Application of direct-sequencing peptide proteomics to the characterization of antagonistic (endogenous and exogenous) proteins in cereal grains

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In a Wheat Field, Evening Shadows
James Edward Hervey MacDonald
Canada, 1929
Abstract

The cereal seed plays a crucial role in society – both in the “food as medicine” paradigm, but also in food security. It is the starch and proteins present in the seed that lend it importance in these dissimilar anthropomorphic activities. This thesis investigation first characterized the post-translational processing of the potential diabetogen, wheat globulin-3. Globulin-3-like peptides were observed primarily in the embryo. These peptides varied significantly in their molecular masses and isoelectric points, as determined by two dimensional electrophoresis and immunoblotting. Five major polypeptide spots were sequenced by mass spectrometry, allowing for the development of a model of the post-translational events contributing to the globulin-3 processing profile. Three separate investigations of starch granules from different cereal species were performed. In the first series of experiments, pathogen-susceptible maize kernels were injected with either conidia of the fungal pathogen *Fusarium graminearum* or sterile water controls. Proteins in the desiccated fungal remnants on the surface of the kernels as well as in the endosperm and embryo tissues of the control and infected kernels were isolated and these proteomes were sequenced using tandem mass spectrometry. Approximately 250 maize proteins were identified. These proteins were classified into functional categories. There was an increased representation of defense proteins in the both the embryo and endosperm tissues of infected maize samples. The proteome of the fungal remnants was composed of 18 proteins. Several of these proteins were categorized as being involved in the metabolism of plant-sourced molecules, or in stress response. The second series of experiments detail the investigation of commercially prepared rice and maize starches using tandem mass spectrometry. The majority of identified proteins,
in both rice and maize samples, were involved in either carbohydrate metabolism or storage. Markers for seed maturity and for starch mobilization were also documented. Finally, the third series of experiments investigated the non-host proteomes present in commercially-prepared starches. Non-host proteins from a variety of species, including *Homarus americanus* were found in the starch samples. This documentation of *H. americanus* proteins in these starch samples may have food safety implications with regards to shellfish allergies.
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# Table of Contents

Abstract ............................................................................................................................... ii
Acknowledgements ............................................................................................................ iv
Table of Contents ................................................................................................................ v
List of Figures .................................................................................................................... xi
List of Tables ..................................................................................................................... xii
Chapter 1 ............................................................................................................................. 1
   Introduction ......................................................................................................................... 1
      1.1 Preamble – Research outline ..................................................................................... 2
      1.2 The Seed .................................................................................................................... 2
         1.2.1 Evolution ............................................................................................................ 2
         1.2.2 Physiology of fruit and seed structures .............................................................. 3
      1.3 Storage proteins ......................................................................................................... 5
         1.3.1 Globulins ............................................................................................................ 6
         1.3.2 Globulins and human health ............................................................................... 9
         1.3.3 Globulin summary ............................................................................................ 12
      1.4 Starch granules ........................................................................................................ 12
         1.4.1 Starch granule surface ...................................................................................... 15
         1.4.2 Starch granule summary ................................................................................... 18
      1.5 Plant/pathogen interactions ..................................................................................... 18
         Summary of plant/pathogen interactions ................................................................... 22
      1.6 Mass spectrometry ................................................................................................... 22
      1.6 Research Hypotheses and Objectives ...................................................................... 30
Chapter 2 ........................................................................................................................... 34
   Seed storage proteins of the globulin family are cleaved post-translationally in wheat embryos ............................................................................................................................. 34
      2.1 Abstract ................................................................................................................... 35
      2.2 Findings ................................................................................................................... 36
      2.3 Results ..................................................................................................................... 38
         2.3.1 Glo-3 antigenically-related proteins co-isolate with wheat globulins .............. 38
         2.3.2 The Glo-3-related proteins are primarily located in the embryo ...................... 38
2.3.3 Identification of selected Glo-3-related polypeptides ........................................ 41
2.3.4 Characterization of selected Glo-3-related polypeptides ........................................ 42
2.4 Discussion .................................................................................................................. 46
  2.4.1 Type 1 diabetes .................................................................................................... 46
  2.4.2 Characterization of Glo-3 ................................................................................ 47
  2.4.3 Post-translational processing of Glo-3 ............................................................ 48
2.5 Conclusion .................................................................................................................. 52
2.6 Methods .................................................................................................................... 53
  2.6.1 Wheat seed protein extraction and sample preparation ..................................... 53
  2.6.2 Sample preparation for 1D separation ............................................................... 54
  2.6.3 Sample preparation for 2D separation ............................................................... 54
  2.6.4 1D SDS-PAGE protein fractionation ............................................................... 54
  2.6.5 Immunoblot analysis ......................................................................................... 55
  2.6.6 Two-dimensional gel electrophoresis (2DE) ..................................................... 56
  2.6.7 Liquid chromatography tandem mass spectrometry (LC-MS/MS) ................. 57
  2.6.8 N-terminal sequencing ..................................................................................... 58

Chapter 3 .......................................................................................................................... 59
Taking stock of the protein remnants on the battlefield between host and pathogen: Maize
CL30-Fusarium interactome .......................................................................................... 59
  3.1 Abstract ................................................................................................................... 60
  3.2 Introduction ............................................................................................................. 61
  3.3 Results ..................................................................................................................... 63
  3.3 Discussion ............................................................................................................... 65
    3.3.1 Percent representation ................................................................................... 68
    3.3.2 Proteinase inhibitors .................................................................................... 68
    3.3.3 Chitinases ...................................................................................................... 69
    3.3.4 Xylanase inhibitors ....................................................................................... 70
    3.3.5 Ribosome inactivating proteins .................................................................... 71
    3.3.6 Peroxidases .................................................................................................. 72
    3.3.7 Fusarium on the surface ............................................................................. 73
    3.3.8 Fusarium metabolism proteins .................................................................. 74
Chapter 3: Fusarium Stress Response

3.3.9 Fusarium stress response .............................................................................................................. 75

3.4 Conclusions ................................................................................................................................. 76

3.5 Methods ......................................................................................................................................... 77

Chapter 4: The Starch Granule Associated Proteomes of Commercially Purified Starch Reference Materials from Rice and Maize

4.1 Abstract ........................................................................................................................................ 81

4.2 Introduction ................................................................................................................................ 82

4.3 Experimental procedures ......................................................................................................... 85

4.3.1 Sampling .................................................................................................................................. 85

4.3.2 Starch granule preparation .................................................................................................... 85

4.3.3 Peptide preparation ................................................................................................................. 85

4.3.4 Chromatography and mass spectrometry ............................................................................. 86

4.3.5 Protein identification ................................................................................................................ 87

4.4 Results ........................................................................................................................................... 88

4.4.1 Starch granule associated proteins in rice ............................................................................ 88

4.4.2 Starch granule associated proteins in maize ....................................................................... 92

4.5 Discussion ..................................................................................................................................... 97

4.5.1 Starch granule associated proteins ......................................................................................... 100

4.5.2 Starch biosynthetic enzymes ................................................................................................. 101

4.5.3 Orthophosphate dikinase ........................................................................................................ 102

4.5.4 Starch mobilization .................................................................................................................. 102

4.5.5 14-3-3 proteins ....................................................................................................................... 103

4.5.6 Storage proteins ...................................................................................................................... 105

4.6 Conclusions ................................................................................................................................. 106

Chapter 5: Commercially Produced Rice and Maize Starches Contain Non-Host Proteins, as Shown by Mass Spectrometry

5.1 Abstract ........................................................................................................................................ 109

5.2 Introduction ................................................................................................................................ 110

5.3 Methods ....................................................................................................................................... 112
List of Abbreviations

1D: one-dimensional  
2D: two-dimensional  
3D: three-dimensional  
ABA: abscisic acid  
ADP: adenine-diphosphate glucose  
AGPase: ADP-glucose pyrophosphorylase  
Beg: barley embryo globulin  
BCA: bicinchoninic acid  
BLAST: basic local alignment search tool  
Bt1: brittle 1  
CBB: Coomassie Brilliant Blue  
CHAPS: 3-([3-cholamidopropyl]dimethylamino)-1-propanesulfonate  
CID: collision-induced dissociation  
DAP: days after pollination  
DON: deoxynivalenol  
DTT: dithiothreitol  
GBSS: granule bound starch synthase  
Glo-3: globulin-3  
ESI: electrospray ionization  
FDR: false discovery rate  
G1P: glucose-1-phosphate  
G6P: glucose-6-phosphate  
HR: hypersensitive response  
HRP: horseradish peroxidase  
IEF: isoelectric focusing  
IgE: immunoglobulin E  
IgG: immunoglobulin G  
IPG: immobilized pH gradient  
KEGG: Kyoto Encyclopedia of Genes and Genomes  
LC: liquid chromatography  
LDS: linoleate diol synthase  
LEA: late embryogenesis protein  
Ma: million years ago  
MS: mass spectrometry  
MS/MS: tandem mass spectrometry  
NCBI: National Center for Biotechnology Information  
NCBInr: National Center for Biotechnology Information non-redundant  
OYE: old yellow enzyme  
OISB: Ottawa Institute of Systems Biology  
PGM: phosphoglucomutase  
PI: proteinase inhibitor  
pI: isoelectric point  
PPDK: orthophosphate dikinase  
PR: pathogenesis-related
PSV: protein storage vacuole
Q: mass-resolving quadrupole
q: radio frequency quadrupole
Reg: rice embryo globulin
RIP: ribosome inactivating protein
ROS: reactive oxygen species
S: Svedberg unit
SBE: starch branching enzyme
SDBE: starch debranching enzyme
SDH: sorbitol dehydrogenase
SGAP: starch granule associated protein
SGS: starch granule surface
SOD: superoxide dismutase
SS: starch synthase
SuSy: sucrose synthase
T1D: type 1 diabetes
TBP: tributylphosphine
TCA: trichloroacetic acid
TMV: Tobacco mosaic virus
TOF: time of flight
UDP: uracil diphosphate
UDPG: uracil diphosphate glucose
UGPase: UTP-glucose-1-phosphate uridylyltransferase
USD: United States dollars
UTMB: University of Texas Medical Branch
WDEIA: wheat-dependent exercise-induced anaphylaxis
List of Figures

Figure 1.1: Ovule morphology................................................................. 4
Figure 1.2: Three-dimensional structure of jack bean 7S globulin trimers........... 7
Figure 1.3: Immunolocalization of globulin-3 in developing wheat embryos......... 10
Figure 1.4: Scanning electron micrographs of wheat starch granules.................... 14
Figure 1.5: Organization of amylopectin and starch granules............................ 16
Figure 1.6: Channels in starch granules may contain proteins............................ 17
Figure 1.7: Damaged starch granules of spring wheat infected with Fusarium......... 23
Figure 1.8: Functional distribution of proteins identified in plastids.................... 24
Figure 1.9: Schematic of a triple quadrupole mass spectrometer........................ 27
Figure 1.10: Types of peptide fragment ions observed in an MS/MS spectrum......... 29
Figure 2.1: SDS-PAGE and immunoblot analysis of AC Barrie salt-soluble proteins. 39
Figure 2.2: 2D gels and immunoblot analysis of AC Barrie salt-soluble proteins...... 40
Figure 2.3: Model of Glo-3 endoproteolytic processing.................................... 45
Figure 3.1: Percent representation of maize functional protein categories............ 64
Figure 4.1: Functional classification of identified proteins.................................. 90
Figure 4.2: Workflow and validation of proteome characterization....................... 104
Figure S1: Silver stained SDS-PAGE of protein extracts.................................. 157
### List of Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Families of PR proteins</td>
<td>20</td>
</tr>
<tr>
<td>2.1</td>
<td>MS/MS sequencing results of selected gel spots of wheat 7S globulins</td>
<td>42</td>
</tr>
<tr>
<td>3.1</td>
<td>Defense proteins in maize samples</td>
<td>66</td>
</tr>
<tr>
<td>3.2</td>
<td>Proteome of desiccated fungi on the surface of infected maize kernels</td>
<td>67</td>
</tr>
<tr>
<td>4.1</td>
<td>Proteins involved in carbohydrate metabolism from <em>Oryza sativa</em> starch</td>
<td>91</td>
</tr>
<tr>
<td>4.2</td>
<td>Proteins involved in cellular processes from <em>Oryza sativa</em> starch</td>
<td>93</td>
</tr>
<tr>
<td>4.3</td>
<td>Seed storage proteins from <em>Oryza sativa</em> starch</td>
<td>94</td>
</tr>
<tr>
<td>4.4</td>
<td>Proteins involved in carbohydrate metabolism from <em>Zea mays</em> starch</td>
<td>96</td>
</tr>
<tr>
<td>4.5</td>
<td>Proteins involved in cellular processes from <em>Zea mays</em> starch</td>
<td>98</td>
</tr>
<tr>
<td>4.6</td>
<td>Seed storage proteins from <em>Zea mays</em> starch</td>
<td>99</td>
</tr>
<tr>
<td>5.1</td>
<td>Non-host protein contaminants associated to rice starch granule</td>
<td>115</td>
</tr>
<tr>
<td>5.2</td>
<td>Non-host proteins identified in maize starch samples</td>
<td>117</td>
</tr>
<tr>
<td>S.1</td>
<td>Proteins isolated from commercially purified wheat starch</td>
<td>158</td>
</tr>
</tbody>
</table>
Chapter 1

Introduction
1.1 Preamble – Research outline

Written in manuscript format, the experiments detailed in this thesis use mass spectrometry techniques (MS) to investigate two separate tissues within the cereal seed: the protein matrix and starch granules. To provide a biological overview for these experiments, the introduction is divided into four distinct sections. The first section provides a summary of seed evolution and physiology, the second section details the components of the protein matrix, with an emphasis on the wheat storage globulins. Starch granule development, morphology, and previous proteomic studies are presented in the third section, while the fourth section provides insight into the mass spectrometry techniques used in the experiments performed for this thesis.

1.2 The Seed

1.2.1 Evolution

*Elksinia polymorpha*, the first known plant to bear seeds, evolved in the Late Devonian (Famennian), 365 million years ago (Ma) (Gerrienne et al., 2004). It has been hypothesized, however, that seeds could have evolved as early as between 385 and 365 Ma in vascular plants that belonged to the paraphyletic group termed Pteridospermae, or seed ferns. The seed ferns evolved from progymnosperms, which employed pteridophytic reproduction (spores), but also featured certain vegetative features common to seed ferns (Linkies et al., 2010). The transition from spores to seeds as a reproduction mechanism required the evolution of three individual traits: heterospory, integuments, and pollen-receiving structures (Taylor and Taylor, 2009). Heterospory is the specialization of spores...
into haploid male-like microspores and female-like megaspores, and integuments are protective layers around the nucellus (Esau, 1977).

1.2.2 Physiology of fruit and seed structures

The common features of all seeds are that, independent of plant lineage, seeds are composed of an embryo, a protective seed coat, and a nutrient source (Linkies et al., 2010). In the case of gymnosperms, seeds are not covered by an ovary, and are attached to the cone scales (< Greek gymnospemos “naked seed”). In contrast, angiosperm seeds are enclosed by the ovary (< Greek angeion “vessel” and sperma “seed”) (Figure 1.1).

The angiosperm cereal *Triticum aestivum* (wheat) is one of the most economically important crop plants, as approximately 60% of the proteins and calories consumed globally by humans are derived from wheat. As the United States Department of Agriculture estimates that more than 356 million metric tons of wheat are consumed annually, wheat is a good model seed system to study. The majority of calories, for both the embryo, and for human consumption, from harvested wheat are derived from the endosperm, which accounts for approximately 83% of total seed weight (Pomeranz et al., 1970). Endosperm tissue arises from double fertilization events that are unique to angiosperms. During the fertilization of the egg cell, a fertilization of a second cell of the megagametophyte by two different sperm cells occurs, leading to the formation of the triploid, nutritive, and supportive tissue that supplies energy to the embryo post-wintering (Floyd and Friedman, 2000). Through an initial cell enlargement and endopolyploidy phase, followed by a cellularization phase, the endosperm tissue is able to grow at very
Figure 1.1: Ovule morphology.

a, Angiosperm ovule. b, Gymnosperm ovule. Legend: i, integument (covering); ii, inner integument; m, micropyle (opening); oi, outer integument; s, stalk. Reproduced from Frohlich and Chase, 2007 with permission Macmillan Publishers Ltd: Nature.
rapid rates (Olsen, 2004). The endosperm cells are composed of 50-90% compact, high
density starch granules, and seed storage proteins.

1.3 Storage proteins

Seed storage proteins serve as sources of carbon, nitrogen, and sulphur source for
germinating embryos (Shewry and Tatham, 1990). Additionally, the nutritional value of
the seeds for human consumption is dependent on the amino acid balance of the storage
proteins. For example, storage proteins high in the amino acids lysine, tryptophan and
methionine, are considered to have a very good amino acid balance (Borlaug, 1983). Seed
proteins are sequestered in an apoptotic, anhydrous protein matrix or in protein bodies
called protein storage vesicles (Hara-Nishimura et al., 1998). Intended to be digested and
subsequently taken up by the sprouting grain, they are now anthropomorphically
classified based on their solubility in certain solvents. Albumins are soluble in water and
dilute buffers, globulins are soluble in salt solutions, prolamins are soluble in 70%
alcohol, and glutelins are soluble in dilute acids or bases (Osborne, 1908). Certain storage
proteins are soluble in more than one solvent or have sequences and/or structures closely
related to proteins of another solubility class. For example, the α-globulins of wheat,
maize and rice are 18-25 kDa proteins that are soluble in saline solutions, but share
significant sequence identity with a high molecular weight glutenin from wheat (Gu et al.,
2004; Shorrosh et al., 1992; Woo et al., 2001). Additionally, rice glutelins are soluble in
NaOH, but have related 3D structures to the 11S globulins of oat (Robert et al., 1985;
Robert et al., 1985).
Depending on the plant species, the relative proportions of the seed storage proteins can be significantly different. In legumes such as chick pea, the majority of the storage proteins are globulins, with distributions of total protein as follows: albumin (8-12%), globulin (53-60%), prolamin (3-6%) and glutelin (19-24%) (Dhawan et al., 1991). This is very different than the distribution of storage proteins in wheat, where albumins and globulins represent 15% of total endosperm proteins, and prolamins (53%), and glutelins (32%) are the most predominant proteins (Shewry and Halford, 2002).

1.3.1 Globulins

The globulin family of seed storage proteins is of particular interest, as dietary exposure to certain members of this protein family have been implicated in the development of type 1 diabetes in certain mammals (MacFarlane et al., 2003). There are two categories of globulins based on their sedimentation coefficients: 7-9S and 11-13S (Danielsson, 1949). Orthologs to the 7S and 11S globulin protein families, vicilins and legumins, respectively, were first identified in the Leguminoseae (Derbyshire et al., 1976). Globulins are encoded by multigene families, and have characteristic β-barrel cupin domains (Dunwell, 1998) (Figure 1.2). The 7-9S globulins are arranged as homo- or hetero-trimers (Lawrence et al., 1994), while the 11-13S globulins are assembled into hexameric configurations (Adachi et al., 2001). Both the globulins and the legumins undergo significant post-translational modifications, including glycosylation and endoproteolytic cleavage prior to their storage in seed protein storage vesicles in the embryo (Hara-Nishimura et al., 1998).
Figure 1.2: Three-dimensional structure of jack bean 7S globulin trimers. The N-terminal and the C-terminal domains, coloured red and blue, respectively, are arranged about the threefold axis. From Ko et al., 2000, reproduced with permission of the International Union of Crystallography.
In cereal crops, there have been several studies characterizing the 7S globulins in barley and maize, (Heck et al., 1993; Kriz, 1989), and more recently in Brachypodium distachyon (Larre et al., 2010). However, these proteins are still poorly characterized in wheat and oats. The lack of information on oat globulins is unusual, as oats are the first foodstuff to be allowed a medical health claim by the U.S. Food and Drug Administration stating that consumption of soluble fiber from whole oats reduces the risk of heart disease. The expression of 7S globulins is primarily in the embryo and aleurone layer (Sun et al., 1996), with little to no evidence of globulin expression in the endosperm (Loit et al., 2009). Expression of globulins is transcriptionally regulated by the phytohormone abscisic acid (ABA) (Rivin and Grudt, 1991). As globulins are expressed primarily in the embryo and contain increased levels of nitrogen-rich amino acids arginine, lysine, aspartic acid and glutamic acid (Zhu et al., 2006), it is hypothesized that the globulins have evolved to be used as an easily exploitable source of amino groups and energy for germinating embryos (Shewry and Tatham, 1990).

There are two members of the 7S globulin protein family present in multiple cereal species: globulin 1 (Glb1) and globulin 2 (Glb2) in maize, barley embryo globulins (Begl and Beg2) in barley and rice embryo globulins (Reg1 and Reg2) in rice (Sun et al., 1996; Yupsanis et al., 1990). Previous studies on Glb1, Beg1 and Reg1 have shown that these proteins are all encoded by single copy genes (Belanger and Kriz, 1989; Heck et al., 1993; Sun et al., 1996). However, there is evidence to suggest that Beg1 may belong to a small multigene family (Zhang et al., 2004). The expression patterns of the members of the protein family can be significantly different, as the rice embryo globulins as well as
Begl transcripts are highly expressed during early germination, whereas Beg2 transcript levels decrease during the germination process (Zhang et al., 2004).

In wheat, there are three members of the globulin protein family: globulin-1, -2 and -3, as well as triticin (Gomez et al., 1988; Singh et al., 1991). Globulin-3A is a potential food allergen (Larre et al., 2011), and has been associated with an increased risk for the development of type 1 diabetes (MacFarlane et al., 2003) and celiac disease (Taplin et al., 2011) in susceptible individuals. The globulin-3 gene family in wheat cultivar Glenlea contains three individual globulin-3 genes: Glo-3A, Glo-3B, and Glo-3C (Loit et al., 2009). These genes share a moderate to high degree of nucleotide sequence identity (73-93%) and are expressed exclusively in the embryo and aleurone layer (Figure 1.3).

Previous investigations into the post-translational endoproteolytic cleavage of the globulin-3 family have shown that these proteins can be processed at internal cleavage sites to yield polypeptides with a range of molecular weights and pI values (Dupont et al., 2011; Singh et al., 2001). To gain a more precise understanding of the processing events that orchestrate our dietary proteomes, a targeted study of these processed proteins is required to understand their role in the development of human diseases.

1.3.2 Globulins and human health

Wheat seed storage proteins, including globulin-3, and the γ-gliadins have been associated with the development of wheat allergies, wheat sensitivity and celiac disease (Tatham and Shewry, 2008). Certain occupations have higher incidences of wheat allergies; baker's asthma and rhinitis can affect as many as 30% of professional bakers
Figure 1.3: Immunolocalization of globulin-3 in developing wheat embryos. Panel A: wheat aleurone (al) cells were positively stained while endosperm (es) cells were unstained when sections were stained with Glo-3A antibodies. Panel (C): wheat embryonic (em) tissue stained with Glo-3A antibodies. Green staining in seed coat (sc) shows unspecific staining. Green (examples indicated with arrowheads) indicates positive staining. Reproduced with permission from Loit et al., 2009.
Though these allergies were recognized by the Romans, it was not until the 1970s that wheat albumins were found to be reactive with immunoglobulin E (IgE) antibodies from individuals suffering from baker’s asthma (Baldo and Wrigley, 1978). The allergies in these individuals are responses to the inhalation of wheat flour and dust particles.

Type 1 diabetes (T1D) is an autoimmune disease characterized by the destruction of the insulin producing β-cells in the pancreatic islets of Langerhans and the loss of glucose homeostasis (Jahromi and Eisenbarth, 2007). It has been hypothesized that a dysfunctional gut barrier may be involved in disease onset in certain individuals (Maurano et al., 2005). While the incidence of T1D in developed countries has risen by 3% annually since 1960 (Onkamo et al., 1999), there is still no definitive cause of this disease. Identified risk genes impart genetic susceptibility to T1D, but there is under 50% concordance in monozygotic twins (Todd, 2010). Therefore, the development of T1D likely relies on environmental triggers, such as diet. Alternatively, the hygiene hypothesis states that the reduced exposure to infectious agents during childhood is responsible for the increased prevalence of T1D (Yazdanbakhsh et al., 2002). In current societies, with heightened standards for sanitation, healthcare and diet, the natural development of the immune system can be repressed by the lack of exposure to T1D protective factors, such as symbiotic prokaryotes and certain parasites (Todd, 1991).

