acid for 1 h followed by overnight destaining (10% isopropanol, 10% acetic acid) on a shaker. The gel images were captured using a Chemidoc MP imager (Bio-Rad).

7.3.8. Primary amine analysis using OPA method

Samples of 10 mg were suspended in 1 mL of 50 mM sodium phosphate buffer (pH 8.3). The OPA reagent was prepared by combining 25 mL of the sodium phosphate buffer, 2.5 mL of sodium dodecyl sulphate (20% w/v), and 40 mg of OPA dissolved in 1 mL of methanol, and 100 µL of β-mercaptoethanol and making the volume to 50 mL with water. Sample solutions of 700 µL were added to a test tube containing 700 µL of OPA reagent and the absorbance was measured at 340 nm after 2 min using a Nova spec-II spectrophotometer (Pharmacia Biotech). Buffer was used as blank and the concentration of free-amino groups was determined using methionine as a standard.

7.3.9. Free-thiol group analysis

Samples prepared for primary amine analysis were also used for the determination of free-thiol using DTNB (Wang *et al*., 2016). The reagent was prepared by mixing 4 mL DTNB (2 mg/mL in n-propanol) and 20 mL of sodium phosphate buffer (pH 8). A sample of 100 µL was mixed with an equal volume of DTNB reagent, shaken for 1 hour and the absorbance was measured at 415 nm using iMark™ Microplate Absorbance Reader (Bio-Rad, Australia). Buffer containing DTNB was used as blank and the concentration of free-thiol was determined using glutathione as a standard.

7.4. Results and discussion

7.4.1. Effect of pH on gluten dispersion and colour

Differences in the dispersibility of WG were observed visually at the different pH tested. The WG-2 sample appeared to be a more homogenous compared to WG-1 and WG-3, both of which settled after shaking. The freeze-dried WG-1 was pale yellow in colour and due to the formation of chromophores as compared to WG-2 and WG-3 which were white. The colour parameters of the freeze-fried powders are presented in **Table 7.1** which shows the lightness (L^*), redness (a^*) and yellowness (b^*) values of CG and treated sample powders. Compared to the CG, the maximum changes in L^*, a^* and b^* values for WG-1 were -8.68, +2.7 and +12 respectively, indicating a significant shift to a more yellow colour. Micard, Belamri et al. (2000) reported significant reduction in L^* and increases in both a^* and b^* values upon heating gluten films to temperature >95°C.

Table 7.1: Colour values (L*, a* and b*) of WG powder as affected by pH

	L*	***a****	***b****
CG	90.03 ± 0.9^{b}	-0.91 ± 0.1^{b}	13.3 ± 2.1^{b}
WG-1	81.35 ± 0.9^{c}	1.78 ± 0.6^{a}	25.27 ± 1.3^{a}
WG-2	93.5 ± 1.2^{a}	-0.68 ± 0.1^{b}	6.4 ± 1.6^{c}
WG-3	89.82 ± 0.5^{b}	-1.20 ± 0.2^{b}	13.15 ± 0.6^{b}

Values are expressed as the mean ± standard deviation. Within a column, different lowercase letters (a–c) indicate a significant difference ($P < 0.05$) based on the Tukey's test.

7.4.2. Effect of pH and heating on structural changes

Figure 7.1 shows the SEM images of the CG and acid treated samples with the CG showing the presence of large solid particles rather than a defined network structure. Conversely, the treated samples showed larger gluten agglomerates that may have resulted via disulphide bond leading to formation of cross-links (Wang, Luo et al. 2016). Interestingly, WG-2 appears to be a more porous and open network structure compared to WG-1 and WG-3, due to differences in hydrophobic interactions existing at the different pH.

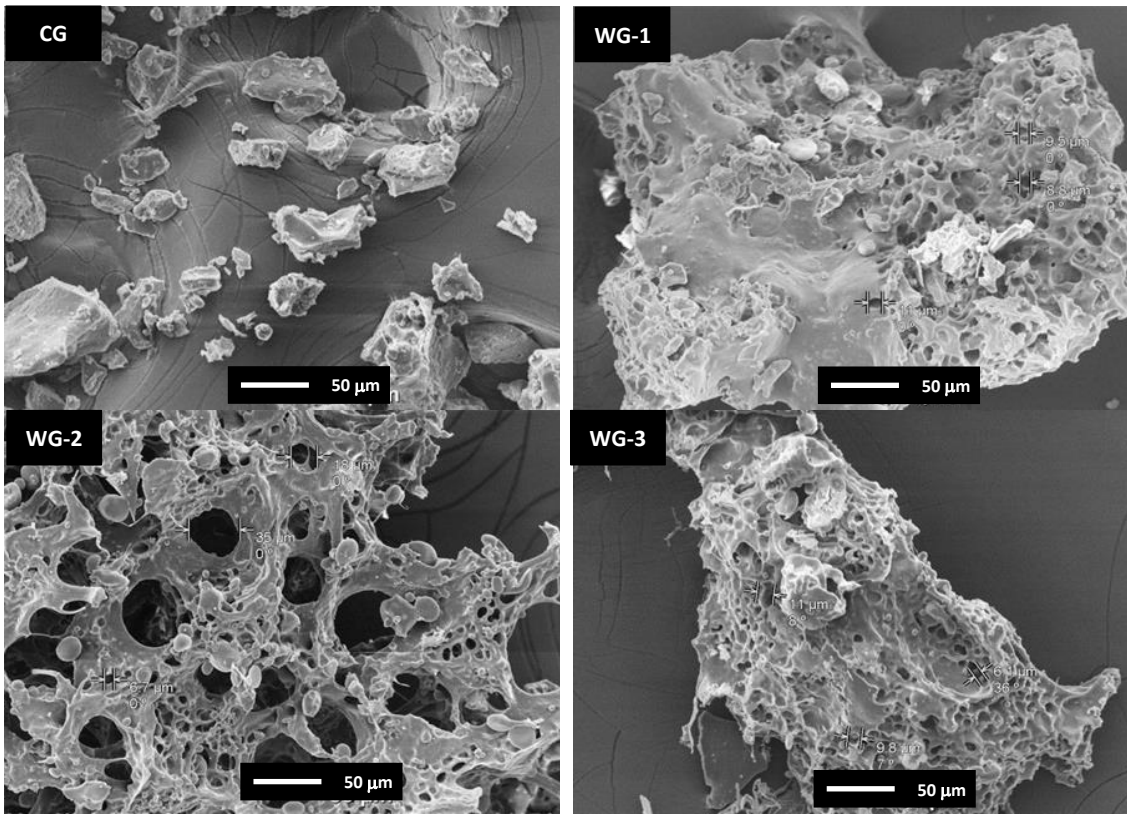

Figure 7.1: SEM images of CG and acid treated WG samples at pH 1 (WG-1), 2 (WG-2) and 3 (WG-3).

The structures of the WG samples dispersed at different pH were significantly different from the CG as shown in the FTIR spectra in Figure 7.2 (a) and (b) for samples without and with heat treatment, respectively. At pH 1, -OH peak shifts were observed at *ca*. 3280 cm^{-1} which were not observed for WG-2 and WG-3, whereas after heat treatment, shifts were observed for both WG-1a and WG-3a. The 3065 cm^{-1} peak present in the CG representing aromatic -CH bonds shifted to 3062 and 3059 cm^{-1} in WG-1 and WG-1a, respectively. The peak at 3011 cm^{-1} due to C=CH alkene bonds present in the CG decreased in intensity in WG-3 and completely disappeared in WG-1 and after heat treatment, the same peaks disappeared in WG-1a and WG-2a samples. The -CH peak at 2856 cm^{-1} present in the CG decreased in intensity in WG-3, WG-2 and WG-1 samples and after heat treatment; the peak almost disappeared in WG-1a. The peak present at 1744 cm^{-1} in the CG due to carbonyl stretching significantly broadened and the maxima shifted to 1723 cm^{-1} in WG-1 and WG-1a samples suggesting an increase in carbonyl bonds and could be due to formation of chromophores imparting yellowness at pH 1.

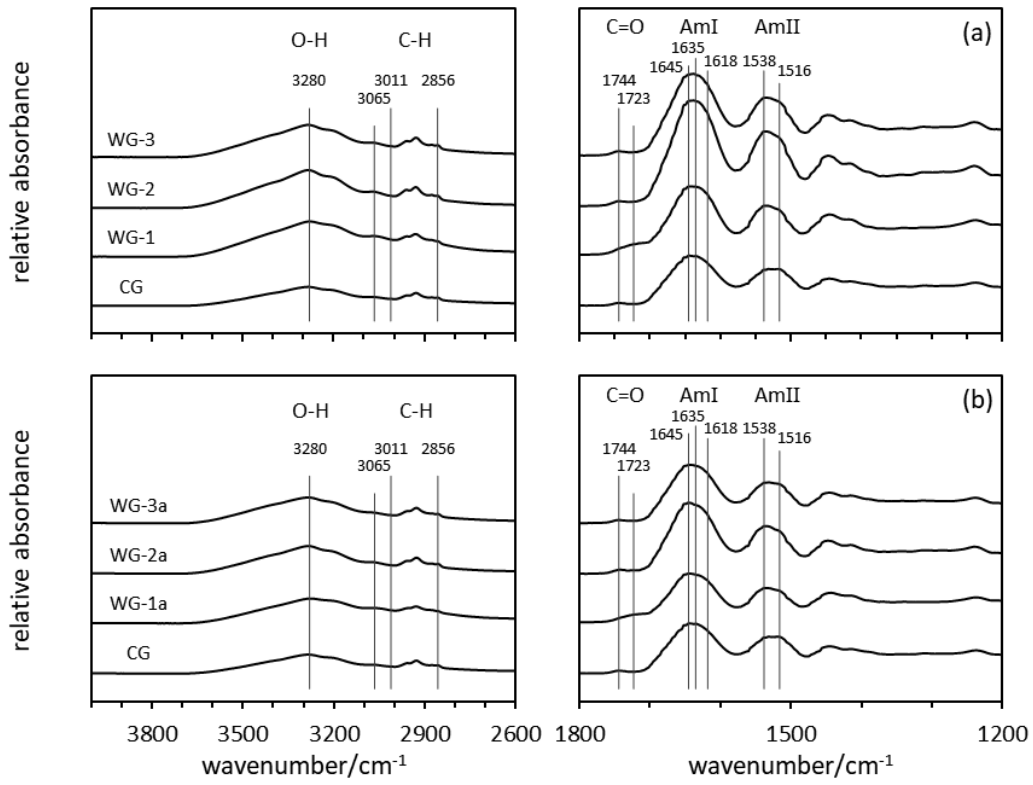

Figure 7.2: FTIR spectra of CG and acid treated WG samples at pH 1 (WG-1), 2 (WG-2) and 3 (WG-3) before (a) and after (b) heat treatment.

The amide I and II regions were further analysed to investigate the differences in the secondary structure of gluten. In the amide I region (1700-1600 cm^{-1}), the peak maxima at 1645 cm^{-1} in the CG shifted to 1640, 1639 and 1638 cm^{-1} in the WG-1, WG-2 and WG-3 samples, respectively. The relative intensities of the peaks at 1635 cm^{-1} decreased whereas those at 1631 and 1618 cm^{-1} increased for all pH treated samples compared to the CG. The amide II (1600-1480 cm^{-1}) region of the CG showed various characteristic peaks which were also present in WG-1, WG-2 and WG-3, but with differences in relative intensities. The CG sample showed peaks at 1540-1516 cm^{-1} with similar relative peak heights, however, all treated samples showed the peak at 1538 cm^{-1} was relatively higher than that at 1516 cm^{-1} in all cases thus showing narrowing of the peaks. Moreover, the peak at 1525 cm^{-1} present in the CG disappeared in all pH treated samples.

The changes in the amide regions are consistent with changes in protein secondary structures as a result of the pH treatment and support the train and loop model which attributes β-sheet conformational changes to hydration (Belton 1999). According to the model, repetitive domains of WG are rich in glutamine residues and tend to form intra- and inter-molecular hydrogen bonds which are associated with β-sheet and described as a "train". Under acidic conditions, glutamine residues are converted into negatively charged glutamic acid thus increasing electrostatic repulsion among the β-sheets and forcing them into an "unzipped form". This creates unbounded mobile regions described as a "loop", which could involve the more flexible β-turn, α-helix and random coils (Belton 1999, Yong, Yamaguchi et al. 2006).

Deconvolution of FTIR spectra is used to analyse quantitative changes in the protein secondary structures. **Figure 7.3** shows the de-convoluted FTIR spectra in the amide-1 (1700-1600 cm^{-1}) region of control and acid treated gluten using FTIR spectroscopy with deconvolution performed on each spectrum in the amide I region. The differences in the secondary structures of WG on treatment at acidic pH are presented in Table 7.2**.** Different pH treatments did not significantly affect the relative concentration of hydrated extended chains (6.02%), β-sheets (32.95%) and β-turns (22.56%) ($p>0.05$), however, acid treated gluten showed lower relative concentration of α-helical structure (13.9–19.7%) and higher relative concentration of random coils (19.6-29.2%) as compared to CG, suggesting deamidation (**Table 7.2**). A reduction in α-helical structure and increase in unordered structures as a result of deamidation has also been reported by Wong, Zhai et al. (2012) previously.

