# Application of a "Glucose Release Index" to assess physical and chemical characteristics of cereal grains that may influence starch digestion and subsequent energy supply to monogastrics

Submitted by

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## Summary

In the pig and poultry production industries, energy forms the largest and the greatest cost pressure when a diet is formulated. In Australia, cereal grains such as barley, sorghum, and wheat are the main dietary energy sources, comprising more than 60% of the diet in many cases. Traditionally, during diet formulation, the energy value of a grain has been represented by a single figure for that particular grain type. However, several studies have indicated that the energy availability from different grains fed to pigs and poultry varies significantly even within one grain cultivar. Given these findings, the use of a single value to represent the energy of each grain type during diet formulation, can lead to inefficient utilisation of dietary resources by animals, and thus decreased animal performance and consequently, a decrease in profit for the pig and poultry production industries.

Thus, there is an opportunity to develop a rapid and reproducible *in vitro* assay to accurately assess the available energy values and nutritional quality of each grain type. In order to develop such an assay, further understanding of factors that affect the available energy values of grains need to be explored.

Starch, which is hydrolysed into glucose by animals, is the most abundant energy component in cereal grains, and there is evidence suggesting that variations in digestible or metabolisable energy values may be related to the extent of starch digestibility. For example in poultry, variations in the *in vitro* digestibility of starch between several wheat cultivars have been shown to correlate with their *in vivo* available metabolisable energy values. However, it is not known to what extent starch digestibility varies between cultivars of other grain types such as barley and sorghum.

There is an increasing body of evidence suggesting that differences in the physical and chemical properties of cereal grains may play an important role in influencing starch digestibility and, consequently, animal performance. Thus, the general hypothesis of this study was that starch digestibility varies between barley, sorghum and wheat, and between cultivars within grain types and this is related to specific chemical and physical characteristics of the grains. To examine this, the following issues were investigated using 18 barley, 15 sorghum and 10 wheat cultivars: 1) an *in vitro* glucose release index (GRI) assay was developed to assess starch digestibility within and between the cereal grain types and, 2) the GRI was correlated to both starch-related (e.g., starch content, starch granule size, the amylose to amylopectin ratio, starch gelatinisation properties) and non-starch-related (e.g., non-starch polysaccharide composition, kernel hardness, the presence of

protein matrix and milling quality) physical/chemical characteristics within and between the cereal grains.

Results revealed significant variations in the GRI both between grains and within a given grain type. The GRI values ranged between 27 - 45%, 25 – 54% and 32 – 53% in barley, sorghum and wheat respectively. Correlation analysis revealed that the GRI in barley, sorghum and wheat was influenced by the physical and chemical characteristics of starch- and non-starch-related grain properties, although the type of characteristic influencing GRI was specific to the grain type. In barley, the ratio of amylose to amylopectin, starch gelatinisation and kernel hardness influenced the GRI. In sorghum, the GRI was influenced by the ratio of amylose to amylopectin, the presence of a protein matrix surrounding starch granules and kernel hardness. Finally in wheat, the presence of protein matrix and milling quality influenced the GRI. It was also shown that the extract viscosity of grains within barley and wheat, but not sorghum, varied significantly.

In conclusion, this study 1) indicated that the GRI may be influenced by some physical and chemical characteristics of cereal grains, and that these characteristics are specific to the type of grain, and 2) identified that future work should establish the relationship between GRI *in vivo* starch digestion and absorption of cereal grains.

The physical and chemical characteristics that may influence starch digestion are discussed in relation to their potential physiological effects on energy digestion, and utilisation in animals. The information generated will provide a basis for future studies that will ultimately assist in the design of *in vitro* assays to predict energy availability from barley, sorghum and wheat grains fed to pigs and poultry, and contribute to the more efficient use of grains in monogastric production systems.

## Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any University or other Tertiary Institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent for this copy of my thesis, when deposited in the University Library, to being available for loan and photocopying.

Mohammad-Reza ZARRINKALAM

Date: 27, 11, 02

# Dedication

This thesis is dedicated to my parents, Mr Hossien Zarrinkalam and Mrs Zahra Zarrindokht Pourjalali for believing in my dreams and giving me encouragement to pursue them.

And to my wife Krystyna, for her love and believing in me.