Celiac disease, also known as gluten enteropathy, or sprue, is a hereditary immune system disorder (Fasano, 2001). The intestinal epithelium and the tight intercellular junctions in healthy individuals are intact and do not permit dietary antigens to pass the gut barrier (Heyman et al., 2012). However, the tight junction system becomes damaged in subjects
with celiac disease (Shan et al., 2002). In these individuals, the immune systems can be stimulated by dietary antigens, such as gliadins that pass through the tight junctions (Tjon et al., 2010). An increased expression of the tight junction modulating protein zonulin may have a role in the degradation of the tight junctions (Lammers et al., 2008). Celiac disease is one of the most common genetic diseases, with worldwide prevalence of approximately 0.3-1% (Catassi et al., 1994).

### 1.3.3 Globulin summary

As the mechanisms of the development of diseases such as type 1 diabetes and celiac disease are still poorly understood, the implication of dietary antagonists is an important avenue to explore. Further research is necessary to discover how globulin-3 is involved with disease development, and whether the post-translation processing of globulin-3 is a factor in the development of these diseases.

### 1.4 Starch granules

Photosynthesis fixes carbon dioxide using the reductant generated through the light reactions of photosynthesis. The reductant can be subsequently oxidized to support the energy needs of plant growth and development. Energy generation and consumption needs vary depending on light levels and the developmental stage of plants. Therefore the capacity to store energy as inert starch granules in the form of complex polysaccharides composed of monomeric glucose molecules linked by glycosidic bonds is a necessity.
Starch typically accumulates in photosynthetically active chloroplasts or in amyloplasts in non-photosynthetic tissues, which specialize in starch storage (Tetlow, 2011).

Starch formation in wheat amyloplasts is associated with stromules (stroma-filled tubules) during the development of the endosperm in cereals (Natesan et al., 2005). It was determined that A-type granules (>15 µM) are initiated in undifferentiated plastids in the coenocytic endosperm (Bechtel and Wilson, 2003). Following cellularization of the endosperm, B-type granules (5-15 µM) form in stromules protruding from plastids containing A-type granules in the subaleurone layer. C-type granules (< 5 µM) are initiated in branched stromules that form cytosolic networks (Bechtel and Wilson, 2003) (Figure 1.4). Similar development of starch granules and stromules was observed in cells of the central endosperm (Bechtel and Wilson, 2003). Starch granules are composed of 98-99% amylose and amylopectin molecules (Buléon et al., 1998). These polysaccharide chains are composed of glucose monomers linked by \( \alpha-1,4 \) or \( \alpha-1,6 \) glycosidic bonds (Ball and Morell, 2003). Amylose contains 99% \( \alpha-1,4 \) bonds and 1% \( \alpha-1,6 \) linkages and therefore is essentially a long, linear molecule compared to amylopectin, which contains 95% \( \alpha-1,4 \) linkages and 5% \( \alpha-1,6 \) linkages (Tester et al., 2004). The crystalline structure of starch granules is attributed to the dense packing of the double helices of amylopectin (Perez and Bertoft, 2010) (Figure 1.5). The ratio of amylose to amylopectin can range significantly between starch granules: from under 1% amylose in waxy starches to greater than 70% amylose in high amylose starches (Buléon et al., 1998). The composition of most starch granules ranges from 20-35% amylose (Tester et al., 2004). The size, shape, and composition of starch granules can vary between species. Wheat starch granules are composed of 36% amylopectin (Morrison et al., 1994), and have a bimodal size
Figure 1.4: Scanning electron micrographs of wheat starch granules. Panels A and B are mature wheat endosperm tissue (Large A-type granules (>15 μm), medium B-type granules (5–15 μm) and small C-type granules (<5 μm) are present in panels A and B. B is a magnified image of the boxed portion in A. C shows starch granules isolated from mature endosperm. D shows a fractured starch granule, revealing the layers of starch. Reproduced from Rahman et al., 2000 with permission from Elsevier.
distribution: type A granules, range from 15 to 35 µm in diameter with a lenticular shape and the smaller type B granules range from 2 to 10 µm with a spherical beach ball shape (Tester et al., 2004). In contrast, rice starch granules have an unimodal size distribution, are approximately 3 to 8 µm in size (Cledat et al., 2004) and are composed of 47-51% amylopectin (Qi et al., 2003). During development, polyhedral compound granules are generated through the interactions of the growing granules (Jane et al., 1994). The shape, size and composition of starch granules all have impacts on the bioutilization, the end use properties as well as on the nutritional benefits of starch for both embryo and predators. The nature of the starch granule surface, and particularly the presence of surface lipids and proteins, has many significant effects on the properties of the starch.

### 1.4.1 Starch granule surface

The starch granule surface is characterized by the presence of certain features, including pores, protrusions, and channels (Fannon et al., 2003; Fannon et al., 2004) (Figure 1.6). The occurrence, size and number of these topological features is dependent on the species, tissue, and starch granule type (Huber and BeMiller, 2000). In addition to these topological features, proteins have been observed on the starch granule surface using atomic force microscopy (Baker et al., 2001) and other techniques, including mass spectrometry (Wall et al., 2010) and immunofluorescence (Lauriere et al., 1986). These starch granule associated proteins (SGAPs) include a number of proteins of varying molecular weight, including several isoforms of the starch granule bound starch synthases (GBSS), starch synthases (SS) and starch branching enzymes (SBE).
Figure 1.5: Organization of amylopectin and starch granules.
(A) Schematic view of a starch granule with its succession of amorphous and crystalline growth rings. (B) Relation between a section of a crystalline growth ring of the granule and the molecular organization of amylopectin. (C) The succession of ten alternating lamellae in context to the primary structure of a portion of an amylopectin molecule. Each line represents an α-1,4 linked glucan chain. The chains are hooked together by α-1,6 branches. The dotted line delimits the sections appearing in the crystalline and amorphous lamellae. (D) Relation of a part of the primary structure depicted in (C) to the secondary structure of a single cluster displaying the double helical structures. Reproduced from Perez et al., 2010 with permission from John Wiley and Sons.
Figure 1.6: Channels in starch granules may contain proteins. Compositional backscattered electron image of sorghum granules treated with a solution of merbromin in methanol to highlight granule topography. Channels and cavities are visible only when they are present within the optical plane. Reproduced from Fannon et al., 2004 with kind permission from Springer Science and Business Media.
(Rahman et al., 2007) as well as several other proteins, many of which are still poorly characterized. For instance, an abundant and poorly characterized unnamed wheat SGAP with a molecular weight of approximately 30 kDa is present both on the surface and within the granule (Baldwin, 2001). The function of the protein is unknown, but it has been hypothesized that it may be metabolized by the germinating embryo. The well documented SGAP puroindoline is the major determinant of wheat grain softness, an important property in determining the end-use quality of wheat (Morris, 2002). Puroindolines have also been shown to have antimicrobial properties and have likely evolved as a defence mechanism (Capparelli et al., 2005; Krishnamurthy et al., 2001).

1.4.2 Starch granule summary

Starch granules can therefore be regarded as unique intracellular compartments of endosperm cells that consist of the metabolic and regulatory proteins for starch synthesis and degradation. The proteins localized to this compartment either contain a specific binding domain for starch or directly interact with a protein or group of proteins bound to granules. Since these proteins and the starch granules store large amounts of energy in their chemical bonds, there are important safeguards, such as the innate immune system, in place to protect them from predation.

1.5 Plant/pathogen interactions

To protect their energy reserves (both starch granules and storage proteins), and, similarly, the embryo from predators and pathogens, plants have evolved layers of
resistance mechanisms. These mechanisms include physical barriers, such as the cuticle, and chemical barriers, including antimicrobial molecules, such as phytoanticipins (Osbourn, 1996). In addition to these “first-line” defenses, plants have inducible resistance mechanisms that are activated upon the sensing of pathogen infection (Durrant and Dong, 2004). These inducible mechanisms include the initiation of the hypersensitive response (HR), the cross-linking of cell walls, and the accumulation of reactive oxygen species (ROS), secondary metabolites, and pathogenesis-related (PR) proteins (Sels et al., 2008). The PR proteins are proteins that are either undetectable or present at low concentrations in healthy tissues, but are induced following pathogen infection (van Loon et al., 2006).

The PR proteins were originally described in *Nicotiana tabacum* (tobacco) as proteins upregulated in the hypersensitive response to Tobacco mosaic virus (TMV) (van Loon and van Strien, 1999). There are now 17 recognized families of PR proteins (Table 1.1). The PR families are numbered based on the order of their discovery (van Loon et al., 2006). While not all PR families have been demonstrated to have direct roles in defense, many of the families have well documented antimicrobial activities. PR families such as PR-3, -4, -8, and -11 (chitinases), PR-5 (thaumatin), PR-12 (defensin), PR-13 (thionin), and PR-14 (lipid-transfer protein) catalyze the degradation of specific microbial macromolecules. Chitinases catalyze the cleavage of the $\beta$-1,4-glycosidic bonds of the fungal cell wall structural molecules chitin and chitosan (Jitonnom et al., 2011). Collectively, PR-5, -12, -13, -14 catalyze the degradation of microbial cell walls, though these families have different target molecules (Dubreil et al., 1998; Lay and Anderson, 2005).
Table 1.1: Families of PR proteins.

<table>
<thead>
<tr>
<th>Family</th>
<th>Properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>PR-1</td>
<td>Unknown</td>
</tr>
<tr>
<td>PR-2</td>
<td>β-1,3-glucanase</td>
</tr>
<tr>
<td>PR-3</td>
<td>Chitinase type I, II, IV, V, VI, VII</td>
</tr>
<tr>
<td>PR-4</td>
<td>Chitinase type I, II</td>
</tr>
<tr>
<td>PR-5</td>
<td>Thaumatin-like</td>
</tr>
<tr>
<td>PR-6</td>
<td>Proteinase-inhibitor</td>
</tr>
<tr>
<td>PR-7</td>
<td>Endoproteinase</td>
</tr>
<tr>
<td>PR-8</td>
<td>Chitinase type III</td>
</tr>
<tr>
<td>PR-9</td>
<td>Peroxidase</td>
</tr>
<tr>
<td>PR-10</td>
<td>Ribonuclease-like</td>
</tr>
<tr>
<td>PR-11</td>
<td>Chitinase, type 1</td>
</tr>
<tr>
<td>PR-12</td>
<td>Defensin</td>
</tr>
<tr>
<td>PR-13</td>
<td>Thionin</td>
</tr>
<tr>
<td>PR-14</td>
<td>Lipid-transfer protein</td>
</tr>
<tr>
<td>PR-15</td>
<td>Oxalate oxidase</td>
</tr>
<tr>
<td>PR-16</td>
<td>Oxalate-oxidase-like</td>
</tr>
<tr>
<td>PR-17</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

Adapted from van Loon et al., 2006.
The functions of the PR proteins are not limited solely to defense. The PR-2 proteins have antimicrobial function through their ability to catalyze the cleavage of 1,3-β-D-glucosidic linkages of the glucans that are prevalent in fungal cell walls (Leubner-Metzger and Meins, 1999). However, a diverse assortment of roles for this family in uninfected plants have been established, including, but not limited to embryogenesis (Helleboid et al., 1998), mobilization of energy reserves (Fincher and Stone, 1993), fruit ripening (Hinton and Pressey, 1980), and cold tolerance (Hincha et al., 1997). Other PR families also have diverse roles unrelated to plant defense, therefore careful study is required to document all the functions of the PR families.

Successful fungal pathogen infection is characterized by the secretion of fungal enzymes that catalyze the degradation of the plant’s physical barriers, such as the cuticle and cell walls (Phalip et al., 2005). Enzymes secreted by Fusarium graminearum, the fungal pathogen responsible for causing Gibberella ear rot in maize, include lipases and cutinases that degrade the cuticle (Feng et al., 2005; Jenczmionka and Schafer, 2005; Kang and Buchenauer, 2000) and pectinases that modify plant cell walls (Kikot et al., 2009), allowing easier access of other fungal enzymes, such as cellulases and xylanases, to plant cell components (Reignault et al., 2008). These fungal cell wall-degrading enzymes facilitate the colonization of wheat spikes prior to fungal grain infection (Wanjiru et al., 2002).

Following the penetration of a cereal plant seed’s physical barriers, the pathogen will target the energy-rich endosperm tissue (Kikot et al., 2009). Fungal enzymes such as proteinases and amylases catalyze the degradation of the seed’s storage proteins and starch granules, respectively (Schwarz et al., 2001). For example, following infection of
endosperm tissue with *F. graminearum*, starch granules were digested to a great degree and the intracellular protein matrix was fully degraded (Jackowiak et al., 2005) (Figure 1.7).

**Summary of plant/pathogen interactions**

Plants and pathogens are continuously evolving new mechanisms for the defense or infection of plant tissues, respectively. This ongoing evolutionary arms race between pathogen and plant can have very large economic impacts. Over $1 billion USD of crops are lost to *F. graminearum* infection every year (Goswami and Kistler, 2004), indicating the study of these plant/pathogen interactions using state of the art tools, such as mass spectrometry, can have both economic impacts and benefits for human health by increasing food security.

**1.6 Mass spectrometry**

The proteomes of wheat endosperm (Vensel et al., 2005), wheat and potato amyloplasts (Andon et al., 2002; Stensballe et al., 2008) and maize starch-granules (Grimaud et al., 2008) have been catalogued using high-throughput mass spectrometry techniques. The proteins identified in these studies were classified based upon their biochemical roles in the cell.

In wheat amyloplasts, many of the identified proteins have roles in metabolism, as well as protein destination and storage (Balmer et al., 2006) (Figure 1.8). It is interesting to note
Figure 1.7: Damaged starch granules of spring wheat infected with *Fusarium*. Reproduced from Jackowiak et al., 2005 with permission Elsevier.
Figure 1.8: Functional distribution of proteins identified in amyloplasts from wheat *endosperm*. Reproduced from Balmer et al., 2006 with permission from Oxford University Press.
(A) Pie chart showing the distribution of gene categories:
- Unknown: 12%
- Carbohydrate Metabolism: 12%
- Cytoskeleton/Division: 1%
- Energetics: 7%
- Nitrogen/sulfur metabolism: 15%
- Nucleic acid related: 11%
- Protein related: 13%
- Transport: 8%
- Signaling: 5%
- Stress Related: 3%
- Miscellaneous: 4%
- Building blocks, Other: 9%
that over one third of the proteins identified in the study were unknown or hypothetical proteins. This underscores the necessity of gene discovery and functional analysis studies for large-scale proteomic analysis. The starch granule associated proteome of water washed soft (pathogen resistant) and hard wheat (pathogen susceptible) cultivars have previously been investigated (Wall et al., 2010). In addition to the many unknown and hypothetical proteins, there were multiple proteins with antimicrobial activities identified. As these samples were collected from field grown plants that were deemed fit for harvest, a natural progression of this work is to study grains intentionally infected with pathogens and investigating the subsequent plant/pathogen interactions at the proteomic level. Furthermore, the proteomes of commercially-available starch has been studied to test the suitability of the starch for patients with celiac disease (Kasarda et al., 2008). The authors discovered many proteins that had not previously been believed to be associated with the starch granule surface, showing that careful study is required when documenting proteins in foods destined for human consumption.

The term mass spectrometry refers to a broad range of analytical techniques used for the analysis of molecules based on their mass to charge ratio. As mass spectrometry covers such a broad range of techniques, only the techniques used in the experiments in this thesis will be covered. These techniques include the electrospray ionization (ESI) delivery system, the time of flight (TOF) mass analyzer and mass selection and detection components, and the QStar QqTOF triple quadrupole tandem mass spectrometer. For this latter instrument, Q refers to a mass-resolving quadrupole, and lower-case q refers to a radio frequency-only quadrupole or hexapole collision cell (Chernushevich et al., 2001). This instrument is a tandem mass spectrometer, as there are two mass selection
quadrupoles (Q1 and Q3) separated by ion fragmentation in a collision chamber (q2). When performing peptide sequencing experiments, mixtures of peptides are first separated by liquid chromatography and ionized using electrospray ionization. This technique involves three separate stages: droplet formation, droplet shrinkage and gaseous ion formation (Smith et al., 1990). To promote droplet shrinkage, peptides are suspended in volatile solvents such as acetonitrile. During droplet formation, liquid is extruded from a capillary tip in a "Taylor cone" shape due to electrostatic forces and is subsequently aerosolized (Figure 1.9). The droplet size of this aerosolized liquid is controlled by the inclusion of certain compounds, such as acetic or formic acid, that increase the conductivity of the peptide mixture as well as the applied potential, the flow rate of the solvent, and the diameter of the capillary. As the droplets shrink, gaseous ions pass into the mass spectrometer (Cech and Enke, 2001).

A characteristic of ESI is that the gaseous ions can each have multiple charged residues. Multiple charges reduce the mass to charge ratio of the protein, allowing for the analysis of high molecular weight proteins that are normally outside the usable mass range of the mass spectrometer (Dongre et al., 1996). For mass analysis, the QStar hybrid QqTOF MS/MS consists of three quadrupoles linked to a TOF mass analyzer (Figure 1.9). The first quadrupole (Q1) is responsible for initial ion selection, allowing for the selection of a specific mass range. The second quadrupole (q2) is a non-mass filtering radio frequency quadrupole that serves as a collision cell where ions selected in Q1 can be broken down into smaller ions through collision-induced dissociation (CID) with an inert gas such as argon, nitrogen or helium. The third quadrupole (Q3) allows for further ion selection before the selected ions are delivered to the TOF mass analyzer (Aebersold and Mann,
**Figure 1.9: Schematic of a triple quadrupole mass spectrometer.**

As ions enter the TOF chamber, the ions are accelerated by an electric field of known strength. Due to this acceleration, all ions with identical charge will have the same kinetic energy. As lighter particles are able to accelerate to higher speeds than heavy particles, an ion’s velocity is dependent on the mass-to-charge ratio. As the time an ion takes to reach an ion detector at a known distance can be measured, this time and the experimental parameters are used to calculate the mass-to-charge ratio of the ion (Takats et al., 2004).

When analyzing peptide samples using a tandem mass spectrometer, the fragmentation of positively charged ions occurs in a predictable pattern due to the tendency for the amide bond of peptides to be cleaved during ion fragmentation (Kelleher et al., 1999). The fragment containing the N-terminus is referred to as a b-ion, while the fragment containing the C-terminus is referred to as a y-ion (Roepstorff and Fohlman, 1984) (Figure 1.10). The differences in mass between the fragmented ions compared to the mass of the ion prior to fragmentation allows for calculation of the amino acid sequence of the peptide. Based on this deduced amino acid sequence, it is possible to identify the protein from which this ion was generated. The MS/MS peptide spectra generated from liquid chromatography (LC) MS/MS analyses are analyzed using search engines such as Mascot (Perkins et al., 1999). The search algorithms used by Mascot compare experimentally obtained fragmentation spectra to theoretical fragmentation spectra calculated from a database of protein sequences, such as the National Center for Biotechnology Information (NCBI) non-redundant protein database. The scoring system used by Mascot is probability-based. The total score is the probability that the observed match is a random event, with the scores reported as -10*LOG_{10}(P), where P is the absolute probability. To
Figure 1.10: Types of peptide fragment ions observed in an MS/MS spectrum. Adapted from Roepstroff and Fohlmann, 1984.
determine whether the calculated protein score is significant, Mascot computes an expectation value for every hit. The expectation value is defined as the number of matches with equal or better scores that are expected to occur by chance alone. This threshold is commonly set at p<0.05, therefore any expectation values below 0.05 are considered significant. Another technique to ensure the significance of the Mascot matches are decoy database searches. These searches use the same protein databases as the standard query, but the sequences have been randomized or reversed. This allows for the calculation of the false discovery rates (FDR) in an experiment (Elias et al., 2005). The FDR is calculated as follows: FDR = FP / (FP + TP), where FP is the number of matches in the decoy database and TP is the number of matches in the target database.

1.6 Research Hypotheses and Objectives

Rationale 1

Previously, globulin-3A in wheat cultivar AC Barrie was shown to have implications in the development of type 1 diabetes in genetically susceptible individuals. As AC Barrie and Glenlea are closely related hexaploid cultivars, it is feasible that the globulin-3 family in AC Barrie is similar to the previously documented globulin-3 proteins in Glenlea. Additionally, the post-translational endoproteolytic processing of globulins have been shown to be similar to the post-translational processing observed in barley.
Hypothesis 1

Globulin-3 in wheat undergoes post-translational processing, including glycosylation and endoproteolytic processing, similar to 7S proteins in other species such as soy and barley.

Objectives

1. Determine the expression pattern of globulin-3 in wheat embryo and endosperm tissues.
2. Sequence the proteolytically-cleaved proteins separated on two-dimensional SDS-PAGE gels.
3. Create a model for proteolytic cleavage of the globulin-3 preproprotein.

Rationale 2

Use of a pathogen-susceptible maize genotype and the mechanical kernel injection technique both promote a fuller expression of the fungal pathogen proteome. Under these optimal pathogen growth conditions, the fungus will take up nutrients from the plant and will express enzymes to metabolize the nutrients. Additionally, the inducible plant defence proteins as well as fungal proteins will be present and interacting within both the endosperm and embryo of the host tissues.

Hypothesis 2

Proteins sequenced from the desiccated fungal remnants on the surface of maize kernels can provide insight into the biochemical processes occurring in the fungus prior to post-
infection desiccation. Host defense-related proteins will be present in both the endosperm and embryo tissues, allowing for a better understanding of plant/pathogen interactions.

Objectives

1. Isolate and purify proteins from desiccated fungal remnants on the surface of infected maize kernels as well as proteins from the endosperm and embryo tissues of infected and control kernels.
2. Sequence the proteomes of these tissues.
3. Classify proteins based on their biochemical roles.

Rationale 3

The certificates of analysis for commercially available starch samples give basic measurements of the non-starch components of the samples, such as pH and percentages of moisture, protein, and lipid, but not the full proteome of the starch samples. There is 1% protein in these starches that is undocumented, thus requiring deeper inspection using mass spectrometry techniques.

Hypothesis 3

The protein component of commercially purified starch samples could affect the outcome of other experiments in which the starches will serve as defined baseline substrates. The
investigation of these trace proteomes will allow for the determination of protein-sourced contributions to the macroscopic characteristics of these starches.

**Objectives**

1. Purify the starch granule-associated proteins in commercially-purified starch.
2. Sequence these proteins using tandem mass spectrometry.
3. Classify the proteins based on biochemical characteristics.

**Rationale 4**

The water used to process the starch during commercial purification can contaminate starch samples with non-host organisms, and can affect the end quality of the starch. For example, proteins from microbial pathogens have been documented on the surface of starch granules in lab-scale purified wheat starch.

**Hypothesis 4**

The source of any non-host proteins present in the commercial starch preparations is the water used during the extraction of the starch granules.

**Objectives**

1. Isolate and sequence the proteins present in commercially prepared rice and maize starches.
2. Document any non-host protein present in the starch samples.
Chapter 2

Seed storage proteins of the globulin family are cleaved post-translationally in wheat embryos

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Contribution of Authors:
AGK performed bioinformatic analyses, analyzed data, assembled all figures and tables, drafted and edited the manuscript. EL helped draft the first manuscript, aided in editing, and helped with experiments. MM performed the electrophoresis experiments, contributed to the initial writing and subsequent editing. AJM, FWS provided the globulin-3 antibodies and edited the manuscript. FWS participated in the study design. IA designed the study, provided all reagents and funding, and edited the manuscript.
2.1 Abstract

**Background:** The 7S globulins are plant seed storage proteins that have been associated with the development of a number of human diseases, including peanut allergy. Immune reactivity to the wheat seed storage protein globulin-3 (Glo-3) has been associated with the development of the autoimmune disease type 1 diabetes in diabetes-prone rats and mice, as well as in a subset of human patients.

**Findings:** The present study characterized native wheat Glo-3 in salt-soluble wheat seed protein extracts. Glo-3-like peptides were observed primarily in the wheat embryo. Glo-3-like proteins varied significantly in their molecular masses and isoelectric points, as determined by two dimensional electrophoresis and immunoblotting with anti-Glo-3 antibodies. Five major polypeptide spots were identified by mass spectrometry and N-terminal sequencing as belonging to the Glo-3 family.

**Conclusions:** These results in combination with our previous findings have allowed for the development of a hypothetical model of the post-translational events contributing to the wheat 7S globulin profile in mature wheat kernels.
2.2 Findings

The 7S globulins, orthologs of the vicilins of the Leguminoseae, are salt-soluble storage proteins that accumulate during seed development (Debiton et al., 2011; Jerkovic et al., 2010). Vicilins were first described by Osborn and Campbell in 1898 as a class of seed storage proteins in *Vicia faba* (horse bean) (Osborne and Campbell, 1898). Both the vicilins and the legumins, distinguishable by their sedimentation coefficients of 7-9S and 11-13S, respectively (Danielsson, 1949), contain characteristic β-barrel cupin domains (Dunwell, 1998). The 7S globulins are translated as preproproteins that, following the cotranslational cleavage of the signal peptide, assemble into homo- or heterotrimers (Lawrence et al., 1994) within the lumen of the endoplasmic reticulum (Chrispeels, 1991). Prior to accumulation in seed protein storage vesicles, the trimers undergo post-translational processing, which includes glycosylation and partial endoproteolytic cleavage (Herman and Larkins, 1999, Heck et al., 1993).