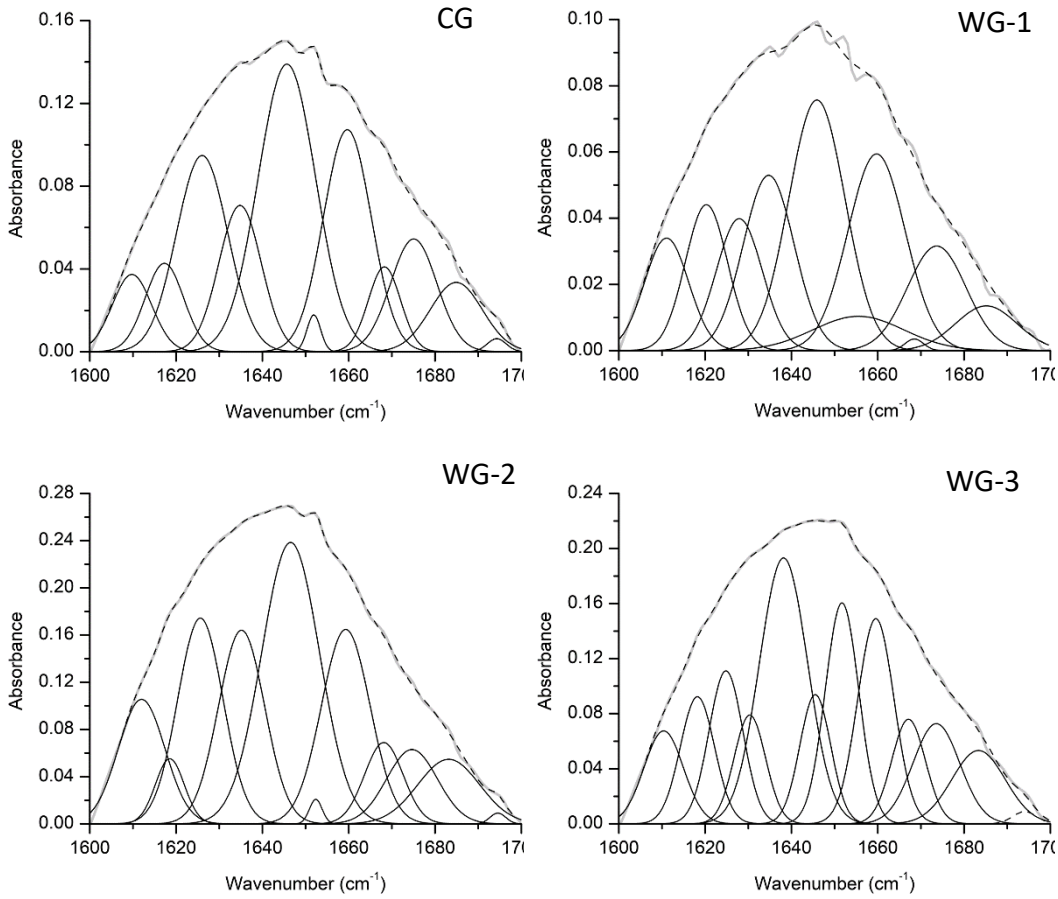

Figure 7.3: De-convoluted FTIR spectra in the amide-1 region of control gluten (CG) and acid treated samples at pH-1 (WG-1), 2 (WG-2) and 3 (WG-3)

Table 7.2: Relative concentration of secondary protein structures of WG at different pH by FTIR

	Hydrated extended chains	β-sheets	β-turns	α-helix	Random coils
CG	5.9±1.8[a]	34.0±1.4[a]	22. 5±0.6[a]	24.2±2.5[a]	13.4±2.9[a]
WG-1	6.4±0.3[a]	32.2±0.1[a]	22.0±1.9[a]	19.7±0.6[ab]	19.6±0.9[ab]
WG-2	6.1±0.1[a]	31.0±0.6[a]	19.5±0.6[a]	20.2±0.6[ab]	23.2±0.6[bc]
WG-3	4.9±1.3[a]	31.8±1.1[a]	20.1±0.2[a]	13.9±0.7[b]	29.2±0.5[c]

Values are expressed as the mean ± standard deviation. Within a column, different lowercase letters (a–c) indicate a significant difference ($P < 0.05$) based on the Tukey's test.

7.4.3. Changes in free-thiol and amino groups

The free-amino group content was determined to monitor changes in -NH_2 chain ends (α-NH_2 at the protein chain ends and ϵ-NH_2 from lysine residues) arising from protein hydrolysis and the results are presented in **Figure 7.4(a)**. The primary amine content of CG was found to be 140.2 µM/g protein which is in agreement with that reported by Langstraat, Jansens et al. (2015). The free-amino content of WG-1 and WG-2 was higher than the CG whereas that of WG-3 was lower ($P < 0.05$) with the increased free-amino content in WG-1 due to protein hydrolysis and changes in the conformation of gluten exposing more amide groups from within the structure. The lower free-amino content of WG-2 and WG-3 suggested a lower degree of hydrolysis and associated conformational changes. Heat treatment (80°C for 15 min) at different pH decreased the free-amino content in comparison to samples without heat treatment at the same pH **Figure 7.4(a)** due to increased deamidation converting glutamine and asparagine into glutamic and aspartic acids, respectively.

The free-thiol group content plays a very important role in the binding of the hydrated gluten network by intramolecular and intermolecular disulphide bonds. The free-thiol group contents of WG-1, WG-2 and WG-3 were significantly lower than 176.6 µM/g protein found of CG ($p<0.05$) **Figure 7.4(b)**. This suggests involvement of free-thiol groups in the cross-linking of gluten proteins (Lagrain, Thewissen et al. 2008). The results are in agreement with SEM images where the gluten network was more pronounced in acid treated samples compared to the control. The higher free-thiol content of WG-1 compared to WG-2 and WG-3 indicates that higher acidity leads to a greater exposure of free-thiol groups. The

free-thiol contents of the heat-treated samples were higher compared to the unheated samples at the same pH due to further breakage of disulphide bonds by heat (**Figure 7.4(b))**.

7.4.4. Changes in SDS-extractability and molecular weight distribution of protein

The SDS extractability of gluten is an indicator of the degree of cross-linking whereby a decrease in SDS extractability is indicative of increased cross-linking. **Figure 7.5** shows the SDS-EP chromatogram of the CG and treated samples which suggest that in WG-1, the relative protein content decreased in the gliadin region (7.5–15 min) and increased in the glutenin region (4.7–7.5 min). The decrease in the gliadin peak at 10.5 min and presence of another broad peak between 4.7 and 7.5 min indicates a protein complex is formed involving gliadin in WG-1. The chromatogram also shows a very small peak at 13.5 min indicating hydrolysis has occurred which is in accordance with the higher concentration of free-amino groups (**Figure 7.4 (a)**). In WG-2 and WG-3, the relative concentrations of proteins at different retention times were not significantly affected compared to the CG sample, however, the SDS extractability decreased due to the formation of intermolecular cross-linking rendering protein un-extractable (Lagrain, Rombouts et al. 2011).

Also shown in **Figure 7.5** are the differences in the SDS-EP chromatograms of samples after heat treatment at different pH. Disappearance of the peak at 5 min and decreased peak intensity at 11 min were evident in all heat-treated samples. In WG-1a, peaks are present at 7.6 and 9 min, however in WG-2a and WG-3a, the

peaks eluted at higher retention times of 8.2 and 9.6 min indicating formation of cross-links in WG-1a. The presence of a small peak between 13-14 min in pH 1 treated samples (both heated and non-heated) and absence of peaks in samples at pH 2 and 3 suggest increased hydrolysis at pH 1 in accordance with the higher free-amino group content (**Figure 7.4(a)**), while no such peak appeared in samples at pH 2 and 3.

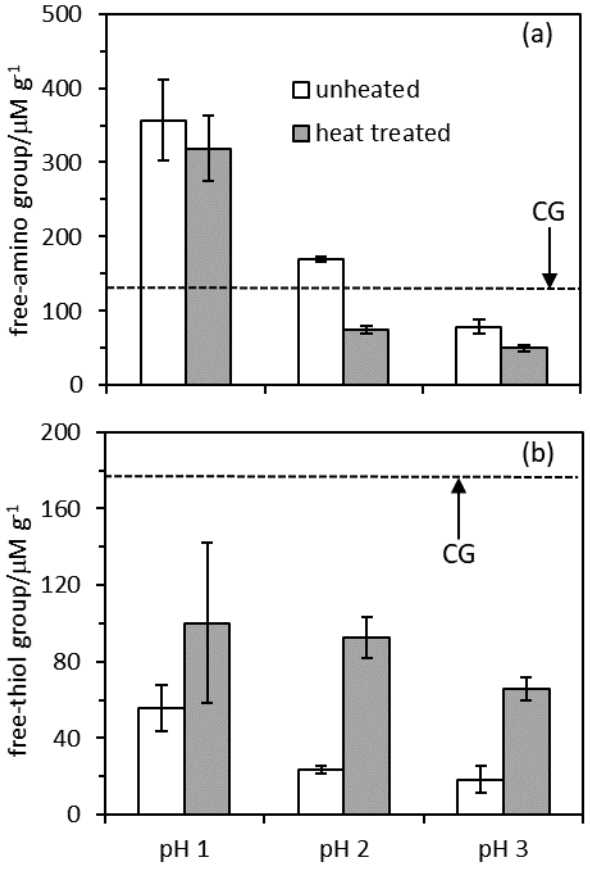

Figure 7.4: Effect of pH on (a) free-amino content and (b) free-thiol content of CG and acid treated WG with and without heat treatment. Values are μM/g of protein.

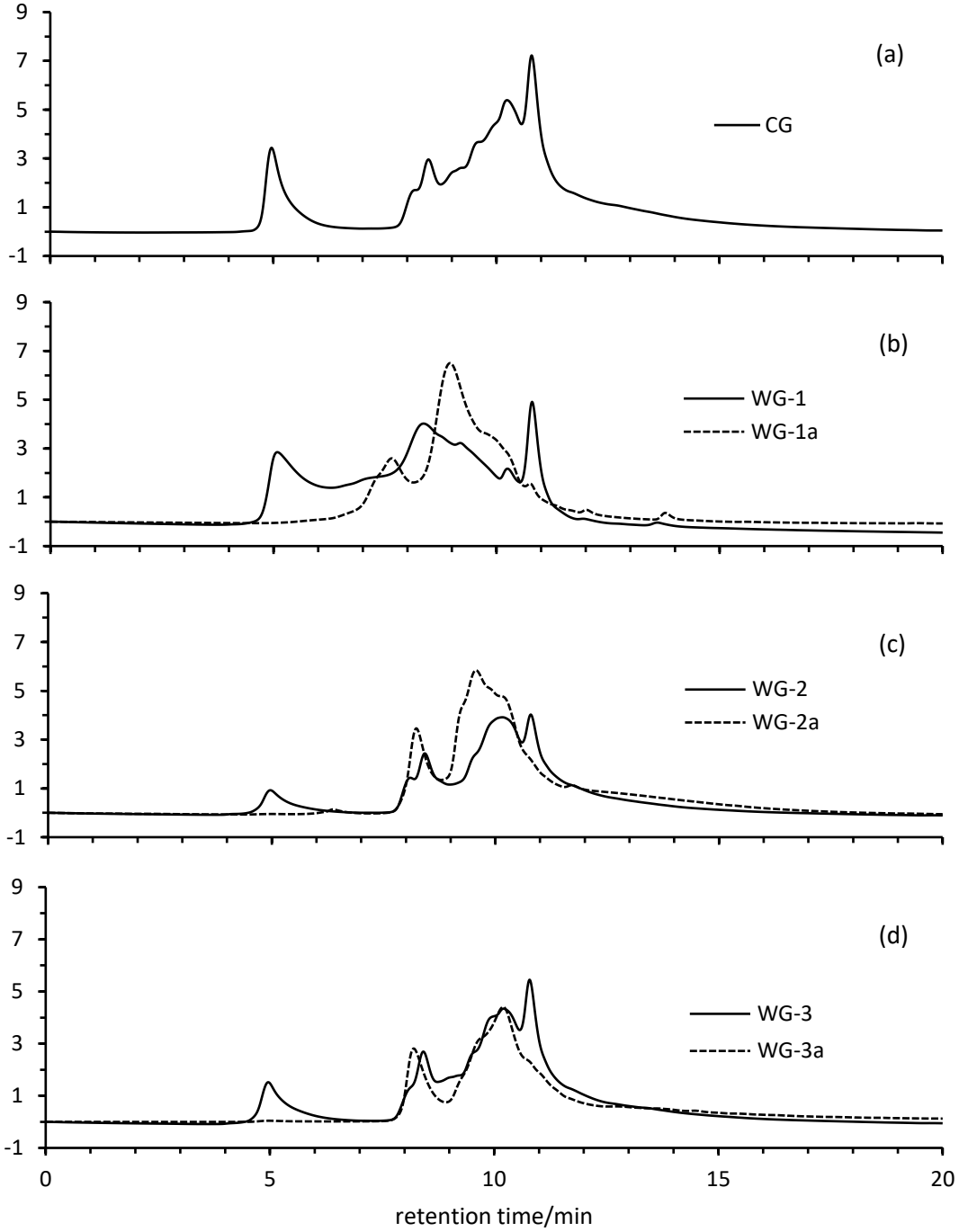

Figure 7.5: SDS-EP chromatograms of CG and acid treated WG at pH-1 with and without heat treatment.

Figure 7.6**(a)** presents the SDS-PAGE gels under non-reducing conditions. Protein bands at the top of each lane in all samples (heated and non-heated) are due to the presence of high MW protein complexes that could not penetrate the gel. Characteristic bands of gluten present in the CG sample were absent in WG-1 and WG-1a and a smear was present between 37 and 50 kDa. A smear at the end of the lane for WG-1a may be due to protein hydrolysis products. However, at pH 2 and 3 for both heated and non-heated samples, the distribution of bands are similar to the CG. These results confirm patterns similar to the SDS-EP chromatogram (**Figure 7.5**).

Under reducing conditions (Figure 7.6**(b)**), the CG bands identified are corresponding to high MW glutenins (HMW-GS), low MW glutenins (LMW-GS) (group B and C); α-, β-, γ- and ω-gliadins, and α-amylase trypsin inhibitors (Liao, *et al*., 2016a). When compared to the non-reducing conditions, the bands in the top of lane in WG-2, WG-2a, WG-3 and WG-3a samples disappeared but these were present in WG-1 and WG-1a under reducing conditions (Figure 7.6**(a and b)**). This suggests that the complex formed at pH 1 involves covalent bonds other than disulphide which cannot be reduced by β-mercaptoethanol and these may involve tyrosyl–tyrosyl cross-links (Tilley, Benjamin et al. 2001, Pena, Bernardo et al. 2006). The bands corresponding to different proteins (glutenins, gliadins and α-amylase trypsin inhibitors) were present at same position as for pH 2 and 3 samples irrespective of heat treatment. In WG-2 and WG-2a, the intensity of the bands decreased, but this was not observed in WG-3 and WG-3a. These results suggest involvement of protein aggregation and hydrolysis due to heating as reported previously using citric acid with temperatures >90°C (Liao *et al*., 2016b).