Exposure to a number of wheat seed proteins can induce a number of immune-mediated diseases including gluten sensitive enteropathy (celiac disease) (Di Sabatino and Corazza, 2009), Baker’s asthma and wheat-dependent exercise-induced anaphylaxis (WDEIA) in predisposed individuals (Tatham and Shewry, 2008). The *Triticum aestivum* (wheat) storage protein WP5212, later named globulin-3 (Glo-3), has been demonstrated to be a potential food allergen (Larre et al., 2011), identified as the first candidate wheat protein associated with the development of type 1 diabetes (T1D) (MacFarlane et al., 2003), and now celiac disease (Taplin et al., 2011). We recently identified the genomic origins of three Glo-3 genes, *Glo-3A, B* and *C* in the wheat cultivar Glenlea (Loit et al., 2009).
Immunofluorescence studies have localized the Glo-3 gene products to the developing wheat seed embryo and aleurone layer (Loit et al., 2009).

Few studies have sought to characterize wheat 7S globulins because they were thought to be minor storage proteins with little contribution to the bread-making properties of wheat flour (Fabijanski et al., 1985; Robert et al., 1985). However, 7S proteins, based on their sedimentation coefficient, have been characterized in barley and maize, and more recently, two Glo-3-like sequences have been identified in the model cereal Brachypodium distachyon (Larre et al., 2010). In addition to cultivar Glenlea, Glo-3 proteins have been observed in cultivars Butte 86 and Recital (Dupont et al., 2011; Tasleem-Tahir et al., 2011), indicating that Glo-3 is well-conserved in wheat, thus deserving more attention.

Due to its documented role in the development of T1D, we initiated the present study to characterize the Glo-3-related proteins and peptides in wheat cultivar AC Barrie, the original source of WP5212 (MacFarlane et al., 2003). We hypothesized that Glo-3 undergoes post-translational processing, including glycosylation and endoproteolytic processing, similar to 7S proteins in other species. Therefore, we sought to characterize the expression and the distribution of Glo-3 antigenically related proteins by Mr and pI in the embryo and endosperm of AC Barrie, and to link observed protein fragments with their corresponding endoproteolytic cleavage events.
2.3 Results

2.3.1 Glo-3 antigenically-related proteins co-isolate with wheat globulins

To characterize the Glo-3 antigenically-related proteins in whole AC Barrie seeds, globulins were extracted, following the classical method (Khavkin et al., 1978; Robert et al., 1985). The globulin-enriched fraction was separated by 1D SDS-PAGE and immunoblots were probed with polyclonal rabbit antibodies specific for Glo-3A (Figure 2.1) (Loit et al., 2009). The four most intense protein bands, as resolved by SDS-PAGE, had relative mobilities of 33-36, 47-53 and 64-65 and 66-68 (doublet) kDa. The Glo-3 antigenically-related proteins had comparable Mr to these intense bands (33-37, 47-53, 64-68 kDa). Pre-immune serum and secondary/tertiary antibody controls were negative for immunoreactivity with the Glo-3-related proteins (Figure 2.1).

2.3.2 The Glo-3-related proteins are primarily located in the embryo

Protein expression levels of 7S globulins have been shown to be highest in the embryo and aleurone layers, while almost absent in the endosperm (Burgess and Shewry, 1986; Sun et al., 1996; Thijssen et al., 1996). To study the expression of Glo-3 proteins, AC Barrie endosperm and embryo salt-soluble protein fractions were compared by two-dimensional (2D) electrophoresis according to pI and Mr, followed by immunoblotting using anti Glo-3 antibodies (Figure 2.2). The embryo protein fraction was noticeably more complex than the endosperm fraction, with 287 spots detected by GE Healthcare ImageQuant TL Colony Version 7.0 in the CBB R-250-stained 2D polyacrylamide gel of the embryo protein fraction compared to the 122 spots detected in the 2D gel of the
Figure 2.1: SDS-PAGE and immunoblot analysis of AC Barrie salt-soluble proteins. The salt-soluble fraction from AC Barrie seeds was separated under reducing conditions by SDS-PAGE (12% polyacrylamide) and stained with CBB R-250. Standard lane (M) is Precision Plus Protein (Bio-Rad). Proteins were immunoblotted with polyclonal anti-Glo-3 antiserum at a 1:10,000 dilution, with pre-immune serum (1:10,000), or with secondary and tertiary antibodies alone.
Figure 2.2: 2D gels and immunoblot analysis of AC Barrie salt-soluble proteins. Salt-soluble globulins were extracted from AC Barrie wheat seed endosperm (panels A, B) and embryo-enriched (panels C, D, E) fractions and separated by 2-DE. Proteins were stained with CBB R-250 (panels A, C) or transferred to nitrocellulose and probed with polyclonal rabbit anti-Glo-3 antiserum (panels B, D, E). Marker lanes (M) are Pre-stained Benchmark (Invitrogen). Molecular masses shown on immunoblots are approximations. Spots chosen for mass spectrometry are labelled 1-5 and marked with arrows (panel D), and represent a sampling of the major observed molecular masses (~30 kDa and ~50 kDa) with isoelectric points in the acidic (pH 3), neutral (pH 6-7) and basic (pH 9-10) regions. Circled spots are non-specific spots common to blots probed with pre-immune serum and anti-Glo-3 antibodies.
endosperm protein fraction (Figure 2.2, panels A, C). Analysis of the immunoblots revealed 91 spots corresponding to antigenically-related Glo-3-related proteins in the embryo protein fraction, and 46 spots in the endosperm protein fraction (Figure 2.2, panels B, D). On the basis of the increased anti-Glo-3 immunoreactivity with the salt-soluble embryo protein fraction, further studies focused on the AC Barrie embryo. One immunoreactive spot, with Mr 57 kDa and pI 5.8, was common between blots probed with anti-Glo-3 specific antibodies (circled in Figure 2.2, panels B and D) and with pre-immune serum (data not shown). This spot was considered non-specific for Glo-3 immunoreactivity.

Of the 91 anti-Glo-3 immunoreactive spots in the salt-soluble embryo protein fraction, 59 spots corresponded to the four dominant bands identified in the 1D immunoblot (Mr 33-37, 47-53, 64-65, and 66-68 kDa) (Figure 2.1 and Figure 2.2, panel D). Twelve spots in the Mr 33-37 kDa range and 23 spots in the Mr 47-53 kDa range were observed with 20 spots having pI values between 7.5 and 9.5. Twenty-four spots were in the Mr range of the 64-65 and 66-68 kDa doublet, with 21 spots having pI values between 7.5 and 9.5. As 71 of the 91 spots had pI values between 7 and 10, the salt-soluble embryo protein fraction was resolved on a 2D gel with a pH range of 7-10 (Figure 2.2, panel E). There were 103 Glo-3 immunoreactive spots identified within this narrower pH range, with 70 of the 103 spots with Mr of 33-37, 47-53, 64-65, or 66-68 kDa.

2.3.3 Identification of selected Glo-3-related polypeptides

To confirm that the antigenic epitopes detected by the anti-Glo-3 antibodies were specific to Glo-3, five anti-Glo-3-immunoreactive spots from the salt-soluble embryo protein
fraction were excised from the 2D gel, and analyzed by mass spectrometry (LC- MS/MS) (Figure 2.2, panel D; numbers indicate location of spots). The spots excised were chosen as they had a wide range of Mr and pl values, and they fell outside the predicted Mr and pl values of proglobulin-3 (GenBank Accession JQ945759) (Mr 66.6 kDa and pl 8.5, as calculated using the Expasy Compute pl/Mw tool). Mass spectrometry results are summarized in Table 1. All five spots were identified as Glo-3A by interrogating the non-redundant NCBI database.

2.3.4 Characterization of selected Glo-3-related polypeptides

To study the post-translational processing of Glo-3, three spots were analyzed with N-terminal sequencing. One sequence (Spot 1) may be N-terminally blocked because no information could be obtained, despite protein visualization after amido black staining. The N-terminal sequence of Spot 3 was determined to be SRDTFNLL, which matched the Glo-3 (GenBank Accession JQ945759) sequence starting at amino acid residue 337. Spot 4 was difficult to visualize following protein transfer and staining. As determined by N-terminal sequencing, the first two residues could not be resolved (X) and the last residue was reported as either arginine (R) or glutamic acid (E). Using arginine as the last residue, the resulting sequence XXHGDSRR matched the findings of Singh et al., and the Glo-3B sequence (GenBank accession FJ439136) starting at residue 117.

The post-translational endoproteolytic cleavage events of preproglobulin-3 that would be required to yield polypeptides with Mr and pl corresponding to the sequenced spots are

Table 2.1: MS/MS sequencing results of selected gel spots of wheat 7S globulins
<table>
<thead>
<tr>
<th>Spot ID</th>
<th>Protein (GenBank accession)</th>
<th>Mascot Score</th>
<th>% coverage</th>
<th>Peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>globulin-3A (JQ945759)</td>
<td>210</td>
<td>17.5</td>
<td>VFGPRSF; DEVSRLL; HTISVPGKF; GRPAREVQEVF; RVAIMEVNYPRAF; TVRQGDVIVAPAGSIMHL; VVPPGHPVVEIAASSRGSSNL; VAQGEGVLTVIENGEKRSY</td>
</tr>
<tr>
<td>2</td>
<td>globulin-3A (JQ945759)</td>
<td>131</td>
<td>12.9</td>
<td>VFGPRSF; DEVSRLL; VVPGLTDADGVGY; RVAIMEVNYPRAF; TVRQGDVIVAPAGSIMHL; VAQGEGVLTVIENGEKRSY</td>
</tr>
<tr>
<td>3</td>
<td>globulin-3A (JQ945759)</td>
<td>38</td>
<td>3.4</td>
<td>VVPPGHPVVEIAASSRGSSNL</td>
</tr>
<tr>
<td>4</td>
<td>globulin-3A (JQ945759)</td>
<td>135</td>
<td>13.8</td>
<td>RPFDEVSRLL; RVAIMEVNYPRAF; VAQGEGVLTVIENGEKRSY; SAKPLLASL; TVRQGDVIVAPAGSIMHL; VVPPGHPVVEIAASSRGSSNL</td>
</tr>
<tr>
<td>5</td>
<td>globulin-3A (JQ945759)</td>
<td>144</td>
<td>17.7</td>
<td>DEVSRLL; RPFDEVSRLL; VVPPGLTDADGVGY; RVAIMEVNYPRAF; EINAERNERVWL; TVRQGDVIVAPAGSIMHL; VVPPGHPVVEIAASSRGSSNL; VAQGEGVLTVIENGEKRSY</td>
</tr>
</tbody>
</table>
summarized in Figure 2.3. In addition to the Mr and pI of the spots in Figure 2.2, panel D, the location of the MS sequenced peptides within globulin-3 (black bars), N-terminal sequence data (purple bars - when available), as well as the location of the epitopes used to generate the polyclonal anti-Glo-3 antibodies (red bars) were considered. The size and location of the signal sequence was predicted by TargetP 1.1 (Emanuelsson et al., 2000; Nielsen et al., 1997), (http://www.cbs.dtu.dk/services/TargetP/). Proteins from previous studies (Dupont et al., 2011; Singh et al., 2001) were included to demonstrate that the methods used for the determination of the processing events in the current study are applicable to previously published findings and that our observed processing corresponds to the processing inferred in the literature. The processing of Spot 3 (Figure 2.3) is supported by the N-terminal sequence that matched cleavage site 3 (SRDTFNLL), as well as the MS sequenced peptide in the C-terminal vicilin domain segment, and an observed Mr 28-30 kDa and pI 6.2-7.0. Spot 3 corresponds to the cleavage of proglobulin-3A at cleavage site 3, with predicted Mr 28.6 kDa and pI 5.76. The processing of Spot 4 is supported by N-terminal sequence data that match the previously documented cleavage site 2 (XXHGDSRR) (Singh et al., 2001). Spot 4 corresponds to cleavage of proglobulin-3A at cleavage sites 2 and 4, with predicted Mr 35.1 kDa and pI 9.14. Spot 2 lacked N-terminal sequence data, but as with Spot 4, contained MS sequenced peptides present in the N-terminal vicilin domain segment and an observed Mr 31-35 kDa. Cleavage at both sites 1 and 3, or 2 and 4 yield products with Mr of approximately 35 kDa. The epitopes used for the generation of the antiglobulin-3 antibody are present in the middle and C-terminal segments. Neither of those epitope sites is present in products created from cleavage sites 1 and 3, so therefore Spot 2 corresponds to cleavage at sites 2.
Figure 2.3: Model of Glo-3 endoproteolytic processing.
The observed M_r, pI, MS/MS sequence data, and N-terminal sequence data from this study and previous studies (Dupont et al., 2011) were reconciled with theoretical peptides created by the endoproteolytic cleavage of preproglobulin-3 (GenBank Accession JQ954759). A linear representation of preproglobulin-3 is shown with approximate locations of potential cleavage sites, labelled 1-4. The protein domains are represented as follows: signal sequence (SS) (white); N-terminal sequence (grey); vicilin domain (divided into three segments by cleavage sites – N-terminal segment (green), middle segment (blue), C-terminal segment (orange). Red boxes correspond to the location of the linear epitopes used when creating the anti-Glo-3 polyclonal antibodies. Black boxes correspond to the location of MS/MS sequenced peptides. Purple boxes correspond to sequence obtained from N-terminal sequencing. The observed and expected molecular masses (kDa) and isoelectric points (pI) of the resulting polypeptides following endoproteolytic cleavage is indicated on the right. Additionally, the expected processing of proteins inferred by previous studies (Dupont et al., 2011) and (Singh et al., 2001) has been demonstrated.
<table>
<thead>
<tr>
<th>Protein</th>
<th>PPI ID</th>
<th>Observed Mr (kDa/pI)</th>
<th>Predicted Mr (kDa/pI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>preproglutinin-3</td>
<td>ss</td>
<td>66.3/8.48</td>
<td>NA</td>
</tr>
<tr>
<td>proglutinin-3</td>
<td></td>
<td>63.6/7.76</td>
<td>63-68/7.1-9.0</td>
</tr>
<tr>
<td>globulin-3</td>
<td></td>
<td>52.1/8.49</td>
<td>47-53/7.3-9.0</td>
</tr>
<tr>
<td>Spot 1</td>
<td></td>
<td>52.1/8.49</td>
<td>48-50/3.0-3.5</td>
</tr>
<tr>
<td>Spot 2</td>
<td></td>
<td>35.1/9.14</td>
<td>31-35/3.0-3.5</td>
</tr>
<tr>
<td>Spot 3</td>
<td></td>
<td>28.6/5.76</td>
<td>28-30/6.2-7.0</td>
</tr>
<tr>
<td>Spot 4</td>
<td></td>
<td>35.1/9.14</td>
<td>31-35/9.4-9.9</td>
</tr>
<tr>
<td>Spot 5</td>
<td></td>
<td>52.1/8.49</td>
<td>48-50/8.7-9.1</td>
</tr>
</tbody>
</table>

**Dupont et al. 2011**

<table>
<thead>
<tr>
<th>Spot</th>
<th>Predicted Mr (kDa/pI)</th>
<th>Observed Mr (kDa/pI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spot 180</td>
<td>35.1/9.14</td>
<td>38-40/&gt;8.5</td>
</tr>
<tr>
<td>Spot 184</td>
<td>35.1/9.14</td>
<td>38-40/&gt;8.5</td>
</tr>
<tr>
<td>Spot 272</td>
<td>17.1/5.84</td>
<td>18-20/4.9-5.2</td>
</tr>
<tr>
<td>Spot 309</td>
<td>52.1/8.49</td>
<td>48-50/8.7-9.1</td>
</tr>
</tbody>
</table>

**Singh et al. 2001**

<table>
<thead>
<tr>
<th>Spot</th>
<th>Predicted Mr (kDa/pI)</th>
<th>Observed Mr (kDa/pI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G4</td>
<td>17.1/5.84</td>
<td>20/NA</td>
</tr>
<tr>
<td>G6</td>
<td>52.1/8.49</td>
<td>54/NA</td>
</tr>
<tr>
<td>G7</td>
<td>35.1/9.14</td>
<td>37/NA</td>
</tr>
<tr>
<td>G8</td>
<td>35.1/9.14</td>
<td>38/NA</td>
</tr>
</tbody>
</table>
and 4. Spots 1 and 5 lack N-terminal sequence data, but had MS sequenced peptides that localized to the N-terminal and C-terminal segments of the vicilin domain, and exhibited relative mobilities of 48-50 kDa (Figure 2.3). The pI of Spot 1 was 3.0-3.5, while the pI of Spot 5 was 8.7-9.1. Both Spot 1 and Spot 5 correspond to the theoretical cleavage of preproglobulin-3 at cleavage site 2 with predicted M_r 52.1 kDa and pI 8.49.

2.4 Discussion

2.4.1 Type 1 diabetes

Inflammation associated with the T-cell-mediated autoimmune disease T1D results in the loss of the insulin-producing β cells in the pancreatic islets of Langerhans (Eizirik et al., 2009). While the incidence of T1D has steadily increased in developed countries over the past 60 years, a definitive cause of T1D has yet to be elucidated (Todd, 2010). While many risk genes for T1D have been identified, it has been proposed that most T1D-related genes are not highly penetrant and that T1D is actually a complex disease requiring both genetic susceptibility and the exposure to environmental risk factors (MacFarlane et al., 2009). Serum IgG antibodies from human patients with T1D, but not from matched control groups, are able to bind Glo-3 in vitro, indicating that Glo-3 may be a candidate risk factor for the development of T1D in certain individuals (MacFarlane et al., 2003). We therefore chose to characterize the Glo-3-related proteins in the wheat cultivar AC Barrie with respect to protein maturation in developed seeds.
When the amino acid sequences of preproglobulin-3A from the wheat cultivars AC Barrie and Glenlea are compared, there are five amino acid substitutions out of 588 residues (Loit et al., 2009). However, the theoretical isoelectric points of these Glo-3 proteins are 8.48, and 7.78 in AC Barrie and Glenlea, respectively. The difference in pI of 0.70 is due to the substitution of two arginine residues (R43Q and R102H) in the AC Barrie Glo-3. The AC Barrie salt-soluble Glo-3-related proteins demonstrate a range of isoelectric points concentrated in the basic range (pH 7-9) when separated by 2DE (Figure 2.2, panel E). As there are three Glo-3 genes in the wheat cultivar Glenlea (Loit et al., 2009), it is feasible that AC Barrie would also have three Glo-3 genes, as both AC Barrie and Glenlea are closely related hexaploid wheat cultivars (McCallum and DePauw, 2008). The charge trains of gel spots with similar Mr and a range of pI values at the major size groups (Figure 2.2) are likely due to post-translational processing and modifications, as previously discussed (Robert et al., 1985), as well as amino acid substitutions between the multiple immunologically related Glo-3 proteins in AC Barrie, or from artifacts generated during the execution of the extraction and examination protocols (Deng et al., 2012).

When the wheat cultivar AC Barrie salt-soluble globulin fraction was resolved by 1D SDS-PAGE under reducing conditions, bands of three major size ranges of approximately 64-70 kDa, 47-53 kDa and 33-37 kDa were visible (Figure 2.1); referred to as 65 kDa, 50 kDa and 35 kDa, respectively. These three major wheat globulin groups are characteristic of the 7S globulins described in other studies (Burgess and Shewry, 1986; Robert et al., 1985). Intriguingly, while Spot 3 had an observed Mr of 28-30 kDa (Figure 2.2D), no distinct band is visible in this region in Figure 2.1, though a faint band is visible just
below the 35 kDa band. The 28-30 kDa band is likely less intense than the major bands, as Spot 3 is the only significant immunoreactive spot at 28-30 kDa, while the doublet at 65 kDa comprises several spots and is still markedly less intense than the 50 and 35 kDa bands, which both include several intense spots.

The 7S globulins are enriched in the embryo and aleurone layer of wheat and other cereals (Heck et al., 1993; Khavkin et al., 1978; Kriz, 1999; Yupsanis et al., 1990). Consistent with our previous findings (Loit et al., 2009), immunoblots probed with anti-Glo-3 polyclonal serum revealed that 7S globulins are expressed at low levels in the salt-soluble fraction of wheat endosperm (Figure 2.1). In the embryo-enriched fraction, Glo-3 related proteins are restricted to each of the major size groups, suggesting that these Glo-3-related proteins have similar post-translational processing patterns as the previously characterized 7S globulins.

2.4.3 Post-translational processing of Glo-3

The 7S storage proteins in other plants undergo a series of post-translational modifications which include limited endoproteolytic events (Gatehouse et al., 1983; Sharma et al., 2010; Spencer et al., 1983). Glycosylation is frequently observed in 7S globulins as N-linked complex glycans (Sturm et al., 1987), but is not required for proper folding or export to the protein storage vacuole (PSV) (Chrispeels et al., 1982). A previous study has shown that wheat 7S globulins bind the lectin concanavalin A, although the exact nature and extent of this binding is unknown (Robert et al., 1985). Additionally, other cleavage events may occur at the N-terminus of Glo-3 as observed for
maize Glb1 (Schwartz, 1979). These post-translational modifications, among others, likely contribute to the heterogeneity of the observed isoelectric points as well as the wide range of observed molecular masses among the Glo-3-related proteins.

The MS/MS sequencing of the numbered spots in Figure 2.2, panel D, all returned Glo-3A sequences when queried against the non-redundant NCBI protein database, indicating that, similar to orthologous 7S storage proteins in other species (Heck et al., 1993), the peptide spots in Figure 2.2, panel D were derived from a common precursor protein. The Glo-3-related proteins found at the acidic end of the pH spectrum, including the proteins in spots 1 and 2, exhibited a pI of approximately 3.0 (Figure 2.2, panel D). The low pI of these proteins could be due to protein precipitation during the rehydration of the IPG strips (Sanchez et al., 1997), as similar spots are visible at pH 7.0 in the immunoblot of the 2D gel of pH 7-10 (Figure 2.2, panel E).

Considering features such as the location of the MS/MS sequenced peptides within Glo-3, N-terminal sequence data (when available), as well as the Mr and pI of the spots, we were able to deduce the likely cleavage pattern of the preproglobulin-3 precursor protein (Figure 2.3) that resulted in the globulin-3 polypeptide spectrum recognized by anti-Glo-3 antibodies (Figure 2.2, panel D). It must be noted that the antibodies used to probe the globulin fraction were raised against two linear epitopes from the vicilin domain of WP5212: SRDTFNLEEQRPKIAN and RGDEAVEAFLRMATA (MacFarlane et al., 2003; Scott, 2007). These epitopes are located in the middle (blue) and C-terminal (orange) vicilin domain segments (Figure 2.3). Therefore, N-terminal polypeptides created by the endoproteolytic cleavage at site 3 may not be recognized by the anti-Glo-3 antibodies used in this study.
The model of endoproteolytic cleavage presented in Figure 2.3 suggests that the Glo-3-related proteins of varying molecular masses and isoelectric points originate from 7S globulin precursors possessing a signal peptide, an N-terminal segment and a vicilin domain (Figure 2.3). These precursors belong to a multigene family whose members differ slightly in sequence, with varying length N-terminal segments (Lawrence et al., 1994). We propose that the Glo-3 protein, prior to co-translational removal of the signal sequence, is a ~66 kDa monomer (Figure 2.3). After signal sequence removal (cleavage site 1), the protein becomes a ~64 kDa holoprotein. Three internal cleavage sites have been identified that, when processed, can yield polypeptides with a range of M_r and pI values (Figure 2.3). Spot 3 (Figures 2, panel D, Figure 2.3) was observed at M_r 28-30 kDa and pI 6.2-7.0, and Spot 4, which has a similar M_r (31-35 kDa), a more basic pI (9.4-9.9), and a different N-terminus than Spot 3. The N-terminal sequence determined for Spot 3 SRDTFNLL represents a novel cleavage site for globulin-3. The location of this cleavage site is consistent with processing by vacuolar processing enzymes (Ariizumi et al., 2011), as an aspartic acid residue precedes the N-terminal sequence of Spot 3. These findings suggest that these ~30 kDa globulin-3-related polypeptides could arise from different processing events of the same precursor, as was observed for barley Beg1 (Heck et al., 1993; Yupsanis et al., 1990). Curiously, there were no proteins identified in the immunoblotting experiments that corresponded to the product of processing at cleavage site 4, as was documented in other studies that sequenced globulin-3 (Figure 2.3 and (Dupont et al., 2011; Singh et al., 2001; Tasleem-Tahir et al., 2011)). Potentially, as the antiglobulin-3 antibodies are polyclonal and were prepared by the co-immunization of rabbits with a mixture of two separate peptides (Sancho et al., 2008), it is possible that the
antibodies recognize only one of the two peptides. However, further study on the binding of these antibodies to processed globulin-3 proteins is still required in order to achieve a more definitive understanding.