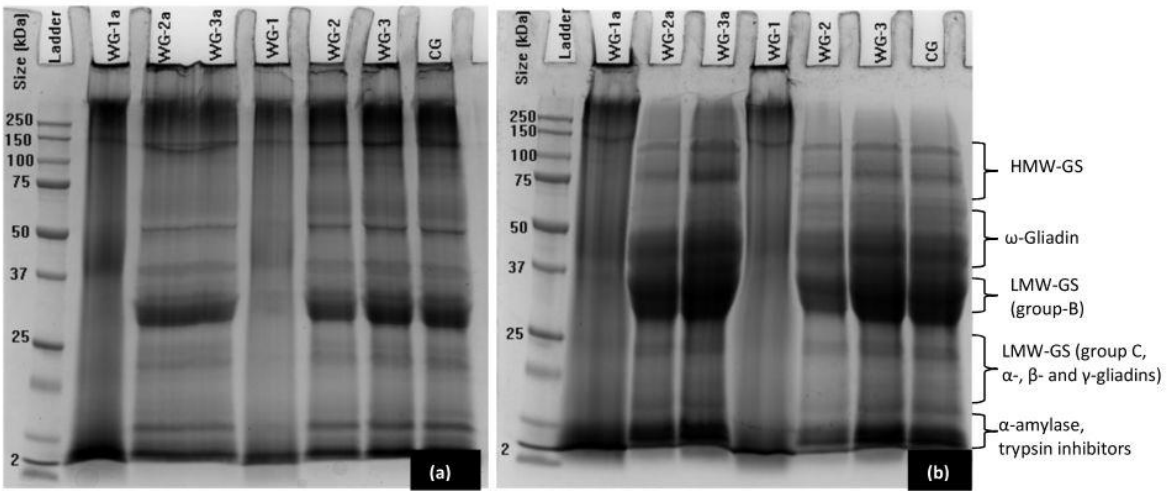

Figure 7.6: SDS-PAGE patterns of CG and acid treated WG with and without heat treatment under (a) non-reducing and (b) reducing conditions.

7.5. Conclusions

This study confirms that suspending WG in aqueous solutions at acidic pH (1, 2 and 3) changes the structure, free functional groups and size distribution of the gluten. At pH 1, significant change in colour, SDS extractability, free-amino content, and thiol content was observed. At higher pH (2 and 3), decreased SDS extractability, decreased free-thiol and free-amino content confirmed lower hydrolysis when compared to samples treated at pH 1. These changes were more pronounced upon heating (80°C for 15 min). In addition to higher hydrolysis at pH 1, the presence of SDS-PAGE bands also confirmed high MW complex formation due to cross-linking. Further research is needed to confirm whether these differences in the structure and size distribution noticed as a result of hydrothermal treatment of gluten at different pH increase or decrease susceptibility of gluten to increased hydrolysis using enzymes and fermentation by lactic acid bacteria to improve proteolysis and yield of opioid peptides.

Chapter 8. General conclusion and future directions

This chapter provides general conclusion of the thesis and recommendations for future work.

Traditional opioids such as morphine and codeine are effective in pain management in patients but cause addiction, sedation, dependence and respiratory depression. Opioid peptides can be a suitable alternative to avoid these effects and assist in more effective pain management. These peptides can be sourced from food proteins which are available readily and relatively cheaper to produce. Plant sources of proteins such as grains can be used for this purpose and this value adding is expected to increase the profitability of agriculture business.

Australia produces about 25 million tonnes of wheat annually and 60 to 85% of production is exported mainly for food and feed applications. The Australian wheat industry is continuously seeking innovative approaches to add value to wheat produced to improve profitability of agriculture industry. Besides its main use as a food and feed crop, wheat is used for production of starch commercially. The total protein content of wheat is in the range of 9 to 14% and consists of soluble protein (albumin and globulin) and insoluble gluten protein. Gluten is a wheat storage protein consisting of gliadins and glutenins and is considered as waste by wheat starch industry. Gliadin and glutenin represent significant differences in composition and functionality. Being readily available, gluten from wheat is of interest for research as a protein source for production of peptides of various bioactivities. This thesis presented results of investigating aspects of gluten with a view to produce peptides that demonstrate opioid activity.

Based on the comprehensive literature review of opioid peptides from food proteins (**Chapter 2)**, the objective of the thesis formulated was to demonstrate and assess whether wheat gluten can be used as a protein source for production of opioid peptides. In pursuit of this objective, the presence of opioid peptides in wheat gluten

was assessed first using *in silico* approach. This was followed by hydrolysis of gluten using lactic acid bacteria commonly used in sourdough fermentation and commercially available proteolytic enzymes. The opioid activity of these hydrolysates and their corresponding less than 3 kDa fractions were assessed. In addition, characterisation of gluten dispersion in acid conditions and controlled heating conditions in water were reported.

It is known from literature that most of opioid peptides should contain tyrosine and proline within them. Keeping this in mind, opioid peptides were identified from wheat gluten on the basis of their amino acid composition, their sequence and higher peptide ranking using bioinformatics approach. Three peptides, YPG, YYPG and YIPP were identified, synthesized and tested for having opioid activity using adenylate cyclase assay and their EC_{50} values were 1.78 mg/mL, 0.74 mg/mL and 1.42 mg/mL. YYPG showed significant opioid activity, and therefore their presence in hydrolysates was used as determinant of opioid peptides. Thus *in silico* approach, a cost effective approach was successfully proved to be useful to screen opioid peptides from protein sources and confirming wheat gluten to be an important protein source for production of these peptides. This form the basis for investigating various methods for hydrolysis of wheat gluten for production of opioid peptides. Two main approaches studied are fermentation by lactic acid bacteria used in sourdough fermentation and commercial proteolytic enzymes.

Lactic acid bacteria have been used in food fermentation for several years. These are used in dairy fermentation for production of yogurt and cheeses, and regarded to be safe and healthy. These are also used for production of sauerkraut

and certain type of pickles. In addition to this lactic fermentation is a major contributor to production of sourdough bakery products. Fermentation by lactic acid bacteria has shown to release bioactive peptides with different functionality from wheat proteins and release of opioid peptides was tested in this thesis. Wheat flour was fermented by selected lactic acid bacteria which are part of sourdough. Focus in this study was to use them for their proteolytic activity. *Lb. acidophilus, Lb. brevis, Lb. fermentum* and *Lb. plantarum* showed proteolysis and increased production of opioid peptides enhanced opioid activity of hydrolysates. Considerable differences in the proteolysis and the production of opioid activity among the bacteria used were noticed. The opioid activity as expressed in terms of EC_{50} of <3 kDa fractions of hydrolysates obtained using *Lb. acidophilus, Lb. brevis, Lb. fermentum* and *Lb. plantarum,* was found to be 6.3, 7.2, 4.9 and 4.3 mg/mL, respectively. *Lb. plantarum* was the most efficient in releasing opioid peptides from wheat proteins as shown in chapter 5. LAB bacteria contain different proteolytic enzymes. The release of opioid peptides therefore was dependent on synergistic or antagonistic effects that are known are specified influencing the yield of opioid peptides and thereby bioactivity. Hence, commercial proteolytic enzymes which are much more specific, were investigated.

Selected commercially enzymes, pepsin, trypsin, alcalase and flavourzyme were investigated for production of opioid peptides from wheat gluten. The results of this study indicated differences in the activities of enzymes even when the reaction conditions used were corresponding to their maximum activity. Flavourzyme proved to be the best of the selected enzymes for gluten hydrolysis. This can be attributed to the fact that Flavourzyme contains a mixture of

endopeptidases and exopeptidases which collectively contributed to increase proteolysis and increased production of opioid peptides. Analysis of enzymatic hydrolysate showed that more peptides with higher bioactive ranking rankings are produced when alcalase and flavourzyme were used for proteolysis as compared to pepsin and trypsin. The potential of different enzymes to produce opioid peptides follow the order: flavourzyme > alcalase > pepsin. The EC_{50} value of flavourzyme hydrolysate was 0.43 mg/mL against μ opioid receptor, which is 10-fold lower than EC_{50} value obtained by *Lb. plantarum* fraction. This clearly shows the advantage of using flavourzyme for release of opioid peptides from wheat gluten compared to fermentation. Proteolysis by flavourzyme should be investigated in the future at conditions other than those presented in this study to identify optimal reaction conditions for maximal yield of opioid peptides. The future work should involve the use of enzyme and bacteria in combination to get better yield of opioid peptides. These studies are restricted to less than 3 kDa hydrolysate fractions which can be further fractionated and tested for opioid activity to make identification of opioid peptides easier. The peptide sequences obtained from flavourzyme hydrolysate, QQPPFW and QPFPQPQPFP, should be further tested for their bioactivity as their peptide ranking was greater than 0.9.

It has also been noticed that most of the opioid peptides appeared to be originating from gliadin preferentially rather than glutenin during enzymatic hydrolysis, and albumin and globulin during lactic acid fermentation. This is because enzymes could not access the glutenin due to its high molecular weight and structural complexity contributing to lower yields of the opioid peptides by influencing proteolysis. A study was designed to investigate if the dispersion of

glutenin can be improved by changing the pH and hydrothermal treatment at acidic pH (1, 2 and 3). Changes in structure, free functional groups and size distribution of the gluten was analysed and significant change in SDS extractability, free-amino content, and thiol content was observed at pH-1. These changes were more pronounced upon heat treatment. The use of thermal treatment at acidic pH before hydrolysis is therefore proposed to increase susceptibility of gluten for proteolysis by both enzymes and proteolytic bacteria for increased production of opioid peptides. This should be further investigated in future studies.

The thesis concludes that it is feasible to use wheat protein for production of opioid peptides which are less likely to have side-effects making them weaker opioids as compared to morphine. Out of gliadin and glutenin proteins, glutenin contributed to opioid active peptide more than gliadin, hence, instead of using whole gluten, glutenin should be separated first and preferred as a substrate to release opioid peptides

A comparison of three different approaches (lactic fermentation, enzymatic hydrolysis and peptides synthesised based on *in silico* study results) for production of opioid peptides is depicted in **Figure 8.1**. Based on the lowest EC_{50} value, it is recommended that the hydrolysis of gluten using flavourzyme should be preferred over other approaches for production of opioid peptides. Opioid peptides, YPG, YYPG, YIPP, QQPPFW and QPFPQPQPFP can be used as supplements in complimentary therapy as part of diet or for pain management in patients suffering from various diseases, particularly cancer. However, further studies are required including *in vivo* experiments and clinical trials to confirm their use in pain management.

Future work should involve using peptides produced through fermentation and enzymatic hydrolysis from gluten should be subjected to opioid receptors other than μ i.e. δ and κ to expand the scope of this study. To comprehensively compare the opioid activity of peptides released from food proteins, including wheat gluten, with the activity of traditional opioids, naloxone reversibility test should be included in the experimental protocol.

It should be noted that cAMP assay was used in this research as the objective was to compare the activities of opioid peptides produced by using three approaches. This assay was simple and cost-effective approach of measurement of activity. We propose to expand the scope of this study by comparing different direct assays (mouse *vas deferens* and guinea pig ileum) with that of cAMP assay (an indirect) used in this work for their effectiveness for measurement of opioid activity. The comparison should be made using standard opioid peptides to establish the method before adoption to hydrolysates that require purification.

In addition, presence of opioid activity in hydrolysates containing peptides that are > 3kDa should also be investigated further.

Lastly, work on using the peptides obtained should also be tested for other bioactivities to expand the use of gluten as a protein source.

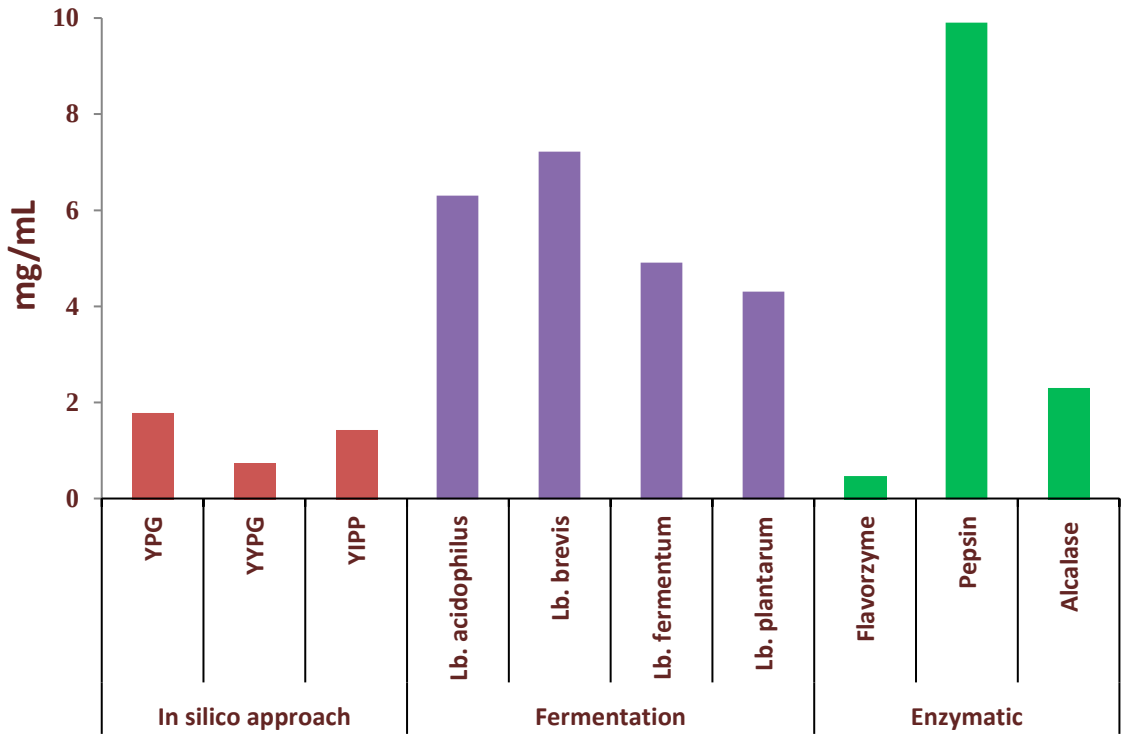

Figure 8.1: Comparison of opioid activity of peptides produced by different approaches.