When compared to the Glo-3A, Glo-3B, and Glo-3C sequences previously published by our group (Loit et al., 2009), each of the spots contained at least one peptide in Table 1 that mapped solely to the Glo-3A coding sequence (GenBank Accession JQ945759 - data not shown). As the Glo-3A protein coding sequence is 99% identical between wheat cultivars Glenlea (GenBank accession ACJ65514) and AC Barrie (JQ945759), the Glo-3B and Glo-3C proteins may share a high percentage identity between these two cultivars. Therefore, we attributed all spots to Glo-3A. However, on close inspection of the Glo-3B (FJ439136) genomic sequence, there are several insertion/deletion events (data not shown) that would yield a protein of similar molecular weight as Glo-3A (66.3 kDa). Further sequencing of Glo-3 cDNA clones from both AC Barrie and Glenlea cultivars is necessary to determine the transcribed sequence of Glo-3.

The endoproteolytic processing events outlined here are likely part of a series of post-translational events that lead to the maturation of the Glo-3-like proteins, also similar to those observed for barley Beg1 (Heck et al., 1993). The identification of wheat Glo-3 holoproteins (~65-70 kDa) by immunoblot analysis suggests that the endoproteolytic modifications described are partial and may not be prerequisites for proper folding, transport, targeting, and storage of the Glo-3 proteins. Alternatively, as the wheat genome has not been sequenced, the spots with Mr 65-70 kDa may correspond to proteins in the globulin-3 family that encode proteins recognizable by the antiglobulin-3 antibodies, but are processed in a different manner than the proteins observed in this study. Further
sequencing and study are required in order to fully catalogue the globulin-3 family. The presence of polypeptides of ~50 kDa and ~30-38 kDa (Singh et al., 2001 and this study) with the same N-termini reinforce the idea that not every hypothesized set of endoproteolytic processing events occurs, and some could occur with varying degrees of processing. Such maturation of vicilins has not only medical implications, but also implications for the quality of foods rich in vicilins following processing and production (e.g. wheat, soybean (Kaviani et al., 2011; Sancho et al., 2008)).

2.5 Conclusion

With a greater understanding of the endoproteolytic processing events that lead to the maturation of the Glo-3 family of proteins observed in the salt-soluble embryo protein fraction, we can now refine the research of Glo-3 to certain domains present only in the polypeptides that are associated with T1D or celiac disease following endoproteolytic processing. In addition, the specific breeding or genetic modification of wheat could be performed to minimize any potential disease- or food quality-related protein or peptide content compared to that of existing wheat cultivars, as is underway for the deletion of the conglycinin α subunit from the soybean proteome (Rayhan et al., 2011; Stanojevic et al., 2011).
2.6 Methods

2.6.1 Wheat seed protein extraction and sample preparation

For each extraction, 4 g of whole *Triticum aestivum* AC Barrie seeds or 50-100 embryos or endosperm, which were dissected as in (Walker-Simmons, 1987) were employed. Seeds were initially ground in a domestic coffee grinder. Finer powder was obtained by hand milling using a mortar and pestle under liquid nitrogen. Wheat powder was mixed with 10 volumes of fresh ice-cold acid denaturing solution (10% (w/v) trichloroacetic acid (TCA) and 0.05% (w/v) dithiothreitol (DTT) in acetone). Samples were stirred at 4°C for 1 h and left at -20°C overnight. The resulting suspension was centrifuged for 30 min at 35,000 x g at 4°C (Beckman Avanti J-25, rotor JA 25.50). The supernatant was decanted and the pellet resuspended in ice-cold acetone containing 0.05% (w/v) DTT. The mixture was extracted by incubation for 1 h at -20°C. The suspension was centrifuged for 20 min at 35,000 x g at 4°C, the supernatant decanted and the pellet dried on ice. Dried powder was extracted with 15 ml of 1 M NaCl solution (1.0 M NaCl; 0.05 M Tris; pH 8.0). The mixture was stirred for 1 h at room temperature and centrifuged (27,000 x g for 30 min at 22°C). Supernatants were collected and the extraction with 1 M NaCl solution was repeated. The supernatants were pooled and dialysed against 5 changes of ddH2O for 24 h at 4°C. Precipitates were collected by centrifugation at 35,000 x g at 4°C for 45 min. Pellets were drained and extracted for either one-dimensional (1D) or two-dimensional protein separation.
2.6.2 Sample preparation for 1D separation

Pellets were resuspended in 1 M NaCl solution. Samples were centrifuged at 20,000 x g for 1 h at 22°C and quantified by the bichinchoninic acid (BCA) assay (Smith et al., 1985). Supernatants were aliquoted and stored at -80°C until use.

2.6.3 Sample preparation for 2D separation

Proteins from pellets were extracted with 1.5 - 3 ml rehydration buffer (8M/2M deionized urea/thiourea, 2% 3-(3-cholamidopropyl)dimethylamino)-1-propanesulfonate (CHAPS), 50 mM DTT, 0.0005% bromophenol blue) containing pH 3-10 ampholytes (Bio-Rad). Following sonication, samples were centrifuged at 200,000 x g for 1 h at 22°C in the Beckman TL-100 ultracentrifuge. Supernatants were collected, quantified by Bradford method (Bradford, 1976), aliquoted and stored at -80°C until use. Throughout the 2D separation process, care was taken not to heat the urea/thiourea-containing solutions above 30°C to avoid carbamylation of amino groups which can lead to artifactual spot heterogeneity (Shaw and Riederer, 2003).

2.6.4 1D SDS-PAGE protein fractionation

Protein extracts were fractionated under SDS-PAGE reducing conditions. Protein samples were combined in a 1:1 (v/v) ratio with 2X sample buffer (4% SDS, 20% glycerol, 0.12 M Tris (pH 6.8), 10% (v/v) β-mercaptoethanol, 0.01% bromophenol blue). Samples were boiled for 5-10 min and centrifuged at 20,000 x g for 10 min. All samples were loaded on
discontinuous (5% stacking, 10%-12% resolving) 1.5 mm SDS polyacrylamide gels (Laemmli, 1970). The SDS running buffer, adjusted to pH 8.3 with NaOH, consisted of 25 mM Tris base, 0.19 M glycine and 0.1% SDS. Electrophoresis was performed in the Mini-PROTEAN 3 System (Bio-Rad) at 100-150 V (400 mA) until dye front reached the bottom of the gel. Gels were either stained with Coomassie Brilliant Blue (CBB) R-250 or were used for immunoblot analysis.

2.6.5 Immunoblot analysis

Proteins from SDS-PAGE were transferred under semi-dry conditions by means of the Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad). Gels were rinsed 5-10 min in semi-dry transfer buffer (24 mM Tris, 192 mM glycine and 15% methanol) and transferred to nitrocellulose membranes (Bio-Rad) also soaked in transfer buffer. Electroblotting proceeded for 1-1.5 h at 11 V (400 mA). Once complete, the transfer was verified by staining membranes with Ponceau S (0.2% (w/v) in 1% glacial acetic acid) followed by several washes of ddH2O. Prior to immunoblotting, membranes were destained with TBST buffer (10 mM Tris-HCl (pH 7.3), 0.1 M NaCl and 0.5% Tween-20) and were incubated 30 min with shaking, in 5% skim milk powder in TBST buffer at room temperature. Membranes were incubated with polyclonal rabbit anti-Glo-3 antibody (MacFarlane et al., 2003) (diluted 1:10,000 in 5% skim milk powder in TBST and 0.05% NaN3) and left overnight at 4°C with gentle rocking. Next day, membranes were washed 4 x 5 min in TBST, and incubated 1 h in biotin-SP-conjugated AffiniPure goat anti-rabbit IgG (H+L) secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) with a dilution of 1:100,000. This incubation was followed by another set of
washes in TBST and a final incubation in affinity purified goat anti-biotin-horseradish peroxidase (HRP)-conjugated tertiary antibody (Cell Signaling Technology, Inc. Danvers, MA) for 1 h. Following 4 x 5 min washes, membranes were treated with ECL™ Western Blotting Detection Reagents (Amersham Biosciences, Piscataway, NJ) for 1 min, exposed to Kodak BioMax Light Film (Fisher Scientific, Nepean, ON). Film was developed by the Kodak X-OMAT 2000A Processor.

2.6.6 Two-dimensional gel electrophoresis (2DE)

Proteomic analysis of the AC Barrie salt-soluble proteins was conducted by 2DE. The proteins were separated in the first dimension by isoelectric focusing (IEF) according to their isoelectric points (pI). Extracts were applied to linear immobilized pH gradient (IPG) 7 cm strips with pH ranges 3-10 and 7-10 (Bio-Rad, Mississauga, ON). A protein load of 100-150 μg was applied to strips for staining and immunoblot applications; 250 μg for mass spectrometry and N-terminal sequencing applications. Strips were covered in mineral oil and rehydrated overnight in a reswelling tray in 125-150 μl volumes of sample diluted in rehydration buffer. For strips in the pH ranges 3-10, DTT was used as a reducing agent; tributylphosphine (TBP) was used for strips in the pH range of 7-10. Rehydrated strips were focused in the PROTEAN IEF Cell (Bio-Rad) according to the following program: step1, 250 V (30 min), linear ramp; step2, 4000 V (2 h), linear ramp; step3, 4000 V (10,000 Vh), rapid ramp. Immediately following isoelectric focusing, the strips were either stored at -80°C for future use or equilibrated prior to running the second dimension. Strips were thawed, if necessary, and reduced for 15 min in 2 ml of equilibration buffer (6 M urea, 2% SDS, 20% glycerol, 0.375 M Tris-HCl, pH 8.8
containing 2% (wt/vol) DTT or 5 mM TBP). A second step, one of alkylation, was performed for 15 min in the same equilibration buffer with the exception that 2.5% (w/v) iodoacetamide was substituted for reducing agents DTT or TBP. Overlay agarose (Bio-Rad) was used to seal the equilibrated strips at the top of 1 mm vertical resolving gels (10% or 12% polyacrylamide). Second dimension SDS-PAGE was performed using the Bio-Rad MiniProtean 3 at 100 V (400 mA); runs were terminated when the bromophenol dye front had reached the end of the gel. Protein spots were counted using ImageQuant TL Colony Version 7.0 (GE Healthcare).

2.6.7 Liquid chromatography tandem mass spectrometry (LC-MS/MS)

Gels were carefully manipulated under a laminar flow hood at all times to reduce keratin contamination. Following separation in the second dimension, proteins were detected with Bio-Safe Coomassie (Bio-Rad) according to manufacturer’s instructions and destained with multiple changes of ddH2O. Selected spots were excised from the gel by means of a clean scalpel blade and subjected to mass spectrometric analysis (Ottawa Institute of Systems Biology, OISB). Proteins were digested in-gel with chymotrypsin as previously described (Wilm et al., 1996). Trypsin digests were avoided because of the high frequency of arginine and lysine residues in the reported wheat globulin sequences (Kriz, 1999; Loit et al., 2009). Peptides were separated by liquid chromatography on an Agilent 1100 Series HPLC System (Agilent Technologies, Palo Alto, CA) and applied by electrospray to a QSTAR Pulsar quadrupole-TOF mass spectrometer (ABI/MDS Sciex, Concord, ON) as described in (Vasilescu et al., 2005). Resulting peptide masses were used to interrogate the non-repetitive NCBI protein database (06/10/2011; 1,432,4397
sequences; 4,906,523,086 residues) using Mascot software (version 2.3) (Matrixscience Ltd.) as in our previous studies (Koziol et al., 2012; Wall et al., 2010). Fixed modifications were set for carbamidomethyl (C) and variable modifications for oxidation (M). One missed cleavage was allowed. Peptide and MS/MS mass tolerances permitted were ±100 ppm and 0.2 Da, respectively.

### 2.6.8 N-terminal sequencing

Gels used for N-terminal sequencing were treated for wet transfer to PVDF membranes. Briefly, the gels were soaked in CAPS (3-(cyclohexylamino)-1-propanesulfonic acid) electroblotting buffer (10 mM CAPS/NaOH pH 11 and 10% methanol) for 5 min. Sequiblot PVDF (Bio-Rad) membranes were wet in 100% methanol, soaked in CAPS electroblotting buffer along with Whatman 3MM paper sheets and transblot sponges. The blotting sandwich was assembled and run in the Mini Trans-Blot Cell (Bio-Rad) at 50 V (170 mA) for 45 min. After disassembly, the membranes were rinsed thoroughly with ddH$_2$O, saturated in 100% methanol and stained for a minimum of 1 min in amido black stain (0.1% amido black, 1% acetic acid and 40% methanol). Membranes were rinsed in multiple changes of ddH$_2$O and air dried before excision of spots. Spots of interest were subjected to Edman degradation using the 494 cLC PROCISE Sequencing System (Applied Biosystems; Foster City, CA) for high sensitivity (femtomole quantities) N-terminal protein sequencing (University of Texas Medical Branch, UTMB, Biomolecular Resource Facility Core).
Chapter 3

Taking stock of the protein remnants on the battlefield between host and pathogen: Maize CL30-Fusarium interactome

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Contribution of authors:

AGK contributed to the study design, performed all experiments, analyzed data, contributed all figures, and wrote and edited the manuscript. LR contributed to study design, provided field samples, and edited the manuscript. IA contributed to the study design, data analysis and editing.
3.1 Abstract

The economic losses associated with gibberella ear rot make necessary a better understanding of both the pathogen, *Fusarium graminearum*, and its host, *Zea mays*. The *F. graminearum* susceptible inbred maize line CL30 was exposed to water controls or conidial suspensions of *F. graminearum* through kernel inoculations, and the host plants were allowed to grow to maturity. Proteins were extracted separately from the maize embryo and endosperm tissues, as well as from the fungal remnants on the surface of the maize kernels. The proteins were sequenced using direct-sequencing LC-MS/MS. We identified 250 maize proteins with high confidence. These proteins were classified into several functional categories, including: late embryogenesis abundant, seed storage, metabolism, and defense. There was a significantly increased representation of defense proteins in both the embryo and endosperm tissues of infected maize samples. The proteome of the fungal remnants on the surface of the maize kernels was composed of 18 proteins that were present after desiccation. Several of these proteins were categorized as being involved in the metabolism of plant-sourced molecules, or in stress response. Taken together, these data should help dissect the interactive system between host and pathogen during the important time period of grain maturation and harvest.
3.2 Introduction

Gibberella ear rot and stalk rot in maize, as well as Fusarium head blight in wheat, barley, and oats are caused through the infection of these crops by the fungal pathogen *Fusarium graminearum* (Holbert et al., 1923; Miller et al., 1985; Reid et al., 1992; Takeda and Heta, 1989; Trail, 2009). Not only do these diseases reduce grain yields (Kazan et al., 2012), but *F. graminearum* also synthesizes mycotoxins, such as deoxynivalenol (DON), fusarins C, autofusarins, and zearalenone (Logrieco et al., 2002; Taylor et al., 2008). One of the best studied mycotoxins, DON, is a virulence factor that aids in fungal colonization, and also poses serious human health risks (Harris et al., 1999). For instance, DON inhibits eukaryotic protein biosynthesis, and upon ingestion, causes nausea, vomiting, and convulsions (Goswami and Kistler, 2004; Vesonder et al., 1981), with long-term exposure to DON being linked to immune system suppression and certain neurological disorders (Bennett and Klich, 2003; Pestka and Bondy, 1994). Consequently, the concentration of DON in food products is tightly regulated, with limits of 1 μg DON per gram of food for human consumption (Trail, 2009).

The sequence of the four chromosomes of the *F. graminearum* strain PH-1 (teleomorph *Gibberella zeae*) genome was published in 2007 (Cuomo et al., 2007). The genome is 36.45 Mb and codes for 13 321 predicted proteins (www.broadinstitute.org/annotation/genome/fusarium_group/GenomeStats.html; last viewed 7/09/2012). Compared to other sequenced fungal genomes, *F. graminearum* has an overrepresentation of certain protein categories, including hydrolytic enzymes (Cuomo et al., 2007), which may have implications in pathogenesis. The infection process is initiated for gibberella ear rot when ascospores or conidia are introduced to exposed silks.
or punctured kernels (Koehler, 1942; Miller et al., 2007; Sutton, 1982). Following germination of the spores, the fungus can then colonize the host plant (Mesterhazy et al., 2012). However, in response to fungal infection, plants have several inducible defense strategies, including the strengthening of cell walls and the production of antimicrobial proteins (Campo et al., 2004; Reid et al., 1992; Reid et al., 1994).

We have previously examined the starch granule-associated proteomes of wheat (Wall et al., 2010), and of commercially prepared rice and maize starches (Koziol et al., 2012; Koziol et al., 2012). In these experiments, it was presumed that the grains used in the preparation of starches were healthy and free of obvious fungal contamination. The use of CL30, a susceptible host genotype of maize, and the mechanical kernel injection technique promotes a fuller expression of the foreign proteome. While there have been proteomic sequencing studies of *F. graminearum* within the host following infection, to our knowledge, the proteome of the fungal remnants on the surface of maize seeds at maturity has not previously been determined. We hypothesized that the proteins sequenced within these fungal remnants could provide insight into the biochemical processes occurring in the fungus prior to desiccation, and that even though CL30 is susceptible to infection by *F. graminearum*, we would be able to identify host defense-related proteins present in both the endosperm and embryo tissues. To that end, we initiated a gel-free protein sequence project using LC-MS/MS to investigate the interplay of the proteomes of host and pathogen.
3.3 Results

Kernels of the inbred maize line, CL30, were injected at 10-15 days post-silk emergence with either water controls or conidial suspensions of *F. graminearum* and allowed to develop to maturity. Following harvest, kernels from both treatments were collected, and the proteomes of discrete tissues from the kernels, as well as the remnants of *F. graminearum* on the surface of the maize seeds were sequenced using LC-MS/MS. Sequences were analyzed and classified by biochemical function.

Cumulatively, 250 plant-sourced proteins were identified in the water control or *Fusarium* treated embryo and endosperm samples. These proteins were classified based upon their molecular function, with the classes as follows: maturation; storage; stress tolerance; metabolism; nucleic acid binding; protein folding, synthesis and transport; oil body-associated; miscellaneous; unknown; and defense. This classification is shown in Figure 3.1. When comparing the percent representation between the control and infected samples, the largest differences in percent representation include nucleic acid binding (11.8% in control and 5.4% in the infected) and oil body associated (5.9% in the control and 2.7% in the infected) in the embryo samples. The storage (4% in the control and 7.5% in the infected), stress tolerance (16% in the control and 7.5% in the infected) and metabolism (10.7% in the control and 18.9% in the infected) proteins in the endosperm also showed differences in percent representation. Additionally, the defense proteins had different representations in both the embryo and endosperm samples.
Figure 3.1: Percent representation of maize functional protein categories.
Proteins were isolated from both the *Fusarium* infected and control embryo and endosperm tissues and sequenced by LC-MS/MS. Functional categories are based on KEGG classifications (Kanehisa and Goto, 2000).
That both the embryo and endosperm samples had different representation of defense proteins was of particular interest. The percentage representation of defense proteins in the control embryo, infected embryo, control endosperm, and infected endosperm samples was 3.5%, 21.6%, 2.7%, and 7.5%, respectively. The defense proteins identified in each sample are shown in Table 3.1, which includes GenBank accession number, protein name, species, Mascot score, percent coverage, and the number of peptide hits (and unique hits). The percent coverage was calculated by adding together the total number of amino acids residues present in all the peptide hits attributed to a particular protein, and dividing this sum by the total length of the protein in GenBank. There were five classes of defense proteins identified: chitinases; ribosome inhibiting proteins; proteinase inhibitors; proteases; and peroxidases.

We discovered 18 proteins that were not degraded upon the desiccation of the fungal remnants on the surface of the maize kernels (Table 3.2). Of the 18 proteins identified, 10 were attributed to *F. graminearum*, and four proteins were attributed to *F. oxysporum*. A putative linoleate diol synthase from *F. graminearum* had the highest Mascot score (349). Other enzymes involved in the processing of raw nutrients include glycoside hydrolase and xylanase. Additionally, we identified ubiquitous proteins, proteins involved with stress tolerance, metabolism, as well as proteins with unknown functions.

### 3.3 Discussion

The economic losses from all *F. graminearum* diseases are in excess of $1 billion annually through the reduction in grain yield and certain traits such as grain quality.
## Table 3.1: Defense proteins in maize samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Accession</th>
<th>Protein</th>
<th>Mascot Score</th>
<th>% Coverage</th>
<th>Peptides, Uniques</th>
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</thead>
<tbody>
<tr>
<td><strong>H₂O treated maize embryo</strong></td>
<td>NP_001147325</td>
<td>subtilisin-chymotrypsin inhibitor CI-1B</td>
<td>139</td>
<td>38.6</td>
<td>2,2</td>
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<td></td>
<td>ACJ62806</td>
<td>ribosome-inactivating protein</td>
<td>106</td>
<td>12.3</td>
<td>3,3</td>
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<td></td>
<td>AAT39992</td>
<td>chitinase, class GH19</td>
<td>101</td>
<td>7.2</td>
<td>2,1</td>
</tr>
<tr>
<td><strong>Fusarium treated maize embryo</strong></td>
<td>ACG36543</td>
<td>peroxidase 12</td>
<td>209</td>
<td>13.6</td>
<td>4,4</td>
</tr>
<tr>
<td></td>
<td>NP_001140805</td>
<td>uncharacterized protein LOC100272880 - pepsin retropepsin-like</td>
<td>202</td>
<td>16.1</td>
<td>4,4</td>
</tr>
<tr>
<td></td>
<td>AAT39991</td>
<td>chitinase, class GH19</td>
<td>159</td>
<td>14.2</td>
<td>4,3</td>
</tr>
<tr>
<td></td>
<td>NP_001105541</td>
<td>chitinase chem5 precursor - contains GH18 hevamine XIP I class III domain</td>
<td>150</td>
<td>8.9</td>
<td>2,2</td>
</tr>
<tr>
<td></td>
<td>NP_001142312</td>
<td>uncharacterized protein LOC100274481 - contains GH18 hevamine XIP I class III domain</td>
<td>147</td>
<td>9.9</td>
<td>3,3</td>
</tr>
<tr>
<td></td>
<td>NP_001232812</td>
<td>trypsin/factor XIIA inhibitor precursor</td>
<td>99</td>
<td>47.8</td>
<td>3,3</td>
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<tr>
<td></td>
<td>NP_001146946</td>
<td>cysteine proteinase inhibitor B</td>
<td>79</td>
<td>14.0</td>
<td>2,2</td>
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<td></td>
<td>CAN72184</td>
<td>hypothetical protein VITISV_042598 - retropepsin like</td>
<td>78</td>
<td>3.1</td>
<td>4,3</td>
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<tr>
<td><strong>H₂O treated maize endosperm</strong></td>
<td>CAA49723</td>
<td>protein b-32 – ribosome inhibiting protein</td>
<td>237</td>
<td>21.5</td>
<td>5,2</td>
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<tr>
<td></td>
<td>NP_001149472</td>
<td>endochitinase A precursor, class GH19</td>
<td>120</td>
<td>11.8</td>
<td>2,2</td>
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<tr>
<td><strong>Fusarium treated maize endosperm</strong></td>
<td>ACJ62815</td>
<td>ribosome-inactivating protein</td>
<td>206</td>
<td>20.6</td>
<td>4,2</td>
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<tr>
<td></td>
<td>ABA34115</td>
<td>maize protease inhibitor</td>
<td>111</td>
<td>38.4</td>
<td>2,2</td>
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<tr>
<td></td>
<td>NP_001142312</td>
<td>uncharacterized protein LOC100274481 precursor - putative xylanase inhibitor XIP-I, or class III plant chitinase</td>
<td>92</td>
<td>7.1</td>
<td>2,2</td>
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<tr>
<td></td>
<td>NP_001142211</td>
<td>uncharacterized protein – contains ricin-type β-trefoil domains</td>
<td>77</td>
<td>5.8</td>
<td>2,2</td>
</tr>
</tbody>
</table>
Table 3.2: Proteome of desiccated fungi on the surface of infected maize kernels.

<table>
<thead>
<tr>
<th>Accession</th>
<th>Protein</th>
<th>Species</th>
<th>Mascot Score</th>
<th>% Coverage</th>
<th>Peptides, Uniques</th>
</tr>
</thead>
<tbody>
<tr>
<td>XP_382844</td>
<td>hypothetical protein FG02668.1 – putative linoleate diol synthase</td>
<td><em>Gibberella zeae</em> PH-1</td>
<td>349</td>
<td>10.1</td>
<td>10,8</td>
</tr>
<tr>
<td>XP_381869</td>
<td>hypothetical protein FG01693.1</td>
<td><em>Gibberella zeae</em> PH-1</td>
<td>220</td>
<td>5.4</td>
<td>5,5</td>
</tr>
<tr>
<td>XP_389762</td>
<td>hypothetical protein FG09586.1 - putative phosphatidylinositol/phosphatidylglycerol transfer protein</td>
<td><em>Gibberella zeae</em> PH-1</td>
<td>194</td>
<td>26.7</td>
<td>5,5</td>
</tr>
<tr>
<td>AAR02398</td>
<td>glycoside hydrolase 7</td>
<td><em>Gibberella zeae</em></td>
<td>183</td>
<td>7.8</td>
<td>4,3</td>
</tr>
<tr>
<td>XP_388897</td>
<td>SODC_NEUCR superoxide dismutase</td>
<td><em>Gibberella zeae</em> PH-1</td>
<td>171</td>
<td>15.8</td>
<td>2,1</td>
</tr>
<tr>
<td>EGU80493</td>
<td>hypothetical protein FOXB_08953 – putative Cu-Zn superoxide dismutase</td>
<td><em>Fusarium oxysporum</em> Fo5176</td>
<td>137</td>
<td>16.9</td>
<td>2,1</td>
</tr>
<tr>
<td>XP_381699</td>
<td>hypothetical protein FG01523.1 – putative aldo-keto reductase</td>
<td><em>Gibberella zeae</em> PH-1</td>
<td>132</td>
<td>17.9</td>
<td>4,4</td>
</tr>
<tr>
<td>XP_381422</td>
<td>hypothetical protein FG01246.1 – putative old yellow enzyme</td>
<td><em>Gibberella zeae</em> PH-1</td>
<td>131</td>
<td>2.6</td>
<td>3,3</td>
</tr>
<tr>
<td>ABU48779</td>
<td>translation elongation factor 1-alpha</td>
<td><em>Wickerhamomyces rhabaulensis</em></td>
<td>126</td>
<td>15.9</td>
<td>2,2</td>
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<tr>
<td>XP_001538961</td>
<td>ubiquitin/79aa fusion protein</td>
<td><em>Ajellomyces capsulatus</em> NAml</td>
<td>121</td>
<td>37.0</td>
<td>3,1</td>
</tr>
<tr>
<td>XP_391289</td>
<td>hypothetical protein FG11113.1 – putative bicupin, oxalate decarboxylase</td>
<td><em>Gibberella zeae</em> PH-1</td>
<td>121</td>
<td>9.6</td>
<td>3,3</td>
</tr>
<tr>
<td>CAD92004</td>
<td>calmodulin</td>
<td><em>Fusarium oxysporum</em></td>
<td>117</td>
<td>32.2</td>
<td>3,2</td>
</tr>
<tr>
<td>EGU72061</td>
<td>hypothetical protein FOXB_17429</td>
<td><em>Fusarium oxysporum</em> Fo5176</td>
<td>110</td>
<td>36.4</td>
<td>2,1</td>
</tr>
<tr>
<td>XP_003044867</td>
<td>predicted protein - ribosomal protein S11</td>
<td><em>Nectria haematococca</em> mpVI 77-13-4</td>
<td>103</td>
<td>22.7</td>
<td>3,3</td>
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<tr>
<td>AAV98254</td>
<td>putative xylanase 2</td>
<td><em>Gibberella zeae</em></td>
<td>99</td>
<td>8.2</td>
<td>2,2</td>
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<td>XP_390596</td>
<td>hypothetical protein FG10420.1</td>
<td><em>Gibberella zeae</em> PH-1</td>
<td>95</td>
<td>21.4</td>
<td>3,3</td>
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<td>EGU83490</td>
<td>hypothetical protein FOXB_05994 - contains stress-induced bacterial acidophilic repeat motif</td>
<td><em>Fusarium oxysporum</em> Fo5176</td>
<td>86</td>
<td>33.8</td>
<td>2,1</td>
</tr>
<tr>
<td>XP_003007386</td>
<td>60S ribosomal protein L26-1</td>
<td><em>Verticillium albo-atrum</em> VaMs.102</td>
<td>82</td>
<td>32.3</td>
<td>3,2</td>
</tr>
</tbody>
</table>
(Goswami and Kistler, 2004). Previous studies have investigated the proteomic profiles of both pathogen and host at various stages of infection (Geddes et al., 2008; Mohammadi et al., 2011; Shin et al., 2011; Zhou et al., 2006), but we were interested in the proteomes of both the field desiccated fungal remnants on the surface of the seed at harvest and in the tissues of the mature seeds themselves.

3.3.1 Percent representation

The sessile nature of plants forces their pathogen defense systems to be particularly robust (The Arabidopsis Genome Initiative, 2000). It has previously been shown that certain defense proteins are upregulated following pathogen infection (Walter et al., 2010). Our data confirms these findings, as there was an increase in the number of sequenced proteins associated with defense in both the infected embryo and infected endosperm tissues compared to the control samples (Figure 1). The number of identified defense proteins increased 617% in the infected embryo, and 278% in the infected endosperm. The lower percentage increase of defense proteins in the endosperm is due to the apoptotic (and therefore less responsive) nature of the endosperm (Young and Gallie, 2000).

3.3.2 Proteinase inhibitors

Proteinase inhibitors (PIs) are abundantly expressed in storage tissues; comprising up to 10% total protein content (Habib and Fazili, 2007). PIs act by forming complexes with proteolytic enzymes (Sels et al., 2008). These complexes reduce the activity of the
proteolytic enzymes, and therefore are important in regulating a number of important biological functions influenced by proteolytic enzymes, including signal transmission, seed dormancy, and apoptosis (Haq et al., 2004). In addition to regulating host biochemical processes, PIs have a proposed role in plant defense. Upon infection, fungi secrete proteases that degrade cell walls to facilitate colonization, as well as digest plant proteins down to the amino acids required for fungal metabolism (Dunaevskii et al., 2005). In response to fungal infection, plant PIs are upregulated, and bind and reduce fungal protease activity (Ryan, 1990). We identified four PIs (Table 3.2). Both the infected embryo and endosperm tissues had more represented PIs compared to the water injected control samples. One PI identified in *Fusarium*-treated embryo was a trypsin/factor XIIA inhibitor (GenBank accession NP_001232812). This PI, in addition to inhibiting proteases, also demonstrates α-amylase inhibitor activity. These dual function inhibitors are particularly effective in defending against fungal pathogens, as the degradation of both proteins and starch granules is inhibited – severely limiting the sources of energy to the growing fungal hyphae.

### 3.3.3 Chitinases

Chitinases hydrolytically cleave the β-1,4-glycosidic bonds in both chitin and chitosan, which are important structural components of fungal cell walls (Jitonnom et al., 2011). The majority of identified plant chitinases are endochitinases that hydrolyze bonds within the chitin, chitosan, or other molecules, as opposed to exochitinases, which catalyze the cleavage of bonds at the non-reducing end of chitin polymers (Huang et al., 2012). Chitinases play an important role in plant defense against fungal pathogens. Following
infection, chitinases are upregulated (van Loon et al., 2006), and hydrolyze exposed chitin polymers in fungal hyphal tips, inhibiting fungal growth (Grover, 2012). Plant chitinases are grouped into two separate families, based on features of their catalytic domains: GH18, which includes chitinase Classes III and V, and GH19, which includes chitinase Classes I, II, and IV (Santos et al., 2008). The GH18 and GH19 families likely do not share a common ancestral protein, as these families have significantly different amino acid sequences and three-dimensional structures (Ubhayasekera, 2011). Class IV chitinases (GenBank accessions AAT39991, AAT39992, NP_001149472) are distinguished by the presence of an N-terminal carbohydrate-binding domain (Ubhayasekera et al., 2009). Class IV chitinases were found in both infected and control samples.

### 3.3.4 Xylanase inhibitors

Xylan is composed of linked β-1,4-linked D-xylose groups that can be substituted by different residues, including L-arabinose (Juge, 2006). Xylans and their substituted derivative molecules are the most abundant hemicellulose molecule in cereals, representing up to 25% of the dry weight of maize (Pordesimo et al., 2005). Secreted fungal endoxylanases catalyze the degradation of xylans into poly- and oligosaccharides by hydrolyzing the β-1,4-linkages between xylopyranoside residues in xylans (Prade, 1996). There are two classes of fungal xylanase: GH10 and GH11 that differ in size, 3D structure and catalytic properties (Biely et al., 1997). We identified a putative xylanase in our sequencing of the *Fusarium* remnants on the seed surface (Table 3.2). This 40 kDa GH10 xylanase is characterized by broad substrate specificity and a resistance to certain
xylanase inhibitor proteins (XIPs) (Goesaert et al., 2004). The two XIP-like proteins we identified (Table 3.2 - GenBank accessions NP_0011005541 and NP_001142312) have been shown to be able to inhibit both GH10 and GH11 xylanase classes. Class GH10 is inhibited through XIPs binding to the xylanase substrate-binding pocket, while class GH11 is inhibited through XIPs binding to the active site (Payan et al., 2004). In addition to sharing identity with XIPs, these XIP-like proteins we identified have sequence identity to class III chitinases, though these proteins have modified active sites that lack the ability to catalyze the degradation of chitin (Payan et al., 2003). The discovery of XIPs in only the *Fusarium* treated samples agrees with previous studies that demonstrated that XIPs in maize were upregulated following *F. graminearum* infection (Mohammadi et al., 2011).

### 3.3.5 Ribosome inactivating proteins

Cereal ribosome inactivating proteins (RIPs) are rRNA N-glycosidases that inactivate ribosomes by catalyzing the irreversible depurination of a single adenine residue in the conserved elongation factor-binding α-sarcin loop of 28S rRNA molecules (Barbieri et al., 1993). Accordingly, protein synthesis is arrested in these depurinated ribosomes.

There are three types of RIPs. The maize RIP1 protein (b-32) is a type 3 RIP, as it is expressed as inactive precursors that, following proteolytic removal of N-terminal, internal, and C-terminal domains, yield two chains, which interact non-covalently to form an active form that is 10 000 times more active than the precursor (Walsh et al., 1991). Unlike many of the other pathogen resistance gene products, b-32 is not upregulated in response to pathogen infection; it is expressed in the endosperm concurrently with the
deposition of storage proteins (Balconi et al., 2010). This is reflected in Table 3.1, as the RIP proteins are found in both the control and *Fusarium* infected samples.

### 3.3.6 Peroxidases

Oxidative burst is the rapid and transient production of reactive oxygen species (ROS) in response to pathogen infection (Angel Torres, 2010). There are three major proposed functions of the ROS during oxidative burst: 1) generating a toxic environment that inhibits pathogen growth, 2) reinforcing cell walls to prevent pathogen spreading, and 3) activating signaling cascades to induce pathogen response genes (Passardi et al., 2005). There are several pathways that work synergistically to produce ROS during oxidative burst. From these intertwined pathways, we identified a single peroxidase in our survey. This class III peroxidase (GenBank accession ACG36543) was present in the *Fusarium* treated embryo sample. Class III peroxidases are thermostable glycoproteins with broad substrate specificity that are localized to vacuoles and cell walls (Almagro et al., 2009). The importance of class III peroxidases in response to pathogen attack was demonstrated in *Arabidopsis*, where transgenic lines with reduced transcript levels of class III peroxidases had a reduced oxidative burst and had an increased susceptibility to pathogen infections (Bindschedler et al., 2006). Our discovery of ACG36543 in only the *Fusarium* treated embryo reinforces previous findings that this particular peroxidase is upregulated in response to pathogen infection (Mohammadi et al., 2011) and may be sufficient to convey protection due to its multifunctional properties (Passardi et al., 2005).
Fungal pathogens can mitigate the effects of the ROS expressed during an infected host’s oxidative burst through the action of several different proteins. These protein families include peroxidases, catalases and superoxide dismutases (Mayer et al., 2001). We identified two copper-zinc (Cu-Zn) superoxide dismutases (SOD) amongst the Fusarium remnants (Table 3.2). There are four classes of SOD, which can be differentiated by their metal co-factors: 1) Cu-Zn, 2) Mn, 3) Fe, and 4) Ni (Mayer et al., 2001). All classes of SOD catalyze the dismutation of \( \cdot \text{O}_2 \) and \( \cdot \text{OH} \) to \( \text{O}_2 \) and \( \text{H}_2\text{O}_2 \) (Hwang et al., 2002). Previously, \textit{F. graminearum} Cu-Zn SOD proteins have been identified in infected \textit{Triticum aestivum} (Paper et al., 2007; Zhou et al., 2006), indicating that this protein is highly expressed by \textit{F. graminearum} during infection.

### 3.3.7 Fusarium on the surface

The proteins identified in the fungal remnants on the surface of the maize kernels could be divided into four main categories depending on their biological functions: 1) Proteins responsible for the metabolism of plant molecules (linoleate diol synthase, glycoside hydrolase), 2) ROS resistance (superoxide dismutase), 3) constitutive expressed proteins (elongation factors, ribosomal proteins, calmodulin, and others), and 4) hypothetical proteins with no assigned function. As we identified individual 18 proteins in our desiccated \textit{Fusarium} samples, these proteins may still be present as they are either very highly expressed or resistant to degradation.
3.3.8 *Fusarium* metabolism proteins

Up to 70% of the total oil content from maize seeds is linoleic acid (Jellum, 1970). This unsaturated n-6 fatty acid can be metabolized by *Fusarium* by the putative linoleate diol synthase (LDS) (Table 3.2) in the following reaction: linoleic acid is dioxygenated to (8R)-hydroperoxylinoleate and subsequently isomerized to (7S,8S)-dihydroxylinoleate (Hornsten et al., 1999). Proteins that share sequence identity with LDS have been found in several filamentous fungi (Soanes et al., 2008) and have been hypothesized to be involved with the mobilization of storage lipids (Huber et al., 2002). Gene deletion studies in the rice-blast disease causing *Magnaporthe oryzae* demonstrated that LDS is not required for successful infection or sporulation, possibly due to the presence of redundant linoleic acid metabolizing pathways (Jerneren et al., 2010).

The degradation of different glycosides is performed by the family of glycoside hydrolases that catalyze the hydrolysis of glycosidic linkages, freeing sugar moieties. The glycoside cellulose is a chain of β-(1→4) linked D-glucose units. There are two classes of fungal glycoside hydrolases that degrade cellulose: 1) endoglucanases that catalyze the cleavage of cellulose polymers at random locations, and 2) cellobiohydrolases that hydrolyze the non-reducing ends of cellulose chains (Munoz et al., 2001). The glycoside hydrolase 7 we identified is a cellobiohydrolase. As the most predominant glycoside in maize stalks (approximately 50% of dry weight (Appenzeller et al., 2004)) is cellulose, glycoside hydrolases are necessary for *Fusarium* to degrade plant cell walls to facilitate colonization and nutrient uptake (Yoshizawa et al., 2002).
3.3.9 *Fusarium* stress response

Old Yellow Enzyme (OYE) is a flavin-dependent NADPH-oxidoreductase that was first described in 1932 by Warburg & Christian in 1932 from brewers’ bottom yeast. Proteins with sequence similarity to OYE have been identified in a broad phylogenetic range of organisms, including bacteria, fungi, plants, and mammals (Williams and Bruce, 2002). While the physiological role of OYE is, as of yet, unknown, a number of hypothesized functions has been proposed, including oxidative stress response (Lee et al., 1999). OYE is upregulated in the presence of hydrogen peroxide (Fitzpatrick et al., 2003) and can be oxidized by many different compounds, including ketones and aldehydes (Trotter et al., 2006). These data together with our discovery of OYE in the fungal remnants indicates that it may play a protective role during plant colonization.

Plant late embryogenesis proteins (LEA) are a diverse group of hydrophilic proteins associated with abiotic stress tolerance - most notably desiccation and cold stress (Tolleter et al., 2010). There are three main groups of LEA proteins in plants that are distinguishable by the presence of sequence motifs (Tunnacliffe and Wise, 2007). The majority of proteins within each group have only minimal secondary structure when in solution, though they have increased folding when in a dry state (Tolleter et al., 2007). It has been shown that LEA proteins accumulate during the final stages of embryo maturation (Galau et al., 1986). This accumulation of LEA proteins in mature seeds is reflected in this study, because in each of the maize samples investigated the percent representation was greater than 20% (Figure 1). While the function of LEA proteins in stress tolerance is unknown, it has been hypothesized that they have several different functions, including stabilizing membranes (Steponkus et al., 1998) or desiccation-
sensitive proteins (Goyal et al., 2005), preventing cellular water loss (Garay-Arroyo et al., 2000), or by binding ions or ROS (Tunnacliffe and Wise, 2007).

In addition to plants, LEA proteins have been discovered in prokaryotes, protists, invertebrates, and certain invertebrates (Tolleter et al., 2007). All fungal LEA-like proteins are group 3 LEAs, which are characterized by the presence of multiple repeats of an 11 amino acid residue motif (Tunnacliffe and Wise, 2007). We identified a single LEA-like protein in the fungal remnants (Table 3.2). This protein also shares 96% sequence identity with a conidiation-specific protein. Conidiation-specific proteins are expressed during conidia (spore) formation, which is triggered by low nutrient or water levels (White and Yanofsky, 1993). It is therefore likely that this LEA-like protein was expressed in the *Fusarium* examined during seed maturation and desiccation in order to form the spores necessary for the biological dispersal of the parental *Fusarium* genomic sequence. As other fungal species propagate in a similar manner, the proteins on the surface of the kernels sourced to fungal species other than *F. graminearum* (Table 3.2) may be present due to the damage from the inoculation of the maize kernels. This damage potentially allowed for the limited colonization by opportunistic fungi that would otherwise not have been able to colonize maize.

### 3.4 Conclusions

The approximately 250 maize proteins identified were classified into functional categories, including: late embryogenesis abundant, seed storage, metabolism, and defense, with an increased representation of defense proteins in both the embryo and
endosperm tissues of infected samples. Most of the proteome of the fungal remnants was categorized as being involved in the metabolism of plant-sourced molecules, or in stress response. Taken together, these data should help dissect the interactive system between host and pathogen during the important time period of grain maturation and harvest.

Understanding the interplay of pathogen and host is important in developing pathogen resistant breeding lines. Greater knowledge of host defense systems will allow for the fine-tuning of the polygenic disease resistance present in maize. Finally, the proteins present in the fungal remnants may be targets for fungicides or for rapid biological screening of grain shipments for presence of pathogen infection.

3.5 Methods

*F. graminearum* susceptible *Zea mays* inbred line CL30 was planted on the Central Experimental Farm in Ottawa, ON, Canada for the 2010 growing season. Conidial suspensions of *F. graminearum* strain DAOM 180378, obtained from the Canadian Collection of Fungal Cultures, Ottawa, ON were prepared to $5 \times 10^5$ conidia/mL as described in (Reid et al., 1996). Maize kernels were inoculated with either the conidial suspensions or sterile water controls using a four-pin automatic kernel inoculator as per Reid, et al., (, 1997). This inoculation was timed to the blister to early milk stages of kernel development; approximately 10-15 days post-silk emergence. Three ears of both the fungal and water treated maize were harvested at maturity.

Kernels were harvested and suspended in protein extraction buffer (10mM Tris-HCl pH 7.8, 0.5 mM EDTA, 10 mM KCl, 1mM PMSF). Kernels were agitated for 30 seconds,
allowed to settle. The supernatant was decanted into a fresh tube, and sonicated on ice at 20 amplitude microns 20 times in three second bursts. Cellular debris was pelleted by centrifugation at 14 000 g for one min at 4 C, and supernatant transferred to a fresh tube and stored at -80 C. The kernels were surface sterilized in a 10% solution of sodium hypochlorite for 5 min, washed 5 times in ddH2O and dried in a laminar flowhood. Kernels were incubated overnight in 0.3% (w/v) sodium metabisulphite and 85% (v/v) lactic acid at 37 C. Embryo and endosperm tissues were manually dissected from the pericarp using a razor blade. Isolated tissues were ground in a mortar and pestle and suspended in protein extraction buffer. The solution was filtered through a 63 μ mesh and centrifuged at 14 000 g for 10 min at 4 C. The pellet was washed two times with protein extraction buffer, two times with ice cold 95% ethanol, and two times with ice cold acetone. The pellets were allowed to dry in a laminar flowhood. Protein extraction buffer was added to the pellets and allowed to incubate for 30 min at room temperature. The solution was centrifuged at 14 000 g for ten min, and the supernatant was collected. Along with the protein samples previously stored at -80 C, the samples were supplemented with four volumes of ice cold acetone and stored at -20 C overnight to precipitate proteins. The samples were centrifuged at 14 000 g for twenty min and washed twice with ice cold acetone and dried in a laminar flowhood.

Pellets were resuspended in 100 μL ddH2O. Protein concentration was determined using the Bradford assay (Bradford, 1976). Two micrograms of each protein sample was subjected to mass spectrometric analysis at the Ottawa Institute of Systems Biology, OISB. Protein samples were digested with trypsin, and digested peptides were separated by liquid chromatography on an Agilent 1100 Series HPLC System (Agilent
Technologies, Palo Alto, CA). Separated peptides were ionized by electrospray to a QSTAR Pulsar quadrupole-TOF mass spectrometer (ABI/MDS Sciex, Concord, ON) as described in (Vasilescu et al., 2005). Mascot software (version 2.3) (Matrixscience Ltd.) was used to interrogate the non-repetitive NCBI protein database (12/20/2011; 16 392 747 sequences; 5 641 810 382 residues) with the taxonomy filter set to either ‘other fungi’ (3 787 334 sequences) or ‘other green plants’ (3 614 282 sequences). Carbamidomethyl (C) and oxidation (M) were set to fixed variable modifications, respectively. One missed cleavage was permitted. Tolerances were set for both peptide (±100 ppm) and MS/MS mass tolerances (0.2 Da). Decoy searches were performed, and false positive rates were approximately 1%.

Molecular function was assigned to proteins using the Kyoto Encyclopedia of Genes and Genomes (KEGG) at http://www.genome.jp/kegg/kegg2.html (last viewed 7/09/2012) (Kanehisa and Goto, 2000; Kanehisa et al., 2006; Kanehisa et al., 2010). Peptides corresponding to unknown or hypothetical proteins in the NCBI database were classified based on sequence similarity by BLAST searches (Altschul et al., 1990).
Chapter 4

The starch granule associated proteomes of commercially purified starch reference materials from rice and maize

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Contribution of authors:

AGK contributed to the study design, performed experiments except LC-MS/MS, analyzed data, contributed all figures, wrote the manuscript, and participated in editing the manuscript. BKM aided in experiments, analyzing data, and editing. MPH performed LC-MS/MS. JS participated in analyzing data and editing. IA participated in study design, data analysis, and editing.
4.1 Abstract

Commercially available reference materials are integral components of many experimental protocols, as it is critical to compare one’s results to those derived from well-characterized standards. Most reference materials are well defined, with all their components being catalogued. However, certain reference materials, such as commercially prepared starch samples, can have undefined components, potentially limiting their usefulness as standards. The proteome of commercially prepared starch has not been documented, and to that end, we initiated a mass spectrometry-based survey of the proteins associated with starch granules in commercially prepared rice and maize starch samples. We performed direct trypsin treatments of starch samples and sequenced both the water-soluble peptides liberated into the aqueous supernatant and the peptides released from the starch granule surface by isopropanol solvent washing. We discovered that the majority of proteins, in both rice and maize samples, were involved in either carbohydrate metabolism or storage. We also documented proteins that are markers for seed maturity and for starch mobilization.
4.2 Introduction

Starch is a versatile and renewable commodity that is exploited as food and biofuel, as well as in industry during the production of chemicals, paper, binders, and adhesives (Roper, 2002). Worldwide, almost 300 billion tons of starch is manufactured per year, with 90% originating from cereal crops (Halford et al., 2011; Tetlow, 2011). These crops, which include rice and maize, produce seeds that contain up to 70% of their dry weight as starch (Dale and Housley, 1986). Having such concentrated reserves of starch so readily available, it is therefore not surprising that as an extractable component, rice starch was used as far back as ancient Egypt for the manufacture of papyrus (Bostock and Riley, 1855). Cereal starch is synthesized in amyloplasts, and stored in the endosperm as semi-crystalline granules consisting of amylose and amyllopectin (Zeeman et al., 2010). Both these polymers contain $\alpha$-(1$\rightarrow$4) glycosidic linkages, however, amyllopectin which makes up 75% of the mass of most starch granules, contains approximately 5-6% of randomly distributed $\alpha$-(1$\rightarrow$6) glycosidic linkages (Grimaud et al., 2008). These linkages introduce branch points in the amyllopectin chains that allow for the clustering of chains into tightly packed arrays, and, consequently, the semi-crystalline nature of starch (Perez and Bertoft, 2010).