Chapter 9. References

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Appendix

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Evaluation of *in silico* approach for prediction of presence of opioid peptides in wheat

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ABSTRACT

Opioid like morphine and codeine are used for the management of pain, but are associated with serious side-effects limiting their use. Wheat gluten proteins were assessed for the presence of opioid peptides on the basis of tyrosine and proline within their sequence. Eleven peptides were identified and occurrence of predicted sequences or their structural motifs were analysed using BIOPEP database and ranked using PeptideRanker. Based on higher peptide ranking, three sequences YPG, YYPG and YIPP were selected for determination of opioid activity by cAMP assay against μ and κ opioid receptors. Three peptides inhibited the production of cAMP to varied degree with EC_{50} values of YPG, YYPG and YIPP were 5.3 mM, 1.5 mM and 2.9 mM for μ-opioid receptor, and 1.9 mM, 1.2 mM and 3.2 mM for κ-opioid receptor, respectively. The study showed that *in silico* approach can be used for the prediction of opioid peptides from gluten.

1. Introduction

Opioids, such as morphine and codeine, are the most common clinically used drugs for pain management (Janecka, Fichna, & Janecki, 2004; Teschemacher, 2003; Trescot, Datta, Lee, & Hansen, 2008). These opioids bind to opioid receptors present in the central and peripheral nervous system. However, they are often associated with side-effects like sedation, dizziness, nausea, vomiting, constipation, addiction, tolerance and respiratory depression (Benyamin et al., 2008). Opioids were considered to be alkaloid (derived from opium) only until discovery of endogenous opioid peptides in 1975 (Goldstein, Goldstein, & Cox, 1975; Hughes et al., 1975). These endogenous peptides and their modified forms have shown activity similar to alkaloids (Giordano et al., 2010; Mollica et al., 2014; Mollica et al., 2005; Mollica et al., 2013b; Mollica et al., 2011). However, exogenous opioid peptides or exorphins are naturally derived from food proteins (Stefanucci et al., 2016; Yoshikawa, 2013). These exogenous peptides are of particular interest as they are naturally derived from food, have possibly less side-effects (compared to synthetic drugs) and are inexpensive to produce (Garg, Nurgali, & Mishra, 2016; Udenigwe, Gong, & Wu, 2013). Most known bioactive peptides are small and non-immunogenic, as compared to larger peptides (6–25 amino acids) (Wang, Mejia, & Gonzalez, 2005). Hence, small peptides are researched more for their bioactivity and considered safe (Shahidi & Zhong, 2008).

Generally, bioactive peptides, including opioids, are produced by hydrolysis of food proteins during food processing (ripening, fermentation), storage (Choi, Sabikhi, Hassan, & Anand, 2012) and during gastrointestinal (GI) digestion (Garg et al., 2016; Stefanucci et al., 2016). The protein hydrolysate is then tested for bioactivity using *in vitro* and *in vivo* methods. Since these hydrolysates are mixtures of several peptides, their bioactivity results from the additive and synergistic effect of various components present. Bioactive hydrolysates containing mixture of peptides needs to be fractionated, purified and then tested for bioactivity (Udenigwe & Aluko, 2012). The whole process of preparing bioactive peptides from native proteins is tedious, time consuming and the peptide yields are low (Udenigwe, 2014), limiting and/or delaying their use in clinical applications.

Alternatively, bioinformatics tools can be used for predicting the presence of bioactive peptides in proteins (*in silico* approach) (Carrasco-Castilla, Hernández-Álvarez, Jiménez-Martínez, Gutiérrez-López, & Dávila-Ortiz, 2012; Holton, Pollastri, Shields, & Mooney, 2013; Lacroix & Li-Chan, 2012). Using this approach, one can search for potential precursors of bioactive peptides and select efficient proteolytic enzymes for their release from native protein sequences (Carrasco-Castilla et al., 2012; Udenigwe et al., 2013). In this approach, protein databases, such as, UniProtKB, SwissProt and TrEMBL can be used to access sequences of a food protein, and presence of bioactive peptides can be predicted using peptide databases BIOPEP and Pepbank (Udenigwe, 2014). The BIOPEP application contains a database of biologically active peptide sequences and a program enabling construction of profiles of the

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potential biological activity of protein fragments, calculation of quantitative descriptors as measures of the value of proteins as potential precursors of bioactive peptides, and prediction of bonds susceptible to hydrolysis by endopeptidases in a protein (Minkiewicz, Dziuba, Iwaniak, Dziuba, & Darewicz, 2008). In fact, it has been successfully used for prediction of bioactive peptides from different food proteins having angiotensin converting enzyme inhibitory (ACE-I) activity (Cheung, Nakayama, Hsu, Samaranayaka, & Li-Chan, 2009; Dellafiora et al., 2015) and dipeptidyl peptidase-IV inhibitors (DPP-IV) (Lacroix & Li-Chan, 2012; Nongonierma, Mooney, Shields, & FitzGerald, 2014). PeptideRanker is a web based application and can predict the probability of a peptide being bioactive according to their score between 0 and 1 and can assist in the discovery of new bioactive peptides across many functional classes. Generally, any peptide over 0.5 threshold is labelled to be bioactive (Mooney, Haslam, Holton, Pollastri, & Shields, 2013; Mooney, Haslam, Pollastri, & Shields, 2012). Increasing the threshold from 0.5 to 0.8 reduces the number of false positive prediction from 16% to 6%, however, true positive rates also decrease (Mooney et al., 2012). If predicted probability is close to 1, the probability of peptide to be bioactive is significantly high (Mooney et al., 2012).

Bioinformatics approach is used for identification of structural patterns of peptides of known bioactivities. Presence of tryptophan in a peptide is associated with antioxidant activity (Chuan-Hsiao, Yin-Shiou, Shyr-Yi, & Wen-Chi, 2014) and carboxyl terminal alanine or proline containing peptides are DPP-IV inhibitors (Lacroix & Li-Chan, 2012). However, there is general lack of information for screening opioid peptides using bioinformatics approach. Wheat gluten contains exorphins; A5, A4, B5, B4 and C, having sequences GYYPT, GYYP, YGGW, YGGWL and YPISL, respectively (Fukudome & Yoshikawa, 1992; Zioudrou, Streaty, & Klee, 1979). Most of food derived opioid peptides have tyrosine and proline residues within them (Yoshikawa, 2013). Tyrosine (Y) is present either at the amino terminal or at the second position (as in gluten exorphins GYYPT and GYYP) and acts as part of the message domain to anchor the opioid peptide within the receptor (Heyl et al., 2003; Li et al., 2005). At position 1, Y acts as a dual hydrogen bond donor/acceptor with less acidic hydroxyl groups exhibiting stronger binding to opioid receptors. Moreover, steric bulk in the Y strengthens receptor binding by either a ligand conformational effect or enhanced van der Waals interactions with a loose receptor site (Heyl et al., 2003). Proline (P) acts as a spacer that fixes the peptide shape and induces other residues to assume proper spatial orientation for interacting with the opioid receptor (Cardillo, Gentilucci, Qasem, Sgarzi, & Spampinato, 2002). Peptides containing P also exhibit enhanced resistance to hydrolysis by enzymes of GI tract (Cardillo et al., 2002; Trivedi et al., 2014) and are therefore more likely to be active upon oral administration (Yang et al., 2001).

For peptides to exert opioid activity, they must bind to opioid receptors present within the central and enteric nervous systems. Opioid receptors belong to the superfamily of G protein coupled receptors (GPCRs) and on activation by opioid ligands, they inhibit adenylate cyclase enzyme (Garg et al., 2016; Gupta, Décaillot, & Devi, 2006) thus decreasing the production of cyclic adenosine monophosphate (cAMP) in the cells (Gupta et al., 2006). This decrease in concentration of cAMP in cells is used for screening opioid ligands (Huang, Kehner, Cowan, & Liu-Chen, 2001). This forms the basis of using cell lines transfected with opioid receptors for assaying the activity of peptides and using it for confirmation of peptides selected using bioinformatics approach.

The objective of this study was to search for opioid peptides in wheat gluten proteins based on the presence of tyrosine and proline, and use bioinformatics tools, BIOPEP and PeptideRanker to identity and rank these peptides for likelihood of having opioid activity. The identified peptides were then assayed for opioid activity by cAMP assay for confirmation of their bioactivity.

2. Materials and methods

2.1. Chemicals and reagents

Cell culture media, Dulbecco's Modified Eagle's Medium (DMEM) containing 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), fetal bovine serum (FBS), antibiotic–antimycotic (100X) and hygromycin-B and phosphate buffer saline (PBS) – pH 7.2, were acquired from Life Technologies (Carlsbad, California, US). Lance cAMP detection reagents, and bovine serum albumin (BSA) stabiliser and optiplate were from Perkin Elmer Life Sciences (Cambridge, MA). 3-Isobutyl-1-methylxanthine (IBMX), trypsin-EDTA, forskolin, [D-Ala^2, N-MePhe^4, Gly-ol^5]-enkephalin (DAMGO) and dynorphin A and all other chemicals were purchased from Sigma Aldrich (Australia) unless otherwise stated. Custom peptides were synthesized from Mimotopes (Melbourne, Australia) at > 95% purity.

2.2. Cell lines

FlpIn CHO (Chinese hamster ovary) cells stably transfected with pOG44 vector encoding Flp recombinase and pDEST vector encoding either human μ or κ receptors were a kind gift from Dr. Meritxell Canals, Monash Institute of Pharmaceutical Sciences, Melbourne, Australia. The cells were transfected using polyethylenimine as transfection reagent and hygromycin-B (200 μg/mL) was used as a selection agent (Burford et al., 2015).

2.3. In silico analysis

2.3.1. Sequences of wheat gluten proteins

The sequences of wheat storage proteins high molecular weight (HMW) and low molecular weight (LMW) glutenins and gliadins (alpha, gamma and omega) were accessed from UniProt database at http://www.uniprot.org/uniprot/ (Boutet, Lieberherr, Tognolli, Schneider, & Bairoch, 2007). These sequences were then searched for the presence of tri and oligo-peptides containing Y and P amino acids either consecutively or separated by a single amino acid.

2.3.2. Peptide ranking and bioactivity prediction

Occurrence of predicted sequences or their structural motif thereof were analysed in known opioids using BIOPEP database (Minkiewicz et al., 2008). The PeptideRanker (Bioware.ucd.ie) was used to rank the predicted sequences according to bioactivity. A peptide having ranking closer to 1, increases its chances to be bioactive so they were selected to be tested for opioid activity.

2.4. Opioid activity assay

Opioid activity of the peptides was determined on the basis of cAMP assay. Cells were grown and maintained at 37 °C in a humidified incubator containing 5% CO_2 in DMEM, 10% FBS, 1% antibiotic–antimycotic and 200 μg/mL hygromycin-B (Burford et al., 2015). Cells were grown to 90% confluency, harvested and resuspended at 2×10^6 cells/mL in the media (DMEM + FBS + hygromycin B) and 100 μL of cells were seeded into sterile 96 well plates and incubated at 37 °C and 5% CO_2 overnight. The culture media in all the wells were replaced with stimulation buffer consisting of PBS, 50 mM IBMX and BSA stabiliser and incubated for 30 min before stimulation. Cells were stimulated at different concentrations of peptides in the presence of 10 μM forskolin for 30 min. The stimulation buffer containing peptides was then removed and 50 μL of ice cold 100% ethanol was added to each well. Ethanol was then evaporated and 75 μL of lysis buffer (0.3% tween-20, 5 mM HEPES and 0.1% BSA) was added to each well and the change in concentration of cAMP in the lysate was determined using Lance cAMP detection kit. 5 μL of lysate containing cAMP was mixed with 5 μL of Alexa flour-647 anti-cAMP antibody (stock antibody

Table 1
Sequence of wheat storage proteins as obtained by UniProt.

Wheat protein	UniProt accession number	Amino acid (AA) sequence	AA residue
HMW glutenin subunit	Q41553	MTKRLVLFAAVVVALVALTAAEGEASGQLQCERELQEH SLKACRQVVDQQLRDVSPECQPVGGGPVARQYEQQVVV PPKGGSFYPGETTPPQQLQQSILWGIPALLRRYYLSVTSP QQVSYYPGQASSQRPGQGQQEYYLTSPQQSGQWQQPGQ GQSGYYPTSPQQSGQKQPGYYPTSPWQPEQLQQPTQGQ QRQQPGQGQQLRQGQQGQQSGQGQPRYYPTSSQQPGQL QQLAQGQQGQQPERGQQGQQSGQGQQLGQGQQGQQPG QKQQSGQGQQGYYPISPQQLGQGQQSGQGQLGYYPTSP QQSGQGQSGYYPTSAQQPGQLQQSTQEQQLGQEQQDQQ SGQGRQGQQSGQRQQDQSGQGQQPGQRQPGYYSTSPQQ LGQGQPRYYPTSPQQPGQEQQPRQLQQPEQGQQGQQPE	815
		QGQQGQQQRQGEQGQQPGQGQQGQQPGQGQPGYYPTS PQQSGQGQPGYYPTSPQQSGQLQQPAQGQQPGQEQQGQ QPGQGQQPGQGQPGYYPTSPQQSGQEQQLEQWQQSGQG QPGHYPTSPLQPGQGQPGYYPTSPQQIGQGQQPGQLQQP TQGQQGQQPGQGQQGQQPGEGQQGQQPGQGQQPGQGQ PGYYPTSLQQSGQGQQPGQWQQPGQGQPGYYPTSSLQP EQGQQGYYPTSQQQPGQGPQPGQWQQSGQGQQGYYPT SPQQSGQGQQPGQWLQPGQWLQSGYYLTSPQQLGQGQ QPRQWLQPRQGQQGYYPTSPQQSGQGQQLGQGQQGYY PTSPQQSGQGQQGYDSPYHVSAEHQAASLKVAKAQQLA AQLPAMCRLEGGDALLASQ	
LMW glutenin	Q8W3V5	MKTFLVFALIAVVATSAIAQMETSCISGLERPWQQQPLPP QQSFSQQPPFSQQQQQPLPQQPSFSQQQPPFSQQQPILSQQ PPFSQQQQPVLPQQSPFSQQQQLVLPPQQQQQQLVQQQIP IVQPSVLQQLNPCKVFLQQQCSPVAMPQRLARSQMWQQ SSCHVMQQQCCQQLQQIPEQSRYEAIRAIIYSIILQEQQQG FVQPQQQQPQQSGQGVSQSQQQSQQQLGQCSFQQPQQQ LGQQPQQQQQQVLQGTFLQPHQIAHLEAVTSIALRTLPT MCSVNVPLYSATTSVPFGVGTGVGAY	303
alpha-gliadin	A0A0E3Z527	MKTFLILALLAIVATTATIAVRVPVPQLQPQNPSQQQPQE QVPLMQQQQQFPGQQEQFPPQQPYPHQQPFPSQQPYPQP QPFPPQLPYPQTQPFPPQQPYPQPQPQYPQPQQPISQQQA QQQQQQQQILQQILQQQLIPCRDVVLQQHNIAHASSQVL QQSTYQLVQQLCCQQLWQIPEQSRCQAIHNVVHAIILHQ QQQQQQQQQQQPLSQVSFQQPQQQYPSGQGSFQPSQQN PQAQGSVQPQQLPQFEEIRNLALETLPAMCNVYIPPYCTI APVGIFGTN	284