The origins of starch synthesis can be traced to the common ancestor of the Archaeplastida (Patron and Keeling, 2005). Following an endosymbiotic event between a heterotrophic eukaryotic host and a cyanobacterium, which gave rise to the Archaeplastida, the genomic reorganization in the new, photosynthetic eukaryotic organism merged polyglucan synthesis pathways of bacterial and eukaryotic origins (Deschamps et al., 2008). Cytosolic starch synthesis pathways evolved from these
reorganization events, and it was with the evolution of the light-harvesting complexes, which are responsible for light capture and photoprotection (Koziol et al., 2007) that starch synthesis was relocated to plastids (Tetlow, 2011). It has been proposed that this relocation was prompted by the requirements for ATP, generated via starch degradation, to overcome protoporphyrin IX-induced oxidative stress (Deschamps et al., 2008).

In addition to starch, the endosperm of cereal seeds contains proteins, lipids, carbohydrates, and other organic and inorganic compounds (Lopes and Larkins, 1993). Most of the proteins found in seeds are storage proteins, which act as sinks of amino acids for germinating embryos (Shewry and Halford, 2002). However, structural and metabolic proteins, and starch granule associated proteins (SGAPs) are present as well (Lowy et al., 1981). Many of these proteins are enzymes involved in starch biosynthesis, and include adenosine 5’ diphosphate-glucose pyrophosphorylase, granule-bound starch synthase (GBSS), soluble starch synthase, starch branching enzyme, starch debranching enzyme, and plastidial starch phosphorylase (Jeon et al., 2010). Other proteins are involved in defense, transport, signaling, protein folding, and stress response (Balmer et al., 2006; Wall et al., 2010).

Despite SGAP being a term in the biochemical lexicon since 1986, SGAPs have only been investigated on a case-by-case approach (Baldwin, 2001; Greenwell and Schofield, 1986). Regardless of this lack of scientific precision, commercial starch samples have nevertheless still been frequently used as reference materials in experiments. The rice and maize starch samples we examined here have been used in a broad range of experiments that, for example, have studied starch granule structure using light and electron microscopy (Baldwin et al., 1994), the enzymatic digestibility of starch granules by
amylases and glucosidases (Erban et al., 2009), the production of ceramic materials with differing levels of porosity through the use of starch additives (Diaz and Hampshire, 2004), the pasting properties of floridean starch in algae (Yu et al., 2002), and the use of sonication treatments during starch separation (Zhang et al., 2005), among others.

The certificates of analysis for these commercially available starch samples give basic measurements of the non-starch components of the samples, such as pH and percentages of moisture, protein, and lipid, but in this current age of systems biology, it is important to be able to catalogue all the detectable components of a sample. It has previously been stated that the presence of proteins and lipids within purified starch samples have an effect on the rate of granule hydrolysis by amylases and glucoamylases (Williamson et al., 1992). Therefore, it is possible that the protein component of commercially purified starch samples could affect the outcome of other experiments in which the starches will serve as defined baseline substrates. It is for this reason that we initiated a tandem mass-spectrometry-based survey of commercially available starch reference materials to catalogue their trace proteomes. We have previously investigated the SGAPs in wheat (Wall et al., 2010; Wall et al., 2010) using strict washing protocols at a laboratory scale. Here, we have investigated the proteomes of rice and maize starch granules following commercial starch purification.
4.3 Experimental procedures

4.3.1 Sampling

Rice and maize starch samples were purchased from Sigma-Aldrich (St. Louis, MO). In order to confirm random sampling, 1 kg rice starch (Cat. No. S7260, Lot No. 107K0052) and 2 kg maize starch (Cat. No. S4126, Lot No. 015K0144) reagent bottles were gently agitated, and four separate 50 mg aliquots from each bottle were removed, with gentle agitation between each sampling step.

4.3.2 Starch granule preparation

To disperse the granules and allow for granule swelling, 350 μL of ddH₂O was added to 200 mg of starch. The samples were incubated for 30 min at room temperature with gentle agitation. To prepare samples for trypsin treatment, ammonium bicarbonate was added to the samples to a final concentration of 100 mM. Trypsin (T6567, Sigma-Aldrich) was suspended in 1 mM HCl to a concentration of 1 μg/μL according to the manufacturer’s directions. Starch samples were incubated with 5 μg of trypsin overnight at 37 °C with gentle mixing.

4.3.3 Peptide preparation

The peptides associated with the starch granule surface were collected according to our modified protocol (Wall et al., 2010). Briefly, the trypsin-treated granules were centrifuged at 18,000 x g for 1 min. This aqueous supernatant was transferred to a fresh
tube. The pellet, which consisted of trypsin-treated starch granules and peptides, was washed five times with a ten-fold excess of ddH₂O to remove any residual water-soluble proteins. Following water washing, proteins remaining on the starch granule surface were extracted by adding 350 μL 50% (v/v) isopropanol, 50 mM NaCl and gently mixing for 45 min at room temperature. The samples were centrifuged at 18,000 x g for 1 min, and the supernatant was collected. The peptides from both this isopropanol fraction and the reserved aqueous supernatant were dried in a Speed Vac (Speed Vac Concentrator model number SVC 100H; Savant Instruments, Inc. Hicksville, NY). Peptide pellets were resuspended in 40 μL of ddH₂O, purified using ZipTips with C18 resin (Millipore, Bedford, MA) to remove salt and residual starch, and dried again in a Speed Vac. Peptides were resuspended in 40 μL of 0.1% formic acid.

4.3.4 Chromatography and mass spectrometry

To determine the number and nature of proteins in the samples, peptides were loaded into 7 cm by 200 μm inner diameter trap columns, fritted, and packed in-house with 5 cm of 5 μm Magic C18AQ reversed-phase packing material (Michrom Bioresources, Auburn, CA) using custom pressure vessels constructed in-house and washed with 100 μL of 0.1% formic acid. The column was then connected to a 75 um inner diameter Picofrit tip (New Objective, Woburn, MA) also packed with 5 cm of Magic C18AQ reversed phase material. Peptides were eluted at a flow rate of approximately 250 nL/min using an HP 1090 HPLC (Agilent Technologies, Santa Clara, CA) and the following gradient of acetonitrile containing 0.1% formic acid over 90 min: 0 min 2%, 3 min 5%, 8 min 10%,
70 min 35%, 75 min 80%, 83.5 min 100% (wash step), 84 min 2%, 90 min 2%
(re-equilibration steps).

Eluted peptides were ionized by nanoelectrospray ionization, and analyzed using a
QSTAR XL QqTOF mass spectrometer (AB Sciex, Foster City, CA) operating in
information-dependent acquisition mode. Mass analysis included a 1 s survey scan
followed by two 2 s and two 3 s tandem mass spectrometric scans on the most intense
peaks in the spectrum. Masses could be sequenced twice before being added to an
exclusion list for 90 s.

4.3.5 Protein identification

Tandem mass spectrometric data were interpreted using Mascot software (version 2.3)
(Matrixscience Ltd., Boston, MA) using a custom database consisting of all proteins from
the 20100704 comprehensive National Center for Biotechnology Information non-
redundant (NCBI_nr) protein database (11 365 658 sequences; 3 876 535 693 residues).
Mass tolerances were set to ±50 ppm and ±0.2 Da for the peptide and fragment ion
spectra, respectively; one missed cleavage was considered. Oxidation of methionine
residues was selected as a variable modification; no fixed modifications were selected
because the tryptic shaving did not involve an alkylation step. An automated decoy search
was performed, and false positive rates were approximately 1%. Each match from the
database was verified manually; data were deemed acceptable if at least three successive
y- or b-ions were present (or y++ or b++ ions if the charge state of the peptide was > 2+).
Only proteins with two or more peptide-based identifications with Mascot scores ≥ 55
were included to minimize the chance of matches occurring due to random events. Peptides corresponding to unannotated sequences in the database were classified based upon sequence homology by BLAST searches (Altschul et al., 1990). Molecular function of the proteins matched from the database was inferred using the Kyoto Encyclopedia of Genes and Genomes (KEGG) at http://www.genome.jp/kegg/kegg2.html (Kanehisa and Goto, 2000; Kanehisa et al., 2006; Kanehisa et al., 2010). For proteins that corresponded to multiple pathways, the most suitable pathway was chosen based upon the context of the protein being found on the starch granule surface.

4.4 Results

Commercial rice and maize starch samples were treated with trypsin. Peptides generated from the proteolytic digestion of proteins associated with the starch granule surface (SGS) were isolated with aqueous or isopropanol/salt solvents. The peptides were sequenced by tandem mass spectrometry, and MS/MS data were interpreted using Mascot software. The molecular functions of proteins identified by Mascot were determined using KEGG.

4.4.1 Starch granule associated proteins in rice

Using Mascot, peptides from 107 proteins were identified in the rice starch sample. We determined that 90 of these proteins were plant-sourced with 60 proteins identified in the aqueous fraction and 30 identified in the isopropanol fraction. The remaining 17 non-plant proteins were from a phylogenetically diverse range of prokaryotes and eukaryotes and may be present in the starch samples as remnants from pathogen attacks (Wall et al.,
2010) or contaminants from the commercial purification of the starch granules. The percentage representation of the molecular functions of the proteins determined using KEGG is shown in Figure 4.1A and are as follows: seed storage proteins (41%), carbohydrate metabolism (34%), cellular proteins (8%), defence proteins (10%), and other metabolism proteins (7%). The carbohydrate metabolism-related proteins can be subdivided into starch and sucrose metabolism (81%), glycolysis/gluconeogenesis (11%), pyruvate metabolism (7%), fructose and mannose metabolism (4%), and glyoxylate and dicarboxylate metabolism (4%).

We identified 21 proteins classified as carbohydrate metabolism proteins in the rice starch samples. Of these 21 proteins, 17 were found in the aqueous fraction and four were found in the isopropanol fraction. These proteins are presented in Table 4.1, which includes GenBank accession number, protein name, Mascot score, number of peptide hits (and unique peptide hits), percent protein coverage of the peptide hits, and the carbohydrate metabolism subcategories. Percent protein coverage of the peptide hits was calculated by dividing the number of amino acid residues spanned by the assigned peptides by the total number of amino acid residues of the full-length protein. Proteins generated from peptides in the isopropanol fraction are presented in **bold** font. Within carbohydrate metabolism subcategories, we found 17 proteins related to starch and sucrose metabolism, including sucrose synthase, granule-bound starch synthase, branching enzyme, ADP-glucose pyrophosphorylase, pullulanase, α-glucosidase, and α-1,4-glucan phosphorylase. Other carbohydrate metabolism-related proteins identified included sorbitol
Figure 4.1: Functional classification of identified proteins.
Panel A) rice and panel B) maize. The functional categories were determined using KEGG. In the pie charts on the left include carbohydrate metabolism, cellular proteins, defence-related proteins, seed storage proteins, and other metabolism proteins. The carbohydrate metabolism category is subdivided into fructose and mannose metabolism, glycolysis/gluconeogenesis, pyruvate metabolism, and starch and sucrose metabolism in the pie charts on the right.
Table 4.1: Proteins involved in carbohydrate metabolism from *Oryza sativa* starch.

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<tr>
<th>Accession</th>
<th>Protein</th>
<th>Mascot Score</th>
<th>Number of Peptides&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Coverage&lt;sup&gt;b&lt;/sup&gt; (%)</th>
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<td>NP_001062412</td>
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<td>Os04g0486600: G3P dehydrogenase</td>
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<tr>
<td>NP_001051330</td>
<td>Os03g0758100: plastidic alpha 1,4-glucan phosphorylase</td>
<td>220</td>
<td>6(4)</td>
<td>12.5</td>
</tr>
<tr>
<td>EAZ04713</td>
<td>hypothetical protein Osl_26874: sucrose synthase</td>
<td>212</td>
<td>6(1)</td>
<td>8.5</td>
</tr>
<tr>
<td>AAD50279</td>
<td>branching enzyme</td>
<td>126</td>
<td>4(1)</td>
<td>4.0</td>
</tr>
<tr>
<td>AAA33891&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ADP-glucose pyrophosphorylase</td>
<td>124</td>
<td>2(2)</td>
<td>5.4</td>
</tr>
<tr>
<td>ABD57308</td>
<td>UDP-glucose pyrophosphorylase</td>
<td>122</td>
<td>2(2)</td>
<td>5.1</td>
</tr>
</tbody>
</table>

<sup>a</sup> Number of total peptides (number of unique peptides). <sup>b</sup> Percent protein sequence coverage by the total peptides. <sup>c</sup> Entries **in bold** were sequenced from the isopropanol extract.
dehydrogenase, glyceraldehyde phosphate, fructose-bisphosphate aldolase, and orthophosphate dikinase.

There were five proteins identified in rice starch with a broad range of functions. These proteins were termed cellular proteins, of which, three were in the aqueous fraction and two were in the isopropanol fraction (Table 4.2). There were four proteins classified as folding, sorting and degradation proteins, including the endosperm lumenal binding protein, which was found in both aqueous and isopropanol fractions, heat shock protein 70, and protein disulfide-isomerase. There was one protein (elongation factor-1 alpha) associated with translation.

The most abundant class of proteins in rice starch was the seed storage proteins. There were 25 seed storage proteins identified in the rice starch samples (Table 4.3). Of these, there were 21 glutelin, one globulin, and three prolamin proteins. There were 14 seed storage proteins found in the aqueous fraction and 11 seed storage proteins in the isopropanol fraction.

### 4.4.2 Starch granule associated proteins in maize

In the maize starch samples, peptides from 96 proteins were identified in the aqueous fraction and 41 proteins were identified in the isopropanol fraction, yielding 137 proteins in total. Of these 137 proteins, 109 were identified as plant-sourced, with the remaining
Table 4.2: Proteins involved in cellular processes from *Oryza sativa* starch.

<table>
<thead>
<tr>
<th>Accession</th>
<th>Protein</th>
<th>Mascot Score</th>
<th>Number of Peptides</th>
<th>Coverage (%)</th>
<th>Molecular Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAB63469</td>
<td>endosperm lumenal binding protein</td>
<td>707</td>
<td>12(7)</td>
<td>21.3</td>
<td>Folding, sorting and degradation</td>
</tr>
<tr>
<td>NP_001066486</td>
<td>Os12g0244100: heat shock 70 protein</td>
<td>178</td>
<td>4(2)</td>
<td>7.2</td>
<td>Folding, sorting and degradation</td>
</tr>
<tr>
<td>AAX85991</td>
<td>protein disulfide isomerase</td>
<td>142</td>
<td>4(4)</td>
<td>8.2</td>
<td>Folding, sorting and degradation</td>
</tr>
<tr>
<td>CAA34756c</td>
<td>elongation factor-1 alpha</td>
<td>104</td>
<td>2(1)</td>
<td>5.0</td>
<td>Translation</td>
</tr>
<tr>
<td>AAA62325</td>
<td>endosperm lumenal binding protein</td>
<td>97</td>
<td>2(2)</td>
<td>3.8</td>
<td>Folding, sorting and degradation</td>
</tr>
</tbody>
</table>

\(^a\) Number of total peptides (number of unique peptides). \(^b\) Percent protein sequence coverage by the total peptides. \(^c\) Entries in **bold** were sequenced from the isopropanol extract.
Table 4.3: Seed storage proteins from *Oryza sativa* starch.

<table>
<thead>
<tr>
<th>Accession</th>
<th>Protein</th>
<th>Mascot Score</th>
<th>Number of Peptides</th>
<th>Coverage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EEC73106</td>
<td>hypothetical protein Osl_07091:glutelin</td>
<td>689</td>
<td>15(14)</td>
<td>42.3</td>
</tr>
<tr>
<td>NP_001046512</td>
<td>Os02g0268100:glutelin</td>
<td>665</td>
<td>12(7)</td>
<td>31.8</td>
</tr>
<tr>
<td>EEC72839</td>
<td>hypothetical protein Osl_06572:glutelin</td>
<td>625</td>
<td>13(1)</td>
<td>35.0</td>
</tr>
<tr>
<td>CAA33838</td>
<td>unnamed protein product:glutelin</td>
<td>567</td>
<td>13(1)</td>
<td>33.5</td>
</tr>
<tr>
<td>EAY75915</td>
<td>hypothetical protein Osl_03835:glutelin</td>
<td>565</td>
<td>12(4)</td>
<td>32.9</td>
</tr>
<tr>
<td>EEC72839</td>
<td>hypothetical protein Osl_06572:glutelin</td>
<td>556</td>
<td>12(7)</td>
<td>28.1</td>
</tr>
<tr>
<td>NP_001046512</td>
<td>Os02g0268100:glutelin</td>
<td>554</td>
<td>11(7)</td>
<td>24.6</td>
</tr>
<tr>
<td>EEC73106</td>
<td>hypothetical protein Osl_07091:glutelin</td>
<td>519</td>
<td>13(12)</td>
<td>38.7</td>
</tr>
<tr>
<td>BAA00462</td>
<td>pre-proglutelin</td>
<td>479</td>
<td>11(0)</td>
<td>26.7</td>
</tr>
<tr>
<td>EEC75527</td>
<td>hypothetical protein Osl_12139:glutelin</td>
<td>470</td>
<td>10(1)</td>
<td>19.0</td>
</tr>
<tr>
<td>EAY85201</td>
<td>hypothetical protein Osl_06564:glutelin</td>
<td>469</td>
<td>9(8)</td>
<td>19.0</td>
</tr>
<tr>
<td>EEC75527</td>
<td>hypothetical protein Osl_12139:glutelin</td>
<td>468</td>
<td>12(9)</td>
<td>24.2</td>
</tr>
<tr>
<td>1311273A</td>
<td>hypothetical protein Osl_0656:glutelin</td>
<td>463</td>
<td>11(0)</td>
<td>26.7</td>
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<tr>
<td>CAA38211</td>
<td>glutelin</td>
<td>457</td>
<td>10(1)</td>
<td>25.4</td>
</tr>
<tr>
<td>BAA00462</td>
<td>prepro-glutelin</td>
<td>413</td>
<td>12(0)</td>
<td>33.1</td>
</tr>
<tr>
<td>1311273A</td>
<td>glutelin</td>
<td>411</td>
<td>12(1)</td>
<td>33.1</td>
</tr>
<tr>
<td>AAO22140</td>
<td>glutelin precursor</td>
<td>382</td>
<td>7(5)</td>
<td>15.8</td>
</tr>
<tr>
<td>EAY85201</td>
<td>hypothetical protein Osl_06564:glutelin</td>
<td>339</td>
<td>9(2)</td>
<td>20.2</td>
</tr>
<tr>
<td>EAY75915</td>
<td>hypothetical protein Osl_03835:glutelin</td>
<td>337</td>
<td>10(3)</td>
<td>28.6</td>
</tr>
<tr>
<td>AAO22140</td>
<td>glutelin precursor</td>
<td>292</td>
<td>6(4)</td>
<td>15.4</td>
</tr>
<tr>
<td>CAA29507</td>
<td>glutelin</td>
<td>306</td>
<td>7(1)</td>
<td>39.4</td>
</tr>
<tr>
<td>BAA11129</td>
<td>prolamin</td>
<td>291</td>
<td>4(4)</td>
<td>49.0</td>
</tr>
<tr>
<td>BAA11129</td>
<td>prolamin</td>
<td>278</td>
<td>5(5)</td>
<td>53.0</td>
</tr>
<tr>
<td>CAA52764</td>
<td>11S globulin</td>
<td>138</td>
<td>3(1)</td>
<td>6.3</td>
</tr>
<tr>
<td>AAV43828</td>
<td>putative prolamin 7</td>
<td>123</td>
<td>2(2)</td>
<td>18.9</td>
</tr>
</tbody>
</table>

* Number of total peptides (number of unique peptides).  
* Percent protein sequence coverage by the total peptides.  
* Entries in bold were sequenced from the isopropanol extract.
28 proteins being attributed to a phylogenetically diverse range of organisms. The molecular functions of the plant-sourced proteins were determined using KEGG, and the percentage representation of the major classifications is shown in Figure 4.1B. The major classifications include: carbohydrate metabolism-related proteins (50%), seed storage proteins (16%), cellular proteins (12%), defence-related proteins (12%), and other metabolism-related proteins (10%). Due to the high percentage representation of carbohydrate metabolism-related proteins, this category was further divided into: starch and sucrose metabolism (47%), glycolysis/gluconeogenesis (34%), pyruvate metabolism (11%), and fructose and mannose metabolism (8%).

In contrast to the 34% in the rice starch samples, we identified 50% of the proteins in the maize starch samples as being involved in carbohydrate metabolism. Maize starch contained 18 proteins associated with starch and sucrose metabolism, including sucrose synthase, granule-bound starch synthase, branching enzyme, ADP-glucose pyrophosphorylase, UTP-glucose-1-phosphate uridylyltransferase, and glucose-1-phosphate adenylyltransferase large subunit 1 (Table 4.4). While the maize starch samples had only one protein, with three isoforms, involved in fructose and mannose metabolism (sorbitol dehydrogenase), there were 13 proteins corresponding to glycolysis/gluconeogenesis, including fructose-bisphosphate aldolase, phosphoglucomutase, triosephosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, 3-phosphoglycerate kinase, and brittle 1. Additionally, there were four proteins found in the maize starch samples involved with pyruvate metabolism. These
Table 4.4: Proteins involved in carbohydrate metabolism from *Zea mays* starch.

<table>
<thead>
<tr>
<th>Accession</th>
<th>Protein</th>
<th>Mascot Score</th>
<th>Number of Peptides</th>
<th>Coverage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>Fructose and Mannose Metabolism</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ABA70761</td>
<td>sorbitol dehydrogenase</td>
<td>164</td>
<td>3(1)</td>
<td>8.5</td>
</tr>
<tr>
<td>NP_001149440</td>
<td>sorbitol dehydrogenase homolog 1</td>
<td>157</td>
<td>3(1)</td>
<td>8.5</td>
</tr>
<tr>
<td><strong>NP_001149440</strong></td>
<td>sorbitol dehydrogenase homolog 1</td>
<td><strong>145</strong></td>
<td>4(1)</td>
<td><strong>8.7</strong></td>
</tr>
<tr>
<td></td>
<td><em>Glycolysis/Gluconeogenesis</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NP_001105336</td>
<td>fructose-bisphosphate aldolase, cytoplasmic isozyme</td>
<td>475</td>
<td>10(7)</td>
<td>32.4</td>
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<tr>
<td>ACG24648</td>
<td>triosephosphate isomerase, cytosolic</td>
<td>305</td>
<td>5(1)</td>
<td>21.7</td>
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<tr>
<td>ACG31393</td>
<td>triosephosphate isomerase, cytosolic</td>
<td>221</td>
<td>4(1)</td>
<td>19.2</td>
</tr>
<tr>
<td>ACF78275</td>
<td>unknown: putative brittle-1 protein</td>
<td>210</td>
<td>5(5)</td>
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<tr>
<td>P48495</td>
<td>triosephosphate isomerase</td>
<td>195</td>
<td>4(0)</td>
<td>17.7</td>
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<tr>
<td><strong>CAB39974</strong></td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
<td><strong>188</strong></td>
<td>4(3)</td>
<td><strong>12.2</strong></td>
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<tr>
<td>ACU23111</td>
<td>unknown: putative fructose-1,6-bisphosphate aldolase</td>
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<td>4(1)</td>
<td>12.8</td>
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<tr>
<td>NP_001105951</td>
<td>cytosolic glyceraldehyde-3-phosphate dehydrogenase GAPC4</td>
<td>167</td>
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<tr>
<td>AAO32643</td>
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<td>CAN70587</td>
<td>hypothetical protein: putative triosephosphate isomerase</td>
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<td>3(2)</td>
<td>12.6</td>
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<td><strong>NP_001105889</strong></td>
<td>brittle-1, chloroplastic/amyloplastic precursor</td>
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<tr>
<td>CAA27681</td>
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<tr>
<td>NP_001105405</td>
<td>phosphoglucomutase, cytoplasmic 2</td>
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<td>2(2)</td>
<td>3.1</td>
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<tr>
<td></td>
<td><em>Pyruvate Metabolism</em></td>
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<td></td>
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<tr>
<td>AAP34174</td>
<td>C4-specific pyruvate orthophosphate dikinase</td>
<td>272</td>
<td>6(2)</td>
<td>8.6</td>
</tr>
<tr>
<td>AAV58858</td>
<td>pyruvate orthophosphate dikinase</td>
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<td>5.1</td>
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<tr>
<td>NP_001146891</td>
<td>2-isopropylmalate synthase B</td>
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<tr>
<td>ACG36184</td>
<td>malate dehydrogenase</td>
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<td>2(2)</td>
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<td></td>
<td><em>Starch and Sucrose Metabolism</em></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>ACG43170</td>
<td>sucrose synthase 1</td>
<td>784</td>
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<td>24.7</td>
</tr>
<tr>
<td><strong>NP_001105001</strong></td>
<td>granule-bound starch synthase 1, chloroplastic/amyloplastic</td>
<td><strong>592</strong></td>
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<td><strong>28.8</strong></td>
</tr>
<tr>
<td>AAQ06291</td>
<td>granule-bound starch synthase precursor</td>
<td>558</td>
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<td>23.5</td>
</tr>
<tr>
<td><strong>AAQ06291</strong></td>
<td>granule-bound starch synthase precursor</td>
<td><strong>500</strong></td>
<td><strong>12(1)</strong></td>
<td><strong>27.1</strong></td>
</tr>
<tr>
<td>NP_001105001</td>
<td>granule-bound starch synthase 1</td>
<td>550</td>
<td>11(1)</td>
<td>24.8</td>
</tr>
<tr>
<td><strong>NP_001105411</strong></td>
<td>sucrose synthase 1</td>
<td><strong>405</strong></td>
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<td><strong>14.1</strong></td>
</tr>
<tr>
<td>NP_001130742</td>
<td>LOC100191846: UTP–glucose-1-phosphate uridylyltransferase</td>
<td>332</td>
<td>7(2)</td>
<td>15.4</td>
</tr>
<tr>
<td>ABK80479</td>
<td>putative granule bound starch synthase</td>
<td>330</td>
<td>6(0)</td>
<td>12.8</td>
</tr>
<tr>
<td>AAB33385</td>
<td>branching enzyme II</td>
<td>258</td>
<td>5(5)</td>
<td>7.1</td>
</tr>
<tr>
<td>NP_001121104</td>
<td>glucose-1-phosphate adenylyltransferase large subunit 1</td>
<td>257</td>
<td>7(4)</td>
<td>12.3</td>
</tr>
<tr>
<td>AAZ82467</td>
<td>ADP-glucose pyrophosphorylase small subunit</td>
<td>181</td>
<td>3(3)</td>
<td>6.0</td>
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<tr>
<td>AAD02955</td>
<td>granule-bound starch synthase</td>
<td>165</td>
<td>4(1)</td>
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</tr>
<tr>
<td>ACA25696</td>
<td>granule-bound starch synthase</td>
<td>160</td>
<td>3(2)</td>
<td>30.9</td>
</tr>
<tr>
<td>AAC33764</td>
<td>starch branching enzyme IIb</td>
<td>157</td>
<td>5(5)</td>
<td>7.1</td>
</tr>
<tr>
<td>ACA25696</td>
<td>granule-bound starch synthase</td>
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<td>3(2)</td>
<td>30.1</td>
</tr>
<tr>
<td>ACA25661</td>
<td>granule-bound starch synthase</td>
<td>131</td>
<td>3(3)</td>
<td>13.7</td>
</tr>
<tr>
<td>AAY42161</td>
<td>ADP-glucose pyrophosphorylase endosperm large subunit</td>
<td>107</td>
<td>4(1)</td>
<td>13.0</td>
</tr>
<tr>
<td>ACA25679</td>
<td>granule-bound starch synthase</td>
<td>77</td>
<td>2(2)</td>
<td>23.8</td>
</tr>
</tbody>
</table>

*a* Number of total peptides (number of unique peptides). *b* Percent protein sequence coverage by the total peptides. *c* **Bold** entries were found in the isopropanol extract.
included pyruvate orthophosphate dikinase, 2-isopropylmalate synthase B, and malate dehydrogenase.

We found nine cellular proteins in the maize starch sample (Table 4.5). There were three proteins identified that were involved with folding, sorting and degradation. These proteins included heat shock protein 70, protein disulfide-isomerase, and thioredoxin H-type. We found one elongation factor, elongation factor 2-gamma 3, which was classified as a translation-related protein. Additionally, we identified histone H4 and two proteins with fasciclin domains associated with cell adhesion. The highest scoring cellular protein in maize starch was the 14-3-3 protein associated with the cell cycle.

There were 12 seed storage proteins identified in the maize starch samples (Table 4.6). Of these, there were four zein, three legumin, two globulin, one vicilin, and two glycinin G1 proteins. Of these 12 proteins, six were found in the aqueous fraction and six were found in the isopropanol fraction.

4.5 Discussion

We have previously examined the starch granule proteome in hard and soft wheats following very strict washing procedures (Wall et al., 2010a; Wall et al., 2010b). Here, we have examined two different species, rice, with starch granules composed of 23.6% amylose (Tan, 2003) and maize with starch granules composed of 23.1% amylose (Uthumporn et al., 2010), whose granules have been processed in large-scale, commercial environments. The protocols for commercial starch processing are optimized for high-throughput processing at low cost, rather than the absolute removal of all non-starch
Table 4.5: Proteins involved in cellular processes from *Zea mays* starch.

<table>
<thead>
<tr>
<th>Accession</th>
<th>Protein</th>
<th>Mascot Score</th>
<th>Number of Peptides</th>
<th>Coverage (%)</th>
<th>Molecular function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q01526</td>
<td>14-3-3-like protein GF14-12</td>
<td>158</td>
<td>2(2)</td>
<td>11.1</td>
<td>Cell cycle</td>
</tr>
<tr>
<td>AAC36132</td>
<td>heat shock protein 70</td>
<td>104</td>
<td>2(0)</td>
<td>3.8</td>
<td>Folding, sorting and degradation</td>
</tr>
<tr>
<td>NP_001105408</td>
<td>endosperm specific protein 1 – contains Fasciclin domain</td>
<td>97</td>
<td>2(2)</td>
<td>7.0</td>
<td>Cell adhesion</td>
</tr>
<tr>
<td>1101277A</td>
<td>histone H4</td>
<td>81</td>
<td>2(0)</td>
<td>17.6</td>
<td>Replication and repair</td>
</tr>
<tr>
<td>ACG29437</td>
<td>thioredoxin H-type</td>
<td>77</td>
<td>2(2)</td>
<td>13.9</td>
<td>Folding, sorting and degradation</td>
</tr>
<tr>
<td>NP_001151465</td>
<td>LOC100285098 – elongation factor 2</td>
<td>66</td>
<td>3(3)</td>
<td>4.2</td>
<td>Translation</td>
</tr>
<tr>
<td>P52588</td>
<td>protein disulfide-isomerase</td>
<td>63</td>
<td>3(3)</td>
<td>8.2</td>
<td>Folding, sorting and degradation</td>
</tr>
<tr>
<td>NP_001151356</td>
<td>fasciclin-like arabinogalactan protein 10</td>
<td>62</td>
<td>2(2)</td>
<td>4.7</td>
<td>Cell adhesion</td>
</tr>
<tr>
<td>ACG36145</td>
<td>elongation factor 1-gamma 3</td>
<td>58</td>
<td>2(2)</td>
<td>4.8</td>
<td>Translation</td>
</tr>
</tbody>
</table>

*a Number of total peptides (number of unique peptides).  
*b Percent protein sequence coverage by the total peptides.*
Table 4.6: Seed storage proteins from *Zea mays* starch.

<table>
<thead>
<tr>
<th>Accession</th>
<th>Protein</th>
<th>Mascot Score</th>
<th>Number of Peptides $^a$</th>
<th>Coverage (%)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAL16994</td>
<td>legumin 1</td>
<td>213</td>
<td>4(1)</td>
<td>10.6</td>
</tr>
<tr>
<td>1802402A</td>
<td>globulin 2</td>
<td>209</td>
<td>4(4)</td>
<td>13.8</td>
</tr>
<tr>
<td>CAA41809</td>
<td>vicilin-like embryo storage protein</td>
<td>200</td>
<td>4(2)</td>
<td>9.1</td>
</tr>
<tr>
<td>P04776</td>
<td>glycinin G1</td>
<td>190</td>
<td>4(4)</td>
<td>9.1</td>
</tr>
<tr>
<td>NP_001104865</td>
<td>legumin 1</td>
<td>180</td>
<td>4(1)</td>
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<tr>
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<td>z1D alpha zein protein</td>
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<tr>
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<td>AAL16994</td>
<td>legumin 1</td>
<td>128</td>
<td>3(3)</td>
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<tr>
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</tr>
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</tr>
<tr>
<td>AAC31468</td>
<td>globulin 1</td>
<td>80</td>
<td>2(2)</td>
<td>7.4</td>
</tr>
<tr>
<td>P04776</td>
<td>glycinin G1</td>
<td>64</td>
<td>2(2)</td>
<td>3.8</td>
</tr>
</tbody>
</table>

$^a$ Number of total peptides (number of unique peptides). $^b$ Percent protein sequence coverage by the total peptides. $^c$ Entries in bold were sequenced from the isopropanol extract.
fractions (Tester et al., 2007). We were therefore interested in determining whether direct trypsinolysis could be used to access and identify the proteins that remain associated with the starch granules following commercial-scale processing (workflow shown in Figure 4.2A). In order to identify hydrophobic peptides on the starch granule surface, we used an isopropanol extraction protocol adapted from Morris et al. (1994). While this protocol was optimized for the isolation of the wheat SGAP puroindoline, an amphipathic protein, we were able to identify certain peptides that remained associated to the SGS following commercial starch processing and subsequent in-lab washing. While it is possible that these associations are artifactual in nature, it is feasible that they are physiologically important and are therefore worthy of further study.

### 4.5.1 Starch granule associated proteins

Thoroughly water washing starch granules from cereal seeds in a laboratory setting can reduce the total protein content to 0.25% (Skerritt et al., 1990), whereas commercial starches have protein contents of approximately 0.4% (Appelqvist and Debet, 1997). The endosperm-sourced proteins of cereal grains can be divided into three distinct categories: 1) seed storage proteins, 2) cellular or amyloplastic proteins, or 3) starch granule associated proteins (SGAPs). The SGAPs are defined as proteins that are located at the surface and/or interior of starch granules (Baldwin, 2001). The most abundant SGAP family, granule-bound starch synthase (GBSS), comprises up to 85% of the total internal SGAPs (Echt and Schwartz, 1981), and is involved in starch synthesis through the addition of uridine diphosphate glucose or adenine diphosphate glucose to growing glucan polymers (Baba et al., 1987; Denyer et al., 1996; Hanashiro et al., 2008). Due to
the abundance of GBSS, it is appropriate that we found five different GBSS proteins in rice starch and an additional ten GBSS proteins in maize starch.

4.5.2 Starch biosynthetic enzymes

The enzymes involved in starch metabolism are well documented. For a comprehensive review, see Tetlow, 2011. Both rice and maize starch samples had a high percentage representation (34% and 50%, respectively) of proteins involved with carbohydrate metabolism (Figures 4.1A and 4.1B). This is consistent with previous studies that examined amyloplast and starch granule-associated proteomes in *Triticum aestivum* (Andon et al., 2002) and *Solanum tuberosum* (Denyer et al., 1996). Many of the proteins in the starch biosynthetic pathway (Keeling and Myers, 2010) were identified in our study (Tables 4.1 and 4.4). These proteins have been assembled in Figure 4.2B to demonstrate how much of the biosynthetic machinery of proteinaceous origin is still present in commercially processed starches. In the endosperm of monocots, other than GBSS, soluble starch synthase, and the starch branching and de-branching enzymes, and a plastidial form of ADP-glucose pyrophosphorylase (AGPase), starch biosynthetic enzymes are located in the cytoplasm (Tetlow, 2011). It is likely that during the dessication of the endosperm, these cytoplasmic enzymes became associated with the remnants of the amyloplast membrane or to the hydrophobic surface of the starch granules (Wall et al., 2010a; Wall et al., 2010b).
4.5.3 Orthophosphate dikinase

It has been proposed that the composition of the storage protein fractions and the starch-protein balance is controlled, in part, by orthophosphate dikinase (PPDK) (Mechin et al., 2007). Given that starch turnover and carbon allocation are integral in coordinating metabolism with growth and that starch content is negatively correlated with biomass (Sulpice et al., 2009), PPDK may also be involved in starch-biomass balance. The classical role of PPDK in both C3 and C4 plants is in catalyzing the reversible reaction of pyruvate, ATP and phosphate to phosphoenolpyruvate, AMP and diphosphate (Chastain and Chollet, 2003). In maize, the expression of PPDK is upregulated at 21 days after pollination (DAP) (Mechin et al., 2007), while the expression of PPDK in rice is highest from 5 – 15 DAP, after which, PPDK is rapidly degraded or inactivated through phosphorylation. This pool of inactivated PPDK is present in mature seeds, and may play a role in developmental processes during seed germination (Chastain et al., 2006). Future analysis of the starch granule-associated phosphoproteome will allow for the determination of phosphorylation levels of PPDK in these starch samples.

4.5.4 Starch mobilization

To utilize their stored carbon reserves, plants must be able to degrade their starch granules to oligosaccharides and monosaccharides. However, of the 59 proteins identified in our survey as being involved with carbohydrate metabolism, only two were involved in the mobilization of starch (Table 4.1). Those enzymes, plastidic α-1,4-glucan α-phosphorylase and α-glucosidase were both found in the rice starch samples.
Glucosidase is involved in the hydrolysis of α-1,4 glucosidic linkages (Smith et al., 2005), while plastidic α-1,4-glucan phosphorylase acts by converting the terminal glycosyl units of the non-reducing ends of linear oligosaccharide glucan chains to glucose 1-phosphate (Duwenig et al., 1997). While the well-studied α-amylase is involved in the direct degradation of the polysaccharides that make up starch granules, α-glucosidase is generally believed to preferentially hydrolyze oligosaccharides (Hanes, 1932). There is, however, evidence that α-glucosidase can act synergistically with α-amylase to enhance granule degradation in barley (Sun and Henson, 1990). While α-glucosidase expression is high during seed development in barley, the expression decreases to basal levels seeds upon maturity (Fincher, 1989). Our ability to detect these starch mobilizing enzymes in only the rice samples may indicate that some of the rice kernels were harvested prior to maturity.

4.5.5 14-3-3 proteins

The 14-3-3 protein family consists of 28–33-kDa acidic polypeptides that are ubiquitously expressed in eukaryotes (Morrison, 2009). The 14-3-3 proteins form homo- or hetero-dimers that are able to bind a broad range of ligands due to the specific phospho-serine/phospho-threonine binding activity of these 14-3-3 dimers (Fu et al., 2000). However, not all 14-3-3 binding is phosphorylation dependent (Aitken, 2006). The ligands with which the dimers of 14-3-3 have been demonstrated to interact include transcription factors, cytoskeletal proteins, signaling molecules, and biosynthetic enzymes, among others (Morrison, 2009). While it has been hypothesized that the
Figure 4.2: Workflow and validation of proteome characterization.
Panel A) For both rice and maize, the purified starch samples came from cereal grains that underwent numerous commercial starch processing steps. Direct tryptic digests and mass spectrometry identified a community of starch granule associated proteins that could be clustered around the granule as a specific proteome. Panel B) Starch biosynthetic pathway reconstruction. To validate the workflow, and characterize the defined proteome, the individual proteins were overlaid on the standard metabolic pathway of starch synthesis (Tetlow, 2011). The congruency validates both the workflow and the nature of the specific proteomes in this case. Presence/absence of protein in the proteomic survey is indicated by black (presence) or white (absence) squares; left-side squares correspond to rice, right-side squares correspond to maize. The starting point of starch biosynthesis in endosperm cells is the conversion of sucrose and uracil-diphosphate (UDP) into uracil-diphosphate glucose (UDPG) and fructose by sucrose synthase (SuSy) (Baroja-Fernandez et al., 2003). The fructose can be converted into sorbitol by sorbitol dehydrogenase (SDH) (de Sousa et al., 2008). The UDPG created by sucrose synthase along with diphosphate is converted to glucose 1-phosphate (G1P) by UTP-glucose-1-phosphate uridylyltransferase (UGPase). The G1P is either converted to adenine-diphosphate glucose (ADPG) by ADP-glucose pyrophosphorylase (AGPase), or converted to glucose 6-phosphate (G6P) by phosphoglucomutase (PGM) and enters glycolysis, where, following glycolysis, the pyruvate can be converted to phosphoenolpyruvate by pyruvate orthophosphate dikinase (PPDK). The ADPG, created from the G1P, is transported into amyloplasts by brittle 1 (Bt1), and can be used to form amylose via granule-bound starch synthase (GBSS) or amyllopectin via starch synthase (SS), starch branching enzymes (SBE) and starch debranching enzymes (SDBE) (Tetlow, 2011).
assembly of the enzyme complexes responsible for starch biosynthesis is mediated by the phosphorylation-dependent binding of 14-3-3 (Tetlow, 2011), there is no record of 14-3-3 being found in mature seeds. Previously, 14-3-3 isoforms have been found in maize starch granules isolated from immature pollen (Datta et al., 2002), as well as in Arabidopsis (Sehnke et al., 2001) and barley (Alexander and Morris, 2006). Isoforms of GBSS, soluble starch synthase, and starch branching enzyme, as well as the starch-degrading enzyme α-amylase can be bound by barley grain 14-3-3 proteins (Alexander and Morris, 2006). The particular isoform of 14-3-3 identified in this study is GF14-12 (Table 4.5), which was first cloned in 1994 and has been shown to form DNA/protein complexes (de Vetten and Ferl, 1994). This discovery of a 14-3-3 protein in mature seeds supports the role of 14-3-3 proteins in the assembly of starch biosynthetic complexes.

4.5.6 Storage proteins

The protein content of cereal endosperm can be divided into four categories based on their solubility in solvents. These Osborne fractions include albumins, soluble in water; globulins, soluble in dilute salt solutions; prolamins, soluble in alcohol/water solutions; and glutelins, soluble in weak acids/bases (Osborne, 1908). Rice endosperm is 7-8% protein (Puchongkavarin et al., 2005), which can be divided into 3.8–8.8% albumin, 9.6–10.8% globulin, 2.6–3.3% prolam and 66–78% glutelin (Cagampan et al., 1966). In maize, the prolamins, represented by the zein family, are more prevalent, while the glutelins are less predominant, depending on the variety of maize used in the production of the maize starch samples. The percentage representation of maize endosperm proteins is usually 4-8% albumin, 3-4% globulin, 47-55% prolam, and 38-45% glutelin (Alais
and Linden, 1991). The protein bodies in rice endosperm are tightly associated with the starch granule surface (Tanaka et al., 1980), and are difficult to remove during starch granule purification, even with extended soaking in solutions of dilute base (Wang and Wang, 2001). The distribution of the seed storage proteins in the rice starch sample favored glutelin, with 84% of the seed storage proteins being identified as glutelins (Table 4.3). Moreover, we found 21 different glutelins associated with the commercial rice starch granule preparations. Given that the current genome sequence database (GenBank) of the rice cultivar Nipponbare contains 22 members of the rice glutelin gene family, it is remarkable that such a high proportion of these are clustered close to the rice granule surface and had not been removed by commercial starch processing and purification. On the other hand, the maize starch samples had an intriguingly high representation of globulins (25%) compared to the prolamins (zeins) (33%) (Table 4.6). This divergence from the expected ratios may be an artefact of the commercial starch purification conditions employed. Future experiments may allow for the determination of the sequence of storage protein isoforms with strong starch-adhering properties. These isoforms could potentially be used to breed varieties of cereals with “customizable” starch adherence that impacts bread-making characteristics or other industrial attributes.

### 4.6 Conclusions

We were able to look at molecular snapshots of the endosperm proteins that remained associated to starch granules following the commercial processing of starch from two different species, both with 23% amylose content. We were, consequently, able to glean insight into the physico-chemical properties of the protein remnants that make up the
intra-granular packing or protein matrix of the mature seeds of two commercially important plants: rice and maize. These proteinaceous remnants could affect the yield of pure starch granules during wet milling and subsequent purification in addition to affecting the experiments in which commercially prepared starches are used as reference materials. Furthermore, the differences inferred between the intracellular architecture and molecular dynamics of rice (C3 plant) and maize (C4 plant) should be instructive to those engineering the maize C4 pathway into rice (Hibberd et al., 2008). Finally, we also found markers of seed maturity in the rice starch samples. These markers indicate that both immature and mature seeds were used in the production of the rice starch samples.
Chapter 5

Commercially produced rice and maize starches contain non-host proteins, as shown by mass spectrometry

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Contribution of authors:

AGK contributed to the study design, performed experiments except LC-MS/MS, analyzed data, wrote the manuscript, and participated in editing the manuscript. BKM aided in experiments, analyzing data, writing the manuscript, and editing. MPH performed LC-MS/MS. JS participated in analyzing data and editing. IA participated in study design, data analysis, and editing.
5.1 Abstract

To evaluate the presence of contaminating, non-host proteins in commercially prepared rice and maize starch samples, we initiated a direct sequencing mass spectrometric proteomics survey. We discovered non-host proteins from a variety of species, including *Phytophthora cinnamomi*, *Homarus americanus*, and *Ovis aries*. Our documentation of *H. americanus* proteins in these starch samples may have food safety implications with regards to shellfish allergies. We hypothesize that these proteins were introduced to the starch samples via process wash water used in the milling and deproteination steps in the commercial preparation of the starches analyzed. The introduction of non-host proteins during commercial processing of starch samples that are used routinely in analytical studies indicates that these studies are using impure materials. Therefore, further study and documentation of the starch samples is required to ensure that all components of the samples are properly catalogued.
5.2 Introduction

In plants, glucose molecules are linked for many reasons, including reducing the osmotic potential of free sugars, sequestering and storing glucose units for diurnal/season metabolic needs, and providing dense granules for gravitropism of statoliths (Ball and Morell, 2003; Caspar and Pickard, 1989; Tetlow, 2011). The linked chains of glucose occur as two distinct polymers: amylose, which is primarily $\alpha(1\rightarrow4)$ linked glucosyl units, with occasional $\alpha(1\rightarrow6)$ branches, and amylopectin, while still predominantly $\alpha(1\rightarrow4)$ linked glucosyl units, contains a much higher proportion of $\alpha(1\rightarrow6)$ branches compared to amylose (Grimaud et al., 2008). Together, amylose and amylopectin are assembled into starch granules (SGs). The SGs in storage organs, such as the endosperm of grains or the storage parenchyma cells of tubers, act as reservoirs for the carbohydrates synthesized during photosynthesis, and have internal semi-crystalline growth rings that are the result of periodic differences in the rate of glucose polymerization during starch synthesis (Perez and Bertoft, 2010).

While the biochemical dissection of SGs in planta has progressed at a fine molecular scale (Graf and Smith, 2011; Yandeau-Nelson et al., 2011), the concomitant analytics of the molecular toolkit for furthering starch-based biofuels or foods has lagged (Shi and Gao, 2011; Shu et al., 2006). Starch extraction on an industrial scale, usually by wet milling, involves several steps, including the removal of the pericarp, germ, fibres, and protein matrix, as well as the purification and drying of the starch granules (Sun, 2005). Generally, wet milling includes a steeping step to allow for easy separation of the germ and to loosen the protein matrix within the endosperm. The steeping process involves fermentation by lactic acid bacteria and yeast (Cabral et al., 2006; Singh et al., 1999).
The seeds are ground gently and the germ is separated from the endosperm based on differences in density in hydrocyclones. The endosperm slurry is ground again, and passed through a series of fine screens to remove fibres and the protein matrix. Starch is purified from any remaining protein based on density in a series of cyclones. The isolated starch granules are then dried prior to being packaged as dry powder and transported (BeMiller and Whistler, 2009).

Commercially extracted starch is ubiquitously present in both industrial and culinary applications. The industrial applications include papermaking, adhesives, bioplastics, biofuel (Kaur et al., 2012; Pitak and Rakshit, 2011; Tasic and Veljkovic, 2011), while modified starches and starch sugars are important food additives (BeMiller, 2011). We have previously documented the remnants of pathogenic microbial proteins on the surface of starch granules in lab-scale purified wheat starch; however, we did not observe proteins from organisms from soil- or water-dwelling organisms (Wall et al., 2010). In our recent study to characterize the proteomes of commercially prepared reference starches from leading biochemical reagent suppliers, we were able to document many host-sourced proteins that remain in starch samples following industrial processing (Koziol et al., 2012). In addition to these host proteins, we discovered proteins from a broad taxonomic range of organisms. It has previously been stated that the purity of the water used to process the starch during commercial purification can contaminate starch samples, and affects the end quality of the starch (Sun, 2005). These findings serve as a caveat for all aspects of biomass research as well as food science and food processing where such biochemical reagents have been assumed to be pure starch. We therefore
hypothesized that the non-host proteins present in the commercial starch preparations were sourced from the water used during the extraction of the starch granules.