(*continued on next page*)

Table 1 (*continued*)

gamma gliadin	P21292	MKTLLILTILAMATTIATANMQVDPSGQVQWPQQQPFPQ PQQPFCQQPQRTIPQPHQTFHHQPQQTFPQPQQTYPHQPQ QQFPQTQQPQQPFPQPQQTFPQQPQLPFPQQPQQPFPQPQ QPQQPFPQSQQPQQPFPQPQQQFPQPQQPQQSFPQQQQPA IQSFLQQQMNPCKNFLLQQCNHVSLVSSLVSIILPRSDCQ VMQQQCCQQLAQIPQQLQCAAIHSVAHSIIMQQEQQQG VPILRPLFQLAQGLGIIQPQQPAQLEGIRSLVLKTLPTMCN VYVPPDCSTINVPYANIDAGIGGQ	302
omega gliadin	Q6PNA3	MKPHHDGYKYTCSIIVTFHYPNFKHQDQKHQFQESIKHK SKMKTFIIFVLLSMPMSIVIAARHLNPSDQELQSPQQQFLE KTIISAATISTSTIFTTTTISHTPTIFPPSTTTTISPTPTTNPPTT TMTIPLATPTTTTTFSPAPTTISLATTTTISLAPTTNSPITTT TIPAATPETTTTIPPATRTNNYASTATTISLLTATTTPPATP TTILSATTTTISPAPTIISPATRTNNSLATPTTIPPATATTIPP ATRTNNSPATATTIPPAPQQRFPHTRQKFPRNPNNHSLCS THHFPAQQPFPQQPGQIIPQQPQQPLPLQPQQPFPWQPEQ RSSQQPQQPFSLQPQQPFS	354

: predicted opioid peptide sequences

diluted at 1:100 in detection buffer provided in kit). Detection mix containing Europium W8044 labelled streptavidin and biotin-cAMP was prepared according to kit instructions and kept at room temperature for 15 min. The detection mix (10 μL) was added to each well and incubated for 1 h before reading. Time-resolved fluorescence (TRF) was detected using an Envision plate reader (Perkin Elmer, Cambridge, MA) with excitation at 337 nm and emission read at 615 nm and 665 nm. DAMGO and dynorphin A were used as positive controls for μ and κ opioid receptors, respectively. Data were analysed and EC_{50} values determined using nonlinear regression analysis to fit a logistic equation using Graph Pad Prism, version 7 (Graph Pad San Diego, California, US).

3. Results and discussions

3.1. Prediction of opioid peptide sequences in wheat gluten

Wheat is one of the most important cereals consumed globally providing protein and carbohydrates to the diet. The main storage protein of wheat gluten consists of glutenins and gliadins. Table 1 shows the sequence of gluten proteins and the relevant sequences containing Y and P are highlighted in each of the wheat proteins. Peptides sequences YPG, YPTSP, YYPG (from HMW glutenin), YIPP, YPH, YPQ, YPS (from alpha-gliadin), YPH, YVPP (from gamma gliadin) and YPN (from omega gliadin) are identified to have opioid activity (Table 1). The occurrence frequencies of these opioid peptides are 17, 7, 2 and 1 in HMW glutenin, alpha gliadin, gamma gliadin and omega gliadin, respectively. Based on this observation, HMW glutenin is by far a superior source of opioid peptides than the rest of tested proteins.

3.2. Selection of opioid peptides using BIOPEP database and PeptideRanker

BIOPEP is a database of biologically active peptide sequences and a tool for evaluation of proteins as the precursor of bioactive peptides. The database was used to compare the presence of structural motifs comprising of di or tripeptides in identified peptides in comparison to known opioid peptides (Table 2). The peptides were classified according to different search terms and for every sequence within the class, EC_{50} values are reported. YYP (part of YYPG) structural motif is present in eight opioid peptides with EC_{50} as low as 60 μM (gluten A5 exorphin) (Table 2). Similarly, five known opioid peptides contain structural motif IP (part of YIPP), whereas Casoxin C, containing YIP (part of YIPP) is an antagonist. YVP is present in only one known opioid peptide. YP (part of YPG, YPQ, YPH, and YPN) was present in 92 out of 156 (nearly 60%) opioid peptides present. It is also realised that YP can be present at amino or carboxyl terminals or in the middle of peptide sequences and may be followed either by aromatic or non-aromatic amino acids. Majority of opioid peptides have aromatic amino acid tryptophan (W), phenylalanine (F) or tyrosine (as in YPYY) and have EC_{50} value as low as 0.01 μM (YPFGFR, YPFGFRG) and 0.02 μM (YPFGFS, YPFGFK, YPFGFG, YPFGFGG, YPFGFKG and YPFGFSG) making these very effective opioids. Non-aromatic amino acids are also present as isoleucine (I) in YPISL, leucine (L) in YPLG and YPLSL, serine (S) in YPSYGLN, YPSF and YPS, valine (V) in YPVSL, alanine (A) in YPASL, threonine (T) in YPTSL and YPTS (Table 2). EC_{50} values of YPISL, YPVSL and YPASL were 13.50 μM, 200 μM and 200 μM, respectively. This implies that peptides having YP structural motif followed by non-aromatic amino acids as predicted in Table 1 (YPG, YPQ, YPH, YPT, YPS and YPN) may have opioid activity. With limited information available of EC_{50} value of the sequences (Table 2), it is also evident that within the search term, the opioid activity is dependent on chain length of the peptide sequence. For example, high EC_{50} values of sequences YYPT (1000 μM) and YYP (800 μM) are suggestive of their weak or negligible opioid activity. Similarly, it can also be stated that casein are superior to gluten as a protein source for the opioid peptides as the EC_{50} values of peptides containing YP motif of casomorphin are significantly lower.

PeptideRanker is a web based application that predicts probability of the peptide to be bioactive (Mooney et al., 2013). In this study, PeptideRanker was used to rank bioactivity of peptides as identified from Table 1 and compared to known gluten exorphins (opioid peptides from wheat). The ranking information is presented in Table 3. PeptideRanking of known gluten exorphins varied from 0.55 to 0.96 (Table 3) and exorphins A4, B4 and B5 show higher likelihood of

Table 2
Opioid peptides as predicted by BIOPEP database.

	Name	Sequence	Molecular mass	EC_{50}(μM)
Search term – YYP by sequence				
1	Gluten A5 exorphin	GYYPT	599.61	60
2	Gluten A4 exorphin	GYYP	498.50	70
3		YYPT	542.56	800
4		YYP	441.45	1000
5		RYYP	597.64	190
6		WYYP	627.67	n.d
7		SYYP	528.53	200
8		GYYPTS	686.69	72
Search term – IP by sequence				
1		FGGFTGRIPKLWDNQ	1735.95	n.d
2		YPFVEPIP	961.10	n.d
3		YPFPGPIP	887.02	n.d
4	Casoxin C *	YIPIQYVLSR	1251.46	50
5	β-Casomorphin-11 (60–70)	YPFPGPIPNSL	1201.37	10
Search term – YVP by sequence				
1	Fragment of human αS1-casein	YVPFP	621.71	n.d
Search term - YP by sequence				
1	VV-hemorphin-7	VVYPWTQRF	1194.60	34.3
2	VV-hemorphin-5	VVYPWTQ	891.43	78.2
3	Oryzatensin *	GYPMYPLPR	1092.52	n.d
4		YPFT	526.23	n.d
5		LVVYPWTQR	1160.62	n.d
6		YPFVEP	750.34	n.d
7		YPFV	524.25	n.d
8		YPFVEPIP	960.48	n.d
9		YPFVE	653.29	n.d
10	Opioid peptide	YPFP	522.23	n.d
11	Opioid peptide	YPFPGPIP	886.44	n.d
12	Opioid peptide	YPF	425.18	n.d
13	Opioid fragment of dermorphin	YAFGYPS	803.33	n.d
14	Opioid fragment of β-lipotropin β-neoendorphin	YGGFLRKYP	1099.56	n.d
15	Opioid fragment of β-lipotropin β/α-neoendorphin	YGGFLRKYPK	1227.66	n.d
16	LVV-hemorphin-7	LVVYPWTQRF	1307.68	29.1
17	LVV-hemorphin-5	LVVYPWTQ	1004.51	80.5
18	Hemorphin-8	YPWTQRFF	1143.53	4.6
19	Hemorphin-7	YPWTQRF	996.46	2.9
20	Hemorphin-6	YPWTQR	849.40	4.3
21	Hemorphin-5	YPWTQ	693.29	46.3
22	Hemorphin-4	YPWT	565.24	45.2
23	Gluten C exorphin	YPISL	591.31	13.5
24	Gluten A5 exorphin	GYYPT	599.24	60
25	Gluten A4 exorphin	GYYP	498.19	70
26	Gliadin 2 exorphin	YPLG	448.22	n.d
27	Casoxin (fr.35–41 of bovine kappa-casein)	YPSYGLN	812.35	n.d
28	Casoxin (fr.33–38 of bovine kappa-casein) *	SRYPSY	771.34	n.d
29	Casoxin	YPYY	604.24	n.d
30	β-Casomorphin	YPSF	512.21	n.d
31	β-Casomorphin-11 (60–70)	YPFPGPIPNSL	1200.60	10
32	β-Casomorphin-7 (60–66)	YPFPGPI	789.39	14
33	β-Casomorphin-5 (60–64)	YPFPG	579.25	1.1
34		YPFGFF~	775.35	0.06
35		YPFGFE~	757.33	0.12
36		YPFGFW~	814.36	0.09
37		YPFGFCQ~	756.34	0.08
38		YPFGFD~	743.31	0.07
39		YPFGFV~	727.35	0.05
40		YPFGFS~	715.32	0.02
41		YPFGFL~	741.37	0.05
42		YPFGFT~	729.33	0.04
43		YPFGFI~	741.37	0.04
44		YPFGFY~	791.35	0.04
45		YPFGFH~	765.34	0.04
46		YPFGFM~	759.32	0.03
47		YPFGFQ~	756.34	0.03
48		YPFGFP~	725.34	0.03
49		YPFGFA~	699.32	0.03
50		YPFGFK~	756.38	0.02
51		YPFGFG~	685.30	0.02
52		YPFGFR~	784.38	0.01
53		YPFGFN~	742.33	0.03
54		YPFGFGG	743.31	0.02
55		YPFGFNG	800.33	0.03
56		YPFGFAG	757.33	0.03
57		YPFGFQG	814.35	0.03
58		YPFGFKG	814.38	0.02
59		YPFGFMG	817.33	0.03
60		YPFGFRG	842.39	0.01
61		YPFGFSG	773.32	0.02
62		YPFGFDG	801.31	0.07
63		YPFGFEG	815.33	0.12
64		YPFGFPG	783.34	0.03
65		YPFGFCQG	814.35	0.08
66		YPFGFFG	833.36	0.06
67		YPFGFVG	785.36	0.05
68		YPFGFLG	799.37	0.05
69		YPFGFTG	787.34	0.04
70		YPFGFIG	799.37	0.04
71		YPFGFYG	849.35	0.04
72		YPFGFHG	823.35	0.04
73		YPFGFWG	872.37	0.09
74		YYPT	542.22	800
75		YYP	441.17	1000
76		YPVSL	577.29	200
77		YPLSL	591.31	200
78		YPASL	549.26	n.d
79		YPTSL	579.27	n.d
80		YPFSL	625.29	70
81		RYYP	597.27	190
82		YPWSL	664.31	70
83		WYYP	627.25	n.d
84		SYYP	528.21	200
85		GYYPTS	686.27	72
86		YPFW~	610.27	n.d
87		YPFWG	668.28	n.d
88		YPFF~	571.26	n.d
89		YPFFG	629.27	n.d
90	Soymorphin-5	YPFVV	623.31	6
91	Soymorphin-6	YPFVVN	737.36	9.2
92	Soymorphin-7	YPFVVNA	808.39	13

* Opioid antagonist, n.d. = not defined.

bioactivity based on higher ranking than exorphins A5 and C. The ranking for the unknown peptides as identified from Table 1 varied between 0.38 and 0.83. Only 3 out of 11 peptides, YPG, YYPG and YIPP were ranked > 0.77. These 3 peptides were used for confirmation of opioid activity.

3.3. Assessment of wheat protein derived peptides for opioid activity

Based on the *in silico* analysis, peptides YPG, YYPG and YIPP are opioid and should bind to opioid receptors to exert bioactivity. The activity of these peptides was confirmed by using cyclic AMP assay, which is based on inhibition of the adenylate cyclase enzyme. The decreasing concentration of cAMP within the cells is taken to be an indication of a positive test and used in determination of activity expressed as EC_{50} values. All three peptides - YPG, YYPG and YIPP inhibited production of cAMP in the presence of 10 μM forskolin in cells expressing μ and κ opioid receptors (Figs. 1, 2). Decreases in concentration of cAMP is graphed against concentration of peptides using

Table 3
Ranking of the predicted peptide sequences obtained by PeptideRanker (bioware.ucd.in) as compared to known exorphins from gluten.

Known peptides	Peptide ranking
GYYPT (exorphin A5)	0.58
GYYP (exorphin A4)	0.8
YGGWL (exorphin B5)	0.96
YGGW (exorphin B4)	0.96
YPISL (exorphin C)	0.55
Predicted sequences from Table 1	
YPG	0.83
YYPG	0.78
YIPP	0.77
YVPP	0.52
YPH	0.59
YPISP	0.52
YPTSP	0.41
YPQ	0.47
YPS	0.44
YPN	0.55
YPT	0.38

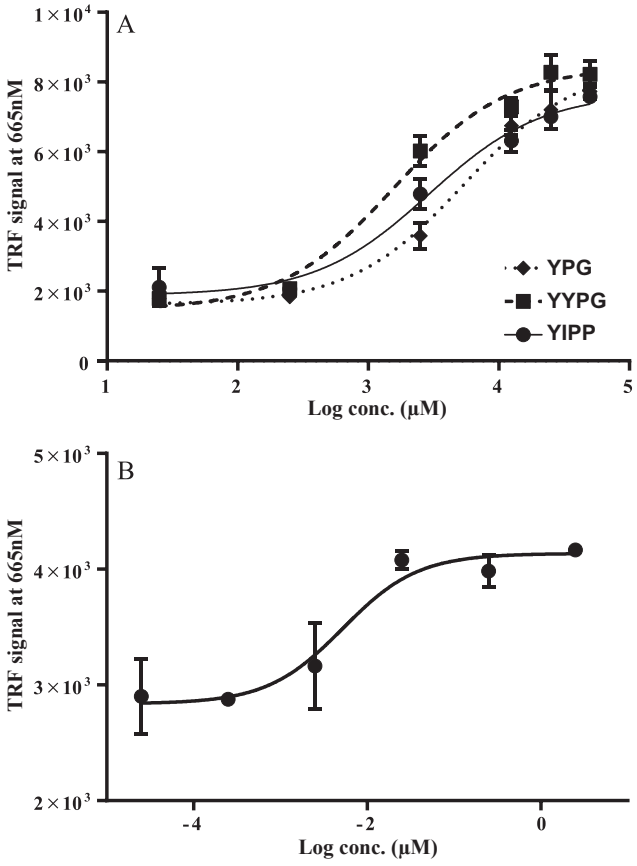

Fig. 1. Dose response curves of three peptides (A) and DAMGO (B) against μ-opioid receptors based on inhibition of adenylate cyclase as depicted by TRF signal measured at 665 nm.

GraphPad Prism 7 software and EC_{50} of the peptides were calculated from a sigmoid response curve. Calculated EC_{50} values of all tested peptides was > 1.0 mM for both μ and κ receptors indicating that a high dose of these peptides is required for them to exert opioid activity. For μ opioid receptor, EC_{50} values of YPG, YYPG and YIPP were 5.3 mM, 1.5 mM and 2.9 mM respectively, while for κ opioid receptor, EC_{50} values were 1.8 mM, 1.2 mM and 3.2 mM, respectively. For both receptors, YYPG had the lowest EC_{50} value, and is more potent opioid peptide than either YPG or YIPP which can be due to presence of 2 Y

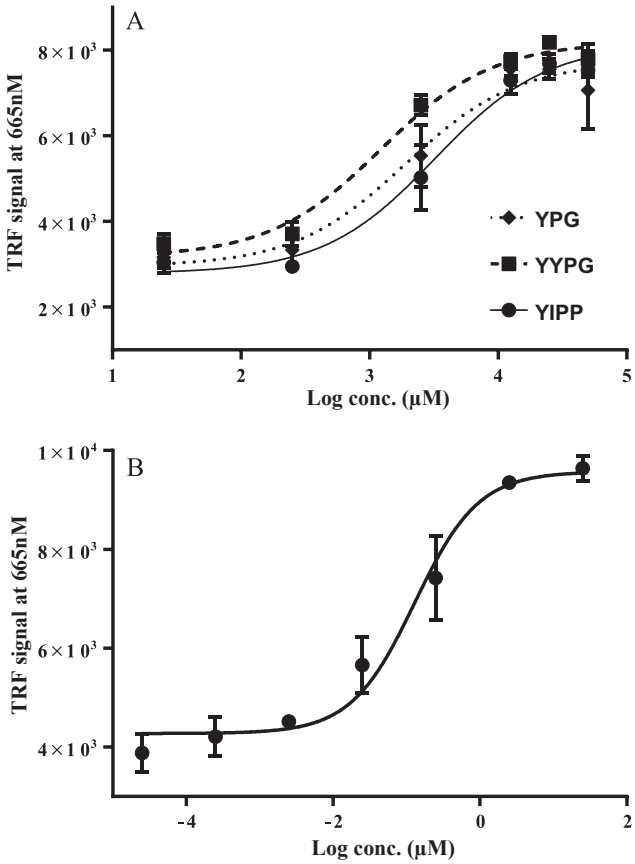

Fig. 2. Dose response curves of three peptides (A) and dynorphin A (B) against κ-opioid receptors based on inhibition of adenylate cyclase as depicted by TRF signal measured at 665 nm.

residues in the peptide. Also, these peptides have higher affinity to κ opioid receptors than for μ opioid receptors. As shown in Table 2, YYP has EC_{50} of 1 mM, and presence of glycine (G) at the amino terminal end in GYYP (gluten exorphin A4) decreased its EC_{50} value to 70 μM making it more effective peptide than YYP. Presence of non-aromatic amino acid (Threonine, T) at the carboxyl terminal (YYPT) also decreased its EC_{50} value to 800 μM but presence of G at the carboxyl terminal (YYPG) did not decrease EC_{50} value. Despite of these peptides binding to opioid receptors (μ and κ), they are not as effective (higher EC_{50} values) in their native form and therefore need modification to improve their binding and agonistic activities. For example, Torino et al. (2010) reported improvement in opioid activity though modification of native endomorphine-2. Further research is required to find if these peptides are adsorbed intact or pass the blood brain barrier (BBB) to make them effective for clinical application. Small size of predicted peptide assures that these peptides can pass the GI tract (De Noni et al., 2009). Use of D-amino acids, β-amino acids, various types of synthetic residues and backbone cyclization can improve stability against enzymatic hydrolysis (Mollica et al., 2013b) and therefore modify their fate in human system. However, even if these peptides may not be able to pass BBB or absorbed, they can also stimulate the brain by brain-gut axis (Stefanucci et al., 2016). They are still worth further investigation for further pharmaceutical development because of their high selectivity and low toxicity (Garg et al., 2016; Mollica, Pinnen, Azzurra, & Costante, 2013a; Stefanucci et al., 2016).

4. Conclusion

Bioinformatics approach was used in identification of opioid peptides from wheat gluten proteins. Structural motifs, as particular amino

acids or their combinations responsible for opioid bioactivity were identified. Using tyrosine and proline residues in the peptide sequences as predictors of opioid peptides, HMW glutenin was found to be the best source of opioid peptides. It was predicted that eleven peptides from wheat gluten could have opioid activity, out of which YPG, YYPG and YIPP were selected as these showed higher ranking of 0.83, 0.78 and 0.77, respectively. The activity of these predicted peptides were determined using cAMP assay in cell lines expressing opioid receptors. Based on the lowest EC_{50} value, YYPG is more potent opioid than YPG or YIPP. The study shows that bioinformatics tools can assist in screening opioid peptides from gluten proteins. This approach can be cost effective for selection and comparison of proteins for production of opioid peptides.

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Conflict of interest

None.

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Original article

Effect of heating and acidic pH on characteristics of wheat gluten suspension

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Summary The effects of different pH treatments with and without heating on the characteristics of wheat gluten suspension was investigated. At pH 1, maximum changes in colour were observed with a concurrent 65% decrease in protein free-thiol content compared to the control gluten. The SDS-Extractability of protein (SDS-EP) chromatogram eluted at lower retention time and the presence of bands at the top lane even during reducing conditions in SDS-PAGE gel suggested complex formation involving bonds other than disulphides. An increase in the free-amino group content and the presence of an additional peak at a higher retention time in the SDS-EP chromatogram was suggestive of hydrolysis. At pH 2 and 3, similar decreases in SDS-EPs and free-thiol content indicated formation of complexes. When heated, the free-thiol content of the dispersions increased compared to the non-heated dispersions indicating disruption of disulphide bonds with changes in gluten structure and size distribution.

Keywords Free-amino, free-thiol, SDS extractable protein, SDS-PAGE, wheat gluten.

Introduction

Wheat gluten (WG) is a heterogeneous mixture of gliadin and glutenin protein subunits which is produced as a by-product of the wheat starch industry. It is widely used in a range of food (bakery products) as well as non-food applications which includes production of bioplastics (Langstraat *et al*., 2015) through to animal feeds and adhesives (Day *et al*., 2006). Gluten can be hydrolysed to produce bioactive peptides with anti-oxidant, anti-microbial, anti-cancer, opioid or anti-hypertensive activities (Korhonen & Pihlanto, 2003; Shahidi & Zhong, 2008; Zhu *et al*., 2011; Janković *et al*., 2015; Garg *et al*., 2016; Wu *et al*., 2016). Enzymatic hydrolysis is the preferred method for the production of bioactive peptides and the extent of hydrolysis depends on the number, nature of amino acids and their sequence. The conformational differences associated with gluten proteins, availability of cleavage sites (Liao *et al*., 2012), product inhibition of enzymes, enzymatic specificity and autopeptidolysis of enzyme (Giesler *et al*., 2014) reduce hydrolysis of gluten and consequent reduction in the yield of peptides.

The inherent complexity and poor water dispersion of WG limits its use for various applications. Poor dispersion results from the presence of high concentrations of proline, leucine and glutamine and low concentrations of lysine, arginine, glutamic acid and aspartic acid (Yildiz, 2010). Heat treatment can change physicochemical and viscoelastic properties of gluten (Falcão-Rodrigues *et al*., 2005; Lagrain *et al*., 2008) via cleavage of intra-molecular disulphide (S-S) bonds and formation of inter-molecular S-S bonds (Lagrain *et al*., 2008). Cross-linking start with glutenins, and at higher temperatures, gliadin also gets incorporated into the network (Singh & MacRitchie, 2004), which decreases protein extractability due to the formation of aggregates. Improvement in the yield of peptides of >10 kDa were observed when thermally treated gluten was hydrolysed by papain (Wang *et al*., 2009). Functional properties of gluten normally improve in both acidic and alkaline pH range away from the isoelectric point of gluten. At alkaline pH, the formation of lanthionine (Jansens *et al*., 2013) and lysinoalanine (Rombouts *et al*., 2010) leads to more aggregates due to cross-linking. On the other hand, the ability of gluten to bind with other polymers is favoured under acidic pH as a result of reduced cross-linking (Langstraat *et al*., 2015) paving way for application of modified gluten in bioplastics. Reddy & Yang (2007) reported the development of gluten based fibre with improved

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mechanical properties similar to that of wool. Other non-food applications of modified gluten are as medical bandages (Quimby *et al*., 1994) and biodegradable substitute for polylactic acid fibres (Li *et al*., 2008).

Hydrothermal treatment of gluten in acidic solutions improves functional properties of gluten including solubility, emulsifying and foaming properties (Liao *et al*., 2010). The extent of these changes depends on the balance between intra-molecular electrostatic repulsion and the non-covalent and S-S bond formation (Liao *et al*., 2011). Consequently, more amino acids are exposed for binding to enzymes and increasing susceptibility to enzymatic hydrolysis (Liao *et al*., 2012) required for increasing the yield of bioactive peptides. When used as pretreatment, increased hydrolysis of gluten by pancreatin increased the number of peptides with MWs below 3000 Da (Cui *et al*., 2013). Similarly, thermal pretreatment improved the yield of peptides with higher antioxidant activity using gluten as a substrate and alcalase as proteolytic enzyme (Elmalimadi *et al*., 2017). Hydrothermal treatment of gluten in acidic solution improves molecular flexibility (Liao *et al*., 2016a). Use of high temperature and acidic concentration hydrolyse peptide bonds, thus impairing the protein quality, however, moderate acid concentration improves emulsifying and foaming properties (Liao *et al*., 2016b). Knowledge of enhancing physicochemical, structural and functional properties of wheat gluten as affected by various treatments is necessary for extending the uses of gluten, which is cheap and readily available natural protein. Hence, this study was carried out to investigate the changes in WG in hydrochloric acid at different pH with and without heating in order to optimise the treatment conditions for improved dispersibility. The changes in the structure of the protein, size distribution, free-thiol content and amino content were determined to evaluate protein aggregation and hydrolysis.

Materials and methods

Materials

Wheat flour with a protein content of 12.5% was obtained from Allied Mills (Australia). Hydrochloric acid (HCl) was purchased from Merck (Melbourne, Australia); mini-PROTEAN TGX gel (12%), 10X Tris/Glycine/SDS buffer and 2X laemmli sample buffer were obtained from Bio-Rad (Melbourne, Australia); o-phthalaldehyde (OPA), methionine, 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB), glutathione and all other chemicals were from Sigma Aldrich (Steinheim, Germany).

Preparation of gluten and heat treatment

Gluten was extracted from the wheat flour by hand-washing using AACC method 38–10, 2000 followed by freeze drying and grinding. The powder was passed through a 212 μm sieve and its protein content was 75% (conversion factor N × 5.7) as measured by Kjeldahl (Qiu *et al*., 2014). Gluten powder (400 mg) was dispersed in 5 mL aqueous HCl solution at pH 1, 2 and 3 in glass tubes to prepare 8% gluten dispersions which were sealed and shaken thoroughly to obtain homogenous dispersions named WG-1, WG-2 and WG-3, respectively. Another set of samples at pH 1, 2 and 3 were heated in an oil bath set at 80 °C for 15 min and were designated WG-1a, WG-2a and WG-3a, respectively. Each sample was then freeze-dried using an Alpha 1–4 LSC freeze dryer (Martin Christ, Germany) before further analysis. All samples were prepared and analysed in triplicate.