5.3 Methods

Methods were as published by Koziol et al (2012). Briefly, rice (cat. no.S7260-1KG, lot no. 107K0052) and maize (cat. no.S4126-2KG, lot no. 015K0144) starches were obtained from Sigma-Aldrich (St. Louis, MO). Prior to sampling, reagent bottles were gently agitated, and four separate 50 mg aliquots from each bottle were removed, with gentle shaking between each sampling step.

5.3.1 Protein extraction

The sampled starches were suspended in 350 μL of ddH2O and incubated for 30 min with gentle agitation. Ammonium bicarbonate was added to the samples to a final concentration of 100 mM. To proteolytically digest the proteins present in the samples, 5 μg of 1 μg/μL trypsin (T6567, Sigma-Aldrich) in 1 mM HCl was added. Samples were incubated overnight at 37°C with gentle agitation. To collect the peptides in the supernatants, samples were centrifuged (Heraeus Sepatech Biofuge A 1302; 18 000 x g, 1 min) and the supernatants were transferred into fresh tubes. Peptides in the supernatants were vacuum-dried (Speed Vac Concentrator SVC 100H; Savant Instruments, Inc. Hicksville, NY) for 3 hours. Starch pellets were washed five times in distilled water, were suspended in protein extraction buffer (50% isopropanol (v/v), 50 mM NaCl), and were gently mixed for 45 min. Samples were centrifuged (2 500 x g, 5 min). Supernatants were transferred to new tubes. Peptides in the supernatants were vacuum-dried as described
above. Starch pellets were discarded. To purify and concentrate the protein fractions, vacuum-dried peptides were resuspended in 40 μL ddH₂O. Peptide suspensions were purified using ZipTips (Millipore, Bedford MA, cat. no. ZTC18M096). The manufacturer’s protocol was repeated eight times per sample. Resulting peptide solutions were vacuum-dried as described above.

5.3.2 Protein separation

Peptide separation was accomplished using a 7 cm by 200 μm inner diameter trap column coupled to a 5 cm by 75 μm PicoFrit nanoelectrospray tip (New Objective, Woburn, MA) packed with 5 μm Magic C18AQ resin (Michrom Bioresources, Auburn, CA). Using a tee union prior to the columns, the flow from an HP 1090 HPLC (Agilent Technologies, Santa Clara, CA) was split to pass the following gradient of acetonitrile containing 0.1% formic acid at a flow rate of approximately 250 nL/min: 0 min 2%, 3 min 5%, 8 min 10%, 70 min 35%, 75 min 80%, 83.5 min 100% (wash step), 84 min 2%, 90 min 2% (re-equilibration steps). Eluted peptides were analyzed using a QSTAR XL QqTOF mass spectrometer (AB Sciex, Foster City, CA) operating in information-dependent acquisition mode. Mass analysis included a 1 s survey scan followed by two 2 s and two 3 s tandem mass spectrometric scans on the most intense peaks in the spectrum for each cycle. Masses could be sequenced twice before being added to an exclusion list for 90 s.

Tandem mass spectrometric data were interpreted using Mascot software (version 2.3) (Matrixscience Ltd., Boston, MA) using a custom database containing all proteins from the 20100704 comprehensive National Center for Biotechnology Information non-redundant (NCBI) protein database (11,365,658 sequences; 3,876,535,693 residues). One missed cleavage was allowed, and mass tolerances were set to ±50 ppm for peptides
and ±0.2 Da for fragment ion spectra. Oxidation of methionine residues was selected as a variable modification, while no fixed modifications were selected. An automated decoy search was performed, and the rate of false positives was approximately 1%. Each match from the database was verified manually and spectra were only used if at least three successive y- or b-ions were present. Only proteins with two or more peptide-based identifications with Mascot scores ≥ 55 (p < 0.05) were included to decrease the chance of matches occurring due to random events.

5.4 Results and Discussion

To better understand the source and nature of the proteins present in commercial starch samples, our approach was applied to both commercially prepared rice and maize starches. These commercial starches have been recently characterized at the level of the host plant proteome (Koziol et al., 2012), but proteins of non-plant origin had not been investigated. Surprisingly, three non-host proteins were identified in the rice samples, and three different non-host proteins were identified in the maize samples (Tables 5.1 and 5.2). Mascot and manual NCBI BLAST searches revealed that these non-host proteins were of microbial and animal origin. The prevalence of the non-host proteins in the samples was approximately 5% calculated by occurrence in Mascot hit tables (data not shown) (Koziol et al 2012). Of the non-host proteins identified, only one (glucose regulated protein/BiP - heat shock protein 70-like) contains a domain that binds carbohydrates (Di Luccio et al., 2007). It is therefore presumed that the other proteins bound non-specifically to the starch granules (Nelson Jr. and Lebrun, 1956).
Microbial remnants from algae and fungi were identified in the starch samples (Table 2).

While the common plant and tree fungus, *Phytophthora cinnamomi*, is not known to

**Table 5.1: Non-host protein contaminants associated to rice starch granule.**

<table>
<thead>
<tr>
<th>Accession</th>
<th>Protein*</th>
<th>Species</th>
<th>Mascot Scoreb</th>
<th>Percent Coverage (%)b</th>
<th>Peptide Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>671058A</td>
<td>glyceraldehydepshosphate dehydrogenase</td>
<td><em>Homarus americanus</em></td>
<td>89</td>
<td>4.5</td>
<td>LTGMAFR IGIDFGGR</td>
</tr>
<tr>
<td>ADB93318</td>
<td>elongation factor 1-alpha</td>
<td><em>Lymanopoda dietzi</em></td>
<td>64</td>
<td>8.4</td>
<td>STTTGHLIYK GSFDYAWVLDK</td>
</tr>
<tr>
<td>P68251</td>
<td>protein kinase C inhibitor protein 1</td>
<td><em>Ovis aries</em></td>
<td>63</td>
<td>14.0</td>
<td>NLLSVAYK KQQMGKEYK DSTLIMQLLR</td>
</tr>
</tbody>
</table>

*a* Protein names produced in Mascot database search. Sequences of unknown or hypothetical proteins were searched in protein BLAST and the protein match with the greatest score is shown.

*b* Percent coverage is the percentage of the full-length protein that is represented by the peptide sequences.
target maize, it causes root rot, stem cankers and crown rot in ornamental and forest species such as cranberry, pear and pine (dos Santos et al., 2011; Hardham, 2005). Being a water mould, it is common for *Phytophthora* species to be spread through cereal crops during irrigation (Hong and Moorman, 2005), allowing for infection of many plants in the same field. Alternatively, *P. cinnamomi* may have been introduced to the starch samples during starch processing. Common steps in wet-milling of cereal grains such as sodium dioxide steeping and lactic acid fermentation may also provide optimal growing conditions for thiophilic and acidophilic bacteria that could potentially contaminate starch samples (Sun, 2005). Such contaminants in wheat starch preparations are known to include microbial enzymes (Wall et al., 2010), so these current results underscore the need to be cautious when using such starch preparations in analytical studies, especially in biomass or biomedical studies, where starch-protein interactions are being queried.

Non-microbial contaminants were also found in the commercial starch samples of rice and maize. These proteins are listed among the other contaminants in Tables 1 and 2. Using Mascot and BLAST searches of the non-redundant NCBI database, the contaminating proteins were found to originate from a variety of species, including *Homarus americanus, Lymanopoda dietzi,* and *Ovis aries.* The proteins found from these species are conserved across the animal kingdom; elongation factor 1-alpha, glyceraldehyde phosphate dehydrogenase, protein kinase C inhibitor, and skeletal actin. The purity of commercial starch is highly dependent on the quality of water used in the processing streams removing the bran and storage components of the starting flour feedstock materials. The unexpected presence of proteins from non-host organisms in the starch samples suggests that cross-contamination between water sources occurred in the
Table 5.2: Non-host proteins identified in maize starch samples.

<table>
<thead>
<tr>
<th>Accession</th>
<th>Protein a</th>
<th>Species</th>
<th>Mascot Score</th>
<th>Percent Coverage (%) b</th>
<th>Peptide Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAA53369</td>
<td>glucose regulated protein /BiP - heat shock protein 70-like</td>
<td>Phytophthora cinnamomi</td>
<td>153</td>
<td>5.3</td>
<td>LSQEEIDR VEIIANDQGNR IINEPTAAIAIAYGIDK AVFPSIVGR</td>
</tr>
<tr>
<td>ACI23566</td>
<td>skeletal muscle actin 3</td>
<td>Homarus americanus</td>
<td>75</td>
<td>9.3</td>
<td>AGFAGDDAPR EEYDESGPGIVHRKCF</td>
</tr>
<tr>
<td>BAE48223</td>
<td>heat shock protein 70</td>
<td>Chlorella pyrenoidosa</td>
<td>55</td>
<td>4.2</td>
<td>YKAEDETHRAR IINEPTAAIAIAYGLDK</td>
</tr>
</tbody>
</table>

a Protein names produced in Mascot database search. Sequences of unknown or hypothetical proteins were searched in protein BLAST and the protein match with the greatest score is shown.

b Percent coverage is the percentage of the full-length protein that is represented by the peptide sequences.
starch mills. As *Chlorella pyrenoidosa* is a fresh water alga (Koziol et al., 2007), it was likely introduced to the starch samples by the water used in the starch processing.

The ease of starch contamination during commercial processing is disconcerting, especially since starch is a consumable product. It is therefore possible that a subset of the contaminants could induce allergic reactions in susceptible individuals. It is the protein component of foodstuffs that is commonly responsible for the development of food allergies (Ramesh, 2008). Recently, the prevalence of shellfish allergies in American children was reported as 17.2% (Gupta et al., 2011). Allergies to shellfish are caused by the protein tropomyosin (Ramesh, 2008). While we did not observe tropomyosin in the starch samples, we documented proteins attributed to *Homarus americanus* (American lobster) in both the rice and maize starch samples. With this discovery of *H. americanus* proteins in commercial starch samples, a risk of triggering shellfish allergies has been identified. It may, therefore, be prudent to screen industrially-extracted starches more rigorously for the presence of any potential allergens.

5.5 Conclusion

We have analyzed commercially prepared starches of two agriculturally important crop species: rice and maize by sampling the starch granule associated proteins via water washing and isopropanol stripping followed by direct sequencing tandem mass spectrometry. Unexpectedly, we identified non-host proteins, which included microbial remnants and non-microbial contaminants on the surface of starch granules of both species. Six non-host proteins were identified in the starch samples. More importantly,
the results demonstrated that this methodology can be used to ensure that potential allergenic proteins are not present in starch samples to be consumed by animals.
Chapter 6

General Discussion of Major Findings
6.1 General Discussion of Chapter 2

The work in this thesis combined data from 2D-electrophoresis, immunoblotting, N-terminal sequencing and MS/MS sequencing of peptides resulting from the endoproteolytic cleavage of salt-soluble globulin-3 in wheat flour preparation enriched in embryo tissue. The results show that the globulin-3 family is subjected to post-translational processing and accumulates in the form of a set of cleavage products. Previous work that described this protein family was performed on the hexaploid wheat cultivar Glenlea (Loit et al., 2009), while the work performed here was on cultivar AC Barrie, the cultivar that was used in the experiments that discovered the putative link between dietary intake of Glo-3A and the development of type 1 diabetes (MacFarlane et al., 2003). Immunoblot analysis of proteins from both embryo and endosperm tissues revealed that the Glo-3 protein family is expressed predominantly in the embryo with very low expression levels in the endosperm (Figure 2.2). Sequencing protein spots extracted from 2D PAGE gels of salt-soluble embryo proteins using mass spectrometry allowed for the development of a model describing the post-translational partial endoproteolytic cleavage of the preproglobulin-3 family (Figure 2.3).

6.1.1 Glo-3 expression pattern and food safety

Our findings that demonstrated that the expression of Glo-3 was predominantly in the embryo confirms immunolocalization data of Glo-3 in Glenlea (Loit et al., 2009) and therefore demonstrates that Glo-3 shares similar expression patterns to 7S globulins in rice, maize and other cereals (Burgess and Shewry, 1986; Sun et al., 1996; Thijssen et al.,
1996). Wheat gluten, first isolated and named by Bartolomeo Beccari in Bologna in 1745 (Beccari, 1745), is today still a generic term that describes a mixture of gliadins and glutelins; seed storage proteins expressed in the endosperm, but not the embryo (van Herpen et al., 2008). However, Glo-3 can be present at very low levels in wheat gluten (MacFarlane et al., 2003); as commercial gluten is often made from whole wheat, which consists of the endosperm and embryo, as well as the aleurone layer. To study the potential diabetogenic effects of Glo-3, an investigation is required that compares wheat gluten that is prepared exclusively from endosperm and therefore does not contain Glo-3 to gluten that is prepared from whole wheat. Stripping or milling off the outer layers of wheat seeds has been demonstrated to reduce the sensitivity of predisposed individuals to wheat flour (Handoyo et al., 2008), as these polished seeds contained lower levels of allergenic proteins. Alternatively, wheat could be genetically transformed to express lower levels of the globulin-3 protein family. A Glo-3 knockdown/knockout line of wheat would allow for increased precision in studying the diabetogenic effects of Glo-3. Using gluten prepared solely from endosperm compared to the entire wheat seed will not only exclude Glo-3, but also other embryo/aleurone layer specific proteins, making it more difficult to definitively prove a role for Glo-3 in the development in T1D. Knockout lines should be identical to the parental lines, with the exception of Glo-3 expression. Maize lines that are Glb1 and Glb2 null have been shown to be able to germinate normally (Kriz, 1989), indicating that knocking out the globulin family is not a lethal mutation.
6.1.2 Post-translational endoproteolytic cleavage of Glo-3

The endoproteolytic processing of some wheat flour protein families has been investigated using N-terminal sequencing (Singh et al., 2001) and mass spectrometry (Dupont et al., 2011), but a concentrated study of the processing of Glo-3 specifically has not been reported before. This present thesis used multiple characteristics of protein spots to determine the location of processing events that occurred. The pI and M_r of the spots, the location of the sequenced peptides within the protein sequence, and the N-terminal sequence data (when available) were all taken into account when assembling the processing model (Figure 2.3). As was demonstrated in Figures 2.2 and 2.3, and in agreement with the literature, the endoproteolytic processing of the Glo-3 protein is partial. It is still unknown how the proteins are prevented from not being completely processed at every protease recognition site; though it has been determined that cleavage is not required for proper folding, transport and storage of these proteins (Heck et al., 1993).

With a better understanding of the processing of the Glo-3 protein family, it is now possible to study the diabetogenic effects of the individual polypeptides generated from the cleavage of proglobulin. The genetic engineering of wheat is now common practice, although the 200 odd traits modified to date are mostly agronomic. Wheat can therefore also be bred or engineered to lack certain protease recognition sites or domains within Glo-3. The wheat lacking either certain domains or cleavage sites can then be used in animal feeding trials in an attempt to locate a specific region within the protein that displays increased diabetogenic activity. Given that this type of engineering already
promises to benefit celiac patients (Gil-Humanes et al., 2010), the same effort should now be expended for a much more expensive disease, T1D.

6.2 General Discussion of Chapter 3

This work has increased the understanding of the protein expression by pathogen and host in water-treated or *Fusarium graminearum*-infected maize kernels. Previous studies have investigated host-*F. graminearum* interactions in actively growing kernels hours or days after infection (Mohammadi et al., 2011; Zhou et al., 2006), but I was interested in the proteins present in the host and pathogen proteomes following the desiccation of mature kernels under field conditions. Our group has previously documented fungal proteins present in healthy, mature wheat seeds (Wall et al., 2010), I therefore decided to extend these investigations by sequencing the proteome of healthy, mature maize seeds, as well as maize seeds with a heavy pathogen load to determine the number and nature of pathogen and host proteins present under these two conditions. In healthy plants, we found no evidence of fungal proteins within the seeds. However, we determined that fungal proteins are present in both the desiccated fungal remnants on the surface of infected seeds as well as within the seeds’ tissues.

6.2.1 Proteins in desiccated fungal remnants

It was intriguing that we only discovered 18 proteins in the fungal remnants despite the fact that silver-stained PAGE gels showed a complex banding pattern (Figure S1), and that there were many fungal proteins sequenced in the endosperm and embryo tissues, it
is possible that increased sampling will reveal an increased number of proteins in the fungal remnants. Alternatively, as the seed contains chaperone proteins that aid in maintaining protein folding and integrity, these chaperone(s) may have allowed for the preservation of the fungal proteins within the seed, while the proteins in the fungal remnants were degraded.

6.2.2 Maize proteome response to fungal infection

This study used a gel-free direct peptide sequencing protocol to investigate the host and pathogen proteomes present in mature kernels. Previous studies have used 2D electrophoresis to separate protein spots, with individual spots being excised from the gel and sequenced with mass spectrometry. These protocols are time- and labour-consuming, though they allow for quantitation of protein levels and the sequencing of a larger portion of the proteome when compared to the gel-free methods used in this study. Nevertheless, the gel-free methods were used here as they are rapid to perform and provide sufficient proteomic sequence data for analysis.

The study of infected and control maize endosperm and embryo tissues independently has not previously been performed. The separation of the tissues allowed for the investigation at a scale smaller than the whole seed. As expected, the endosperm tissue had a small increase in the number of defense-related proteins present in the infected samples compared to the controls, while the embryo had a much larger increase in the number of proteins when the two conditions were compared (Figure 3.1). It would be interesting to compare rates of appearance of host defense proteins in the two respective tissues, diploid
embryo and triploid endosperm now that we know more about how apoptosis is governed in developing cereal seeds (Chaban et al., 2011; Wang et al., 2012).

6.3 General Discussion of Chapter 4

A previous study investigated the proteomes of wheat starch purified by several different commercial companies to investigate the suitability of these starches for celiac patients (Kasarda et al., 2008). In that study, non-seed storage proteins such as α-amylase inhibitors as well as enolases were identified. We were therefore interested in determining whether commercially prepared rice and maize starches also contained these non-seed storage proteins. Commercial preparation of a bulk commodity such as starch implies that removal of protein is not absolutely complete. This work demonstrated that the contaminating 1% protein content of commercially prepared rice and maize starches includes several biochemical classes of proteins, including many proteins involved with starch metabolism (Figure 4.1A,B).

Additionally, in an unpublished analysis of commercially purified wheat starch with results shown in the Supplemental Materials of this thesis, I discovered that Glo-3 proteins were present in these starch samples (Table S1). The wheat starch was prepared and analyzed using the same protocols as the rice and maize starches in Chapter 3 of this thesis. Although these particular commercially-prepared starches are not intended for human consumption, the presence of Glo-3 could invalidate certain studies if these starches are considered “pure starch controls” in animal feeding trials to study type 1 diabetes or celiac disease. Furthermore, starches that are intended for animal feed, such as
Peking duck force-feedings, are known to be watched by federal surveillance authorities for presence of *Fusarium* toxins, both small molecules like vomitoxin as well as proteins (Danicke et al., 2004).

### 6.4 General Discussion of Chapter 5

Using mass spectrometry, I show that there are non-host proteins present in commercially-prepared rice and maize starch samples. When starches from healthy or infected wheat or maize seeds are prepared in the lab I discovered that there are certain non-host proteins present in the wheat starch (Wall et al., 2010), but not in maize starch (This thesis, chapter 3). This is potentially due to the manner in which the starches are prepared. The wheat was stored in paper bags after it had been hulled, a procedure that removes the protective palea and lemma from the seed, allowing for organisms to colonize the seed surface. During the starch extraction, wheat seeds were sterilized in hypochlorite and rinsed with distilled water prior to grinding and starch extraction (Wall et al., 2010). While adequate to remove microorganisms and large debris, it is unlikely that this protocol would strip away all foreign proteins present on the surface of the seeds. Conversely, the protocol for extracting maize starch in the laboratory involves a long steeping step in lactic acid, followed by the careful dissection of the endosperm from the pericarp and embryo tissues. The removal of the pericarp, and therefore any non-host surface proteins attached to this tissue, should reduce the number of non-host proteins in the starch samples. When comparing the commercially prepared starches to these control starches prepared in the laboratory, the presence of proteins from *Homarus americanus*, *Chlorella pyrenoidosa*, and *Ovis aries* can therefore be attributed to the commercial
starch preparation protocols. While these proteins are present in low concentrations in the samples, their presence, like Glo-3 in wheat starch, must be taken into account when interpreting studies using these starches as controls. Low levels of wheat proteins have been detected in the urine of schizophrenia patients so it is important to develop more such refined techniques to monitor the presence, biotransformation and migration of food-derived peptides (Samaroo et al., 2010).

6.5 Translational medicine

With the current high-throughput technologies available today, the phrase “you are what you eat” is more relevant than ever. Cereal proteins and their degradation products in the diet are being shown to be involved with an ever increasing number of biochemical pathways. For instance, studies on celiac disease are able to show, with great precision, that in predisposed individuals, the partial proteolytic degradation of gliadins by gastrointestinal enzymes can produce the so-called toxic peptides and the immunodominant peptides that damage epithelial cells, or activate mucosal T-cell-mediated adaptive immunity, respectively (Lindfors et al., 2012). Additionally, “nutropioids”, the food-derived opioid oligopeptides, have been studied for many years e.g. (Samaroo et al., 2010; Zioudrou et al., 1979). These oligopeptides with µ-opioid activity are recognized by the µ-opioid receptors (MOR) that are expressed exclusively in the brain and the small intestine, where they are involved with controlling food intake levels (Pfluger et al., 2012) and gut motility and bowel movements (Moughan et al., 2007), respectively. Recently, satiety effects of protein rich diets have been associated with the antagonism of MORs (Duraffourd et al., 2012).
Given that dietary cereal proteins can have effects that range from the development of diseases and allergies to cravings and satiety, the use of mass spectrometry to study these proteins is imperative. In this thesis, I used this tool to gain an understanding of the post-translational endoproteolytic cleavage of Glo-3 and the proteomes of cereals grown under normal or pathogen-infected conditions. The data generated in this thesis not only allowed me to test my hypotheses, but will ultimately allow for a greater understanding of the proteins that are ingested on a daily basis worldwide.


Koehler, B. (1942). Natural mode of entrance of fungi into corn ears and some symptoms that indicate infection. Journal of Agricultural Research 64, 0421-0442.


granule surface as judged by tryptic shaving and mass spectrometry. J. Cereal Sci. 52, 115-120.


Appendix A: Supplemental Tables and Figures
Figure S1: Silver stained SDS-PAGE of protein extracts.
Lane 1: Bio-Rad molecular weight standards. Lane 2: proteins extracted from desiccated *Fusarium* on the surface of CL-30 maize kernels. Lane 3: water washed endosperm proteins from *Fusarium*-infected maize. Lane 4: water washed embryo proteins from *Fusarium*-infected maize. Lane 5: crude endosperm proteins from *Fusarium*-infected maize. Lane 6: crude embryo proteins from *Fusarium*-infected maize. Lane 7: crude endosperm proteins from control maize. Lane 8: water washed endosperm proteins from control maize. Lane 9: crude embryo proteins from control maize. Lane 10: water washed embryo proteins from maize.
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Appendix B: *Curriculum Vitae*

**Adam Koziol**

*Academic History*


*Theses and Supervisors*


*Areas of Academic Interest*
Food safety, mass spectrometry, bioinformatics, molecular biology, molecular evolution, antimicrobial peptides, recombinant protein expression.

*a. publications in refereed journals*


**Koziol AG**, Marquez BK, Huebsch MP, Smith JC and Altosaar I. (2012). Commercially produced rice and maize starches contain non-host proteins, as shown by mass


Koziol AG and Durnford DG. (2008). Euglena light-harvesting complexes are encoded by multifarious polyprotein mRNAs that evolve in concert. Molecular Biology and Evolution. 25: 92–100. (MSc work)


b. Articles submitted to refereed journals

c. Non-refereed contributions


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| Figures | Box 1 Figure 1: Diagrammatic ovules |
| Author of this NPG article | no |
| Your reference number | |
| Title of your thesis / dissertation | Application of direct-sequencing peptide proteomics to the characterization of antagonistic (endogenous and exogenous) proteins in cereal grains |
| Expected completion date | Jan 2013 |
| Estimated size (number of pages) | 171 |
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