Colour measurement

A portable Konica Minolta (Tokyo, Japan) Chroma Meter CR-300 with illuminant D65 and a 2° standard observer was used to measure the colour. A measuring head area of 8 mm diameter was used with diffuse illumination and 0° viewing angle, and a white chromameter standard tile ($L = 97.47$, $a = 0.13$, $b = 1.83$) was used for calibration. Samples were placed on the white standard plate to measure the CIE $L^*a^*b^*$ colour values which were taken in triplicate.

Scanning electron microscopy (SEM)

Images were obtained using a JOEL NeoScope SEM (JCM-5000, Frenchs Forest, NSW, Australia). Freeze-dried samples were mounted on an aluminium sample holder and coated with gold for 6 min using JOEL NeoCoater (MP19020NCTR). The images were acquired at a scale of 10 μm under high vacuum using an accelerating voltage of 10 kV.

Fourier transform infrared (ftir) analysis

The FTIR absorbance spectra were acquired using Perkin Elmer Frontier™ FTIR spectrophotometer (Waltham, USA) in attenuated total reflectance (ATR) mode using a diamond ATR crystal. Background spectra were recorded at the beginning of the measurements with a blank diamond ATR cell. For each sample, 16 scans were collected at a resolution of 4 cm^{-1}.

Size-exclusion high performance liquid chromatography (SE-HPLC)

The SE-HPLC analysis was performed using a Prominence-i, LC-2030C instrument (Shimadzu, Kyoto,

Japan). Samples were prepared by dissolving 1 mg of sample in 1 mL of 50 mM sodium phosphate buffer (pH 6.8) containing 2% SDS (extractability buffer) for 60 min followed by centrifugation (20 min at 10 000 ***g***) (Langstraat *et al.*, 2015). The supernatant (10 μL) was loaded onto a Yarra SEC 3000 column (Phenomenex, Torrance, United States) and eluted with 50 mM sodium phosphate buffer (pH 6.8) containing 300 mM NaCl at flow rate of 0.4 mL min^{-1} with detection set at 214 nm. The elution profiles were divided into higher and lower MW protein fractions that were identified as SDS-extractable glutenin and SDS-extractable gliadin, respectively (Jansens *et al.*, 2013).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Samples were dissolved in the SDS buffer (10 μg μL^{-1}) and mixed with a volume of 2× sample buffer containing β-mercaptoethanol followed by heating at 95 °C for 5 min for the reducing SDS-PAGE analysis. For the non-reducing SDS-PAGE analysis, all the conditions were the same except that the sample buffer did not contain β-mercaptoethanol and it was not heated. Samples were then loaded on the gel (20 μL) and electrophoresis was conducted at 200 V for 45 min. The gels were stained using 0.15% Coomassie Brilliant Blue R250 dye in 72% isopropanol and 3% acetic acid for 1 h followed by overnight destaining (10% isopropanol, 10% acetic acid) on a shaker. The gel images were captured using a Chemidoc MP imager (Bio-Rad).

Primary amine analysis using OPA method

Samples of 10 mg were suspended in 1 mL of 50 mM sodium phosphate buffer (pH 8.3). The OPA reagent was prepared by combining 25 mL of the sodium phosphate buffer, 2.5 mL of sodium dodecyl sulphate (20% *w*/*v*), 40 mg of OPA dissolved in 1 mL of methanol, and 100 μL of β-mercaptoethanol and making the volume to 50 mL with water. Sample solutions of 700 μL were added to a test tube containing 700 μL of OPA reagent and the absorbance was measured at 340 nm after 2 min using a Novaspec-II spectrophotometer (Pharmacia Biotech). Buffer was used as blank and the concentration of free-amino groups was determined using methionine as a standard.

Free-thiol group analysis

Samples prepared for primary amine analysis were also used for the determination of free-thiol using DTNB (Wang *et al.*, 2016). The reagent was prepared by mixing 4 mL DTNB (2 mg mL^{-1} in n-propanol) and 20 mL of sodium phosphate buffer (pH 8). A sample of 100 μL was mixed with an equal volume of DTNB reagent, shaken for 1 h and the absorbance was measured at 415 nm using iMark™ Microplate Absorbance Reader (Bio-Rad, Australia). Buffer containing DTNB was used as blank and the concentration of free-thiol was determined using glutathione as a standard.

Results and discussion

Effect of pH on gluten dispersion and colour

Differences in the dispersibility of WG were observed visually at the different pH tested. The WG-2 sample appeared to be a more homogenous compared to WG-1 and WG-3, both of which settled after shaking. The freeze-dried WG-1 was pale yellow in colour due to the formation of chromophores as compared to WG-2 and WG-3 which were white. The colour parameters of the freeze-fried powders are presented in Table 1 which shows the lightness (L^*), redness (a^*) and yellowness (b^*) values of CG and treated sample powders. Compared to the CG, the maximum changes in L^*, a^* and b^* values for WG-1 were −8.68, +2.7 and +12, respectively, indicating a significant shift to a more yellow colour. Micard *et al.* (2000) reported significant reduction in L^* and increases in both a^* and b^* values upon heating gluten films to temperature >95 °C.

Effect of pH and heating on structural changes

Figure 1 shows the SEM images of the CG and acid treated samples with the CG showing the presence of large solid particles rather than a defined network structure. Conversely, the treated samples showed larger gluten agglomerates that may have resulted *via* disulphide bonds leading to formation of cross-links (Wang *et al.*, 2016). Interestingly, WG-2 appears to be a more porous and open network structure compared to WG-1 and WG-3, due to differences in hydrophobic interactions existing at the different pH.

The structures of the WG samples dispersed at different pH were significantly different from the CG as

Table 1 Colour values (L^*, a^* and b^*) of WG powder as affected by pH

	L^*	a^*	b^*
CG	90.0 ± 0.9^b	−0.91 ± 0.1^b	13.3 ± 2.1^b
WG-1	81.4 ± 0.9^c	1.78 ± 0.6^a	25.3 ± 1.3^a
WG-2	93.5 ± 1.2^a	−0.68 ± 0.1^b	6.4 ± 1.6^c
WG-3	89.8 ± 0.5^b	−1.20 ± 0.2^b	13.2 ± 0.6^b

Values are expressed as the mean ± standard deviation. Within a column, different lowercase letters (a–c) indicate a significant difference ($P < 0.05$) based on the Tukey's test.

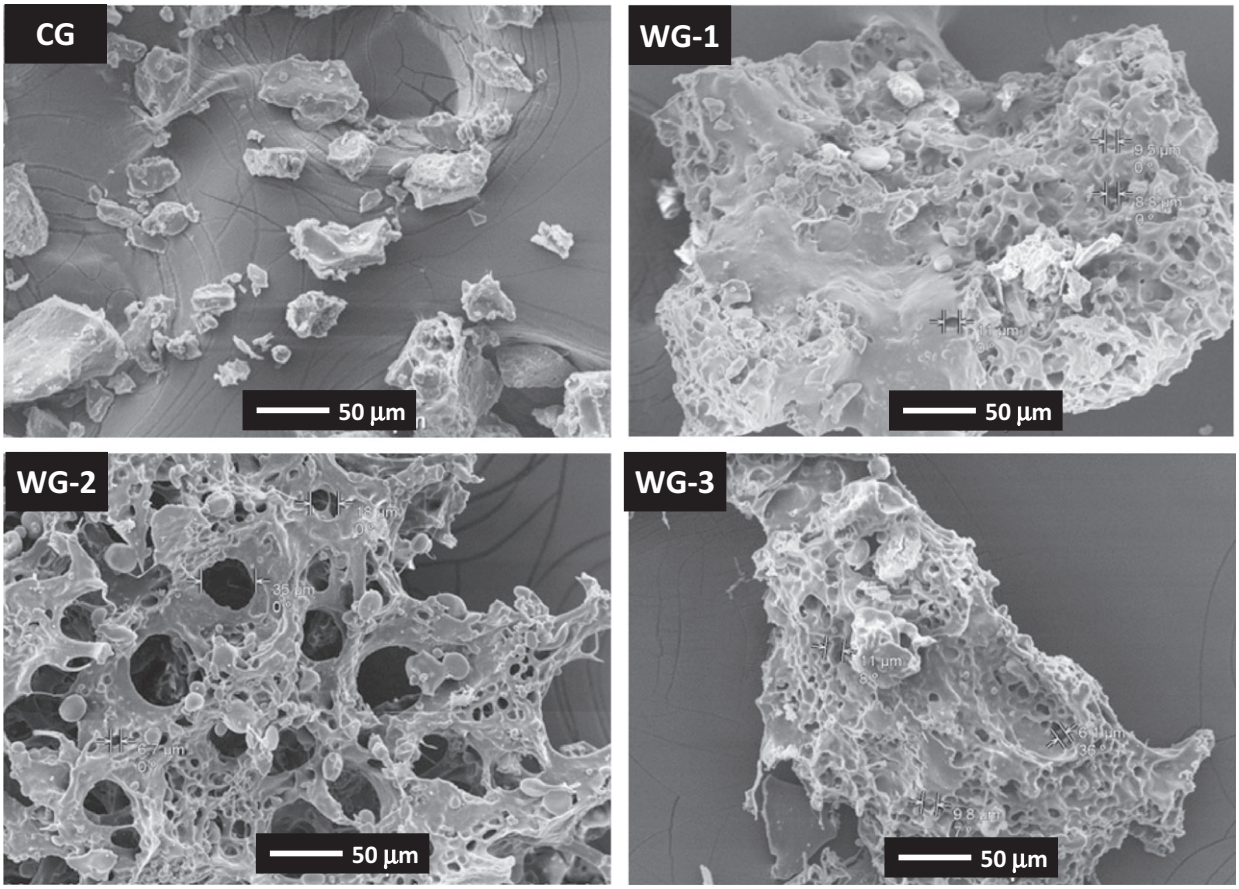

Figure 1 SEM images of CG and acid treated WG samples at pH 1 (WG-1), 2 (WG-2) and 3 (WG-3).

shown in the FTIR spectra in Fig. 2(a) and (b) for samples without and with heat treatment, respectively. At pH 1, -OH peak shifts were observed at *ca.* 3280 cm^{-1} which were not observed for WG-2 and WG-3, whereas after heat treatment, shifts were observed for both WG-1a and WG-3a. The 3065 cm^{-1} peak present in the CG representing aromatic -CH bonds shifted to 3062 and 3059 cm^{-1} in WG-1 and WG-1a, respectively. The peak at 3011 cm^{-1} due to C=CH alkene bonds present in the CG decreased in intensity in WG-3 and completely disappeared in WG-1 and after heat treatment, the same peaks disappeared in WG-1a and WG-2a samples. The -CH peak at 2856 cm^{-1} present in the CG decreased in intensity in WG-3, WG-2 and WG-1 samples and after heat treatment; the peak almost disappeared in WG-1a. The peak present at 1744 cm^{-1} in the CG due to carbonyl stretching significantly broadened and the maxima shifted to 1723 cm^{-1} in WG-1 and WG-1a samples suggesting an increase in carbonyl bonds and could be due to formation of chromophores imparting yellowness at pH 1.

The amide I and II regions were further analysed to investigate the differences in the secondary structure of gluten. In the amide I region (1700-1600 cm^{-1}), the peak maxima at 1645 cm^{-1} in the CG shifted to 1640, 1639 and 1638 cm^{-1} in the WG-1, WG-2 and WG-3 samples, respectively. The relative intensities of the peaks at 1635 decreased whereas those at 1631 and 1618 cm^{-1} increased for all pH treated samples compared to the CG. The amide II (1600–1480 cm^{-1}) region of the CG showed various characteristic peaks which were also present in WG-1, WG-2 and WG-3, but with differences in relative intensities. The CG sample showed peaks at 1540–1516 cm^{-1} with similar relative peak heights, however, all treated samples showed the peak at 1538 cm^{-1} was relatively higher than that at 1516 cm^{-1} in all cases thus showing narrowing of the peaks. Moreover, the peak at 1525 cm^{-1} present in the CG disappeared in all pH treated samples.

The changes in the amide regions are consistent with changes in protein secondary structures as a result of the pH treatment and support the train and loop model which attributes β-sheet conformational changes

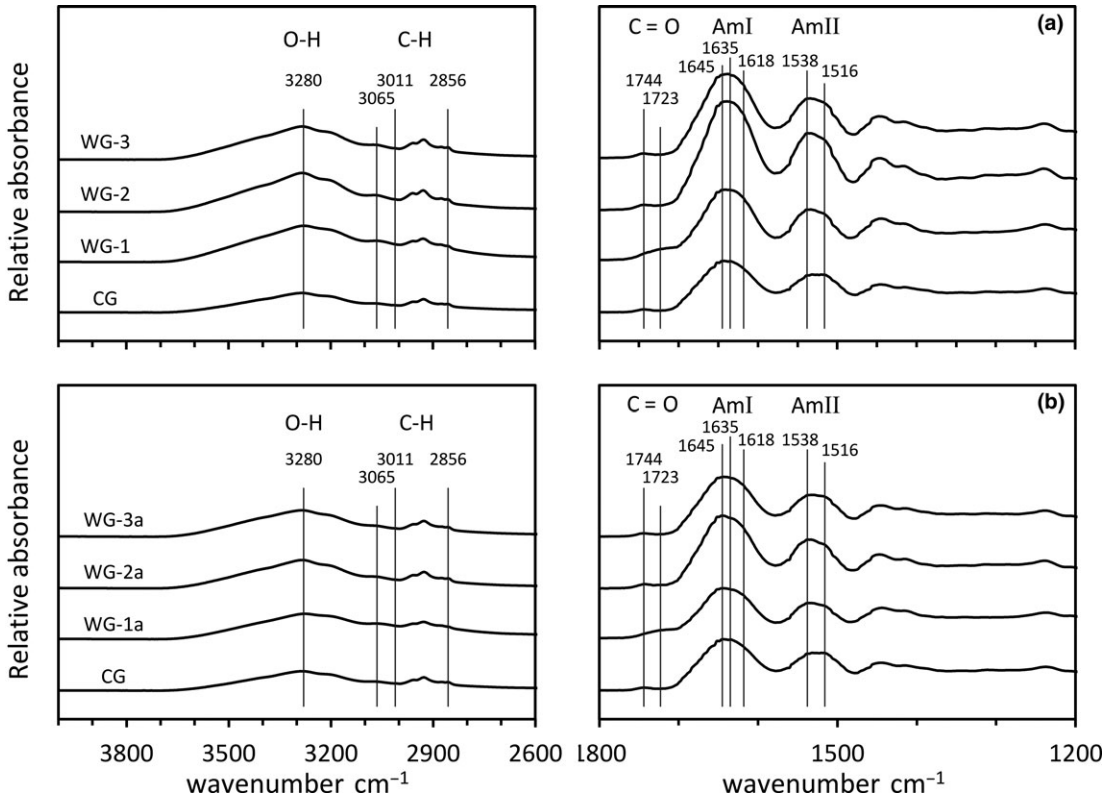

Figure 2 FTIR spectra of CG and acid treated WG samples at pH 1 (WG-1), 2 (WG-2) and 3 (WG-3) before (a) and after (b) heat treatment.

to hydration (Belton, 1999). According to the model, repetitive domains of WG are rich in glutamine residues and tend to form intra- and inter-molecular hydrogen bonds which are associated with β-sheet and described as a 'train'. Under acidic conditions, glutamine residues are converted into negatively charged glutamic acid thus increasing electrostatic repulsion among the β-sheets and forcing them into an 'unzipped form'. This creates unbounded mobile regions described as a 'loop', which could involve the more flexible β-turn, α-helix and random coils (Belton, 1999; Yong *et al.*, 2006).

Changes in free-thiol and amino groups

The free-amino group content was determined to monitor changes in -NH_2 chain ends (α-NH_2 at the protein chain ends and ϵ-NH_2 from lysine residues) arising from protein hydrolysis and the results are presented in Fig. 3(a). The primary amine content of CG was found to be 140.2 μM g^{-1} protein which is in agreement with that reported by Langstraat *et al.* (2015). The free-amino content of WG-1 and WG-2 was higher than the CG whereas that of WG-3 was lower ($P < 0.05$) with the increased free-amino content in WG-1 due to protein hydrolysis and changes in the conformation of gluten exposing more amide groups from within the structure. The lower free-amino content of WG-2 and WG-3 suggested a lower degree of hydrolysis and associated conformational changes. Heat treatment (80 °C for 15 min) at different pH decreased the free-amino content in comparison to samples without heat treatment at the same pH (Fig. 3(a)) due to increased deamidation converting glutamine and asparagine into glutamic and aspartic acids, respectively.

The free-thiol group content plays a very important role in the binding of the hydrated gluten network by intra- and inter-molecular disulphide bonds. The free-thiol group contents of WG-1, WG-2 and WG-3 were significantly lower than 176.6 μM g^{-1} protein found of CG ($P < 0.05$) (Fig. 3(b)). This suggests involvement of free-thiol groups in the cross-linking of gluten proteins (Lagrain *et al.*, 2008). The results are in agreement with SEM images where the gluten network was more pronounced in acid treated samples compared to the control. The higher free-thiol content of WG-1 compared to WG-2 and WG-3 indicates that higher

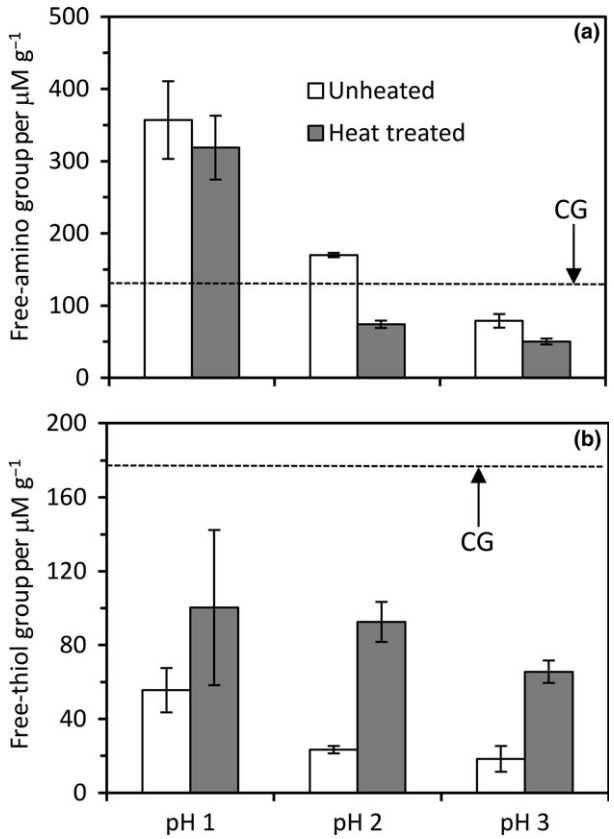

Figure 3 Effect of pH on (a) free-amino content and (b) free-thiol content of CG and acid treated WG with and without heat treatment. Values are μM g^{-1} of protein.

acidity leads to a greater exposure of free-thiol groups. The free-thiol contents of the heat-treated samples were higher compared to the unheated samples at the same pH due to further breakage of disulphide bonds by heat (Fig. 3(b)).

Changes in SDS-extractability of protein and molecular weight distribution

The SDS extractability of gluten is an indicator of the degree of cross-linking whereby a decrease in SDS extractability is indicative of increased cross-linking. Figure 4 shows the SDS-EP chromatogram of the CG and treated samples which suggest that in WG-1, the relative protein content decreased in the gliadin region (7.5–15 min) and increased in the glutenin region (4.7–7.5 min). The decrease in the gliadin peak at 10.5 min and presence of another broad peak between 4.7 and 7.5 min indicates a protein complex is formed involving gliadin in WG-1. The chromatogram also shows a very small peak at 13.5 min indicating hydrolysis has occurred which is in accordance with the higher concentration of free-amino groups (Fig. 3(a)). In WG-2 and WG-3, the relative concentrations of proteins at different retention times were not significantly affected compared to the CG sample, however, the SDS extractability decreased due to the formation of intermolecular cross-linking rendering protein un-extractable (Lagrain *et al.*, 2011).

Also shown in Fig. 4 are the differences in the SDS-EP chromatograms of samples after heat treatment at different pH. Disappearance of the peak at 5 min and decreased peak intensity at 11 min were evident in all heat-treated samples. In WG-1a, peaks are present at 7.6 and 9 min, however, in WG-2a and WG-3a, the peaks eluted at higher retention times of 8.2 and 9.6 min indicating formation of cross-links in WG-1a. The presence of a small peak between 13 and 14 min in pH 1 treated samples (both heated and non-heated) and absence of peaks in samples at pH 2 and 3 suggest increased hydrolysis at pH 1 in accordance with the higher free-amino group content (Fig. 3(a)), while no such peak appeared in samples at pH 2 and 3.

Fig. 5(a) presents the SDS-PAGE gels under non-reducing conditions. Protein bands at the top of each lane in all samples (heated and non-heated) are due to the presence of high MW protein complexes that could not penetrate the gel. Characteristic bands of gluten present in the CG sample were absent in WG-1 and WG-1a and a smear was present between 37 and 50 kDa. A smear at the end of the lane for WG-1a may be due to protein hydrolysis products. However, at pH 2 and 3 for both heated and non-heated samples, the distribution of bands are similar to the CG. These results confirm patterns similar to the SDS-EP chromatogram (Fig. 4).

Under reducing conditions (Fig. 5(b)), the CG bands identified are corresponding to high MW glutenins (HMW-GS), low MW glutenins (LMW-GS) (group B and C); α-, β-, γ- and ω-gliadins, and α-amylase trypsin inhibitors (Liao *et al.*, 2016a). When compared to the non-reducing conditions, the bands in the top of lane in WG-2, WG-2a, WG-3 and WG-3a samples disappeared but these were present in WG-1 and WG-1a under reducing conditions (Fig. 5(a and b)). This suggests that the complex formed at pH 1 involves covalent bonds other than disulphide which cannot be reduced by β-mercaptoethanol and these may involve tyrosyl–tyrosyl cross-links (Tilley *et al.*, 2001; Pena *et al.*, 2006). The bands corresponding to different proteins (glutenins, gliadins and α-amylase trypsin inhibitors) were present at same position as for pH 2 and 3 samples irrespective of heat treatment. In WG-2 and WG-2a, the intensity of the bands decreased, but this was not observed in WG-3 and WG-3a. These results suggest involvement of protein aggregation and hydrolysis due to heating as reported previously using citric acid with temperatures >90 °C (Liao *et al.*, 2016b).

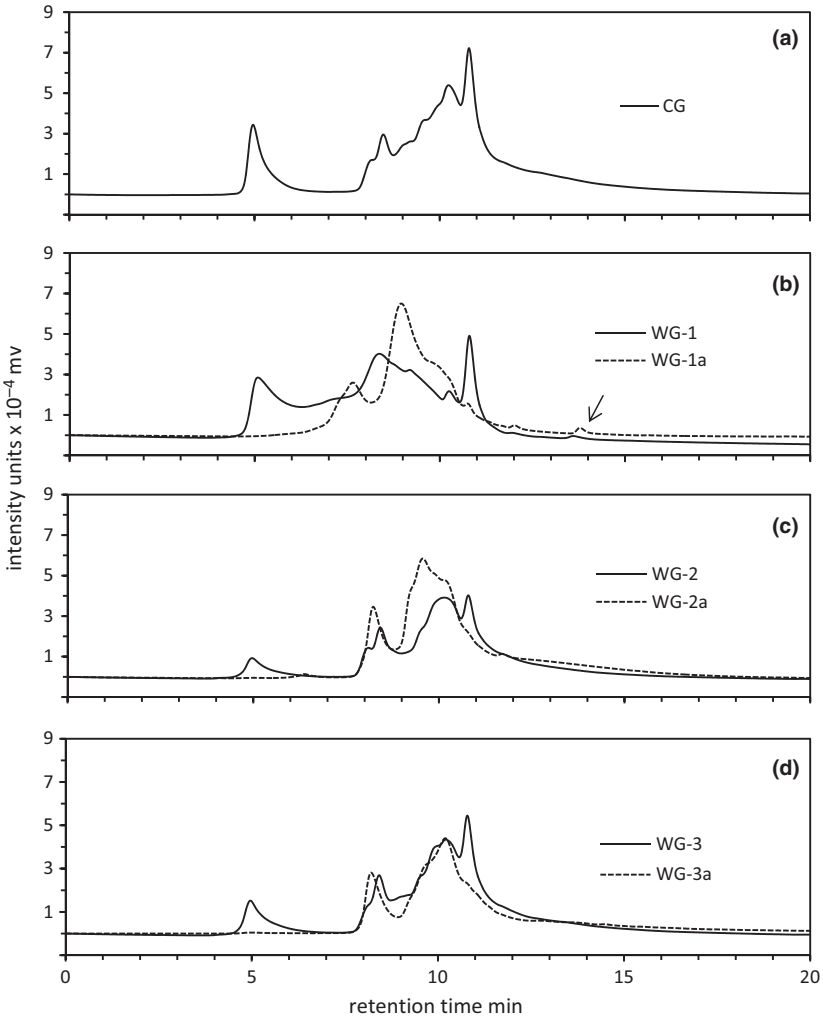

Figure 4 SDS-EP chromatograms of CG and acid treated WG with and without heat treatment, (a) control gluten, (b) acid treated wheat gluten at pH-1, (c) acid treated wheat gluten at pH-2, (d) acid treated wheat gluten at pH-3.

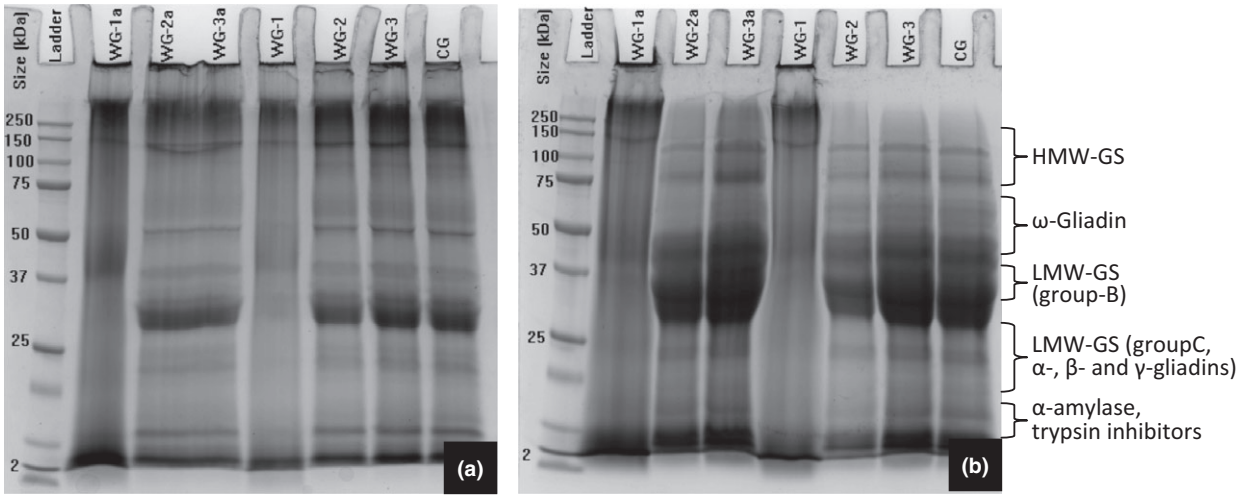

Figure 5 SDS-PAGE patterns of CG and acid treated WG with and without heat treatment under (a) non-reducing and (b) reducing conditions.

Conclusions

This study suggests that suspending WG in aqueous solutions at acidic pH (1, 2 and 3) changes the structure, free functional groups and size distribution of the gluten. At pH 1, significant change in colour, SDS extractability, free-amino content and thiol content was observed. At higher pH (2 and 3), decreased SDS extractability, decreased free-thiol and free-amino content confirmed lower hydrolysis when compared to samples treated at pH 1. These changes were more pronounced upon heating (80 °C for 15 min). In addition to higher hydrolysis at pH 1, the presence of SDS-PAGE bands also confirmed high MW complex formation due to cross-linking. Further research is needed to confirm whether these differences in the structure and size distribution during hydrothermal treatment of gluten at different pH increase or decrease susceptibility of gluten to increased hydrolysis using enzymes.

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