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# Abbreviations

| | |
|---|---|
| AME | apparent metabolisable energy |
| DE | digestible energy |
| GRI | glucose release index |
| LSD | least significant differences |
| ME | metabolisable energy |
| MJ | megajoule |
| MPa.s | millipascal seconds |
| NSP | non-starch polysaccharides |
| RVA | rapid visco analyser |
| RVU | rapid visco analyser units |
| SGs | Starch granules |

# Chapter 1 General introduction

## 1.1 General Introduction

Dietary energy is the largest single economic input in pig and poultry production, and the greatest cost pressure when a diet is formulated (Meyer Strategy Group, 1995; Wiseman, 1997). In Australia, cereal grains such as barley, sorghum and wheat are the main dietary energy sources for conventional intensive pig and poultry industries, comprising more than 60% of the diet (Meyer Strategy Group, 1995). Traditionally, during diet formulation, a single figure has been applied to represent the energy value for each grain type. However, several studies have indicated that the available energy values of cereal grains vary significantly within each grain type (Hughes and Choct, 1999; van Barneveld, 1999a) (Table 1.1). These variations in available energy are thought to arise from differences in the physical and chemical characteristics of grains, and have a significant impact on animal performance and the cost of production, thereby affecting the overall profit of the pig and poultry industries (Black, 1997). Assessing the nutritional quality (energy availability) of cereal grain prior to diet manufacturing will help maximise profitability for the pig and poultry industries (Black, 1997; Wiseman, 1997).

Starch represents 50 to 80% of total grain weight (Stone, 1996), and thus is the most abundant energy component present in cereal grains. However, marked differences in starch digestion and absorption in pigs and poultry occur between different feedstuffs, between different cultivars or within batches of the same feedstuff (Johansen and Bach Knudsen, 1994; Meulen *et al.*, 1997; Martin *et al.*, 1998; Noah *et al.*, 1999; Weurding *et al.*, 2001b). The physiological and nutritional importance of such variations in monogastric animals is profound (Rerat *et al.*, 1984a; Higgins *et al.*, 1996; Lerer-Metzger *et al.*, 1996; Weurding *et al.*, 2001a). In poultry, for example, there is a direct relationship between starch digestibility and the metabolisable energy value of wheats (Wiseman *et al.*, 2000). Differences in starch digestibility may result from alterations in the physicochemical characteristics of starch itself such as its chemical composition, molecular structure and starch granule size (Blakeney, 1993). In addition, it may also be influenced by the physicochemical characteristics of the non-starch components present in cereal grains such as non-starch polysaccharides (NSP), the protein matrix surrounding starch granules, and the hardness of grain cell walls (Blakeney, 1993; Wiseman *et al.*, 2000). Although the mechanisms by which these components exert their effects need to be further defined, it is believed that they act to alter digestive enzyme activity and/or the accessibility of the digestive enzymes to starch (Blakeney, 1993; Wiseman *et al.*, 2000).

**Table 1.1 The range of available energy values observed for common Australian feed grains when feed to pigs and poultry (Hughes and Choct, 1999; van Barneveld, 1999a)**

| Type of grain | DE[1] (MJ/kg DM[3]) | AME[2] (MJ/kg DM[3]) |
|---|---|---|
| **Barley** | 11.7 – 16 | 10.4 - 12.2 |
| **Sorghum** | 15.8 – 17.4 | 13.5 - 17.7 |
| **Wheat** | 13.3 – 17 | 10.4 - 15.9 |

[1] Faecal digestible energy based on dry matter (kg/MJ) in pigs.
[2] Apparent metabolisable energy based on dry matter (kg/MJ) in poultry.
[3] Dry matter

In Australia, a number of R&D and commercial organisations (Grain R&D Corporation, Australian Pork Limited, Rural Industries R&D Corporation, Meat and Livestock Australia and Ridley AgriProducts) set up a joint research program known as the "Premium Grains for Livestock Program" (PGLP). The aims of the Program are to understand the causes of variation in the nutritive value of grains fed to livestock, and to develop strategies to maximise the economic returns to the producers to grains and livestock. This thesis is part of the PGLP that has focussed on developing rapid assays that can be used to assess the relative importance of physical and chemical characteristics of cereal grains that may ultimately influence starch digestion. Some physical and chemical data generated by various researchers involved in this program on barley, sorghum and wheat has been used throughout the thesis with their permission. The assays used to generate this data and their place of conduct has been listed in appendix 1.1.

The main objectives of the thesis were to:

1. Develop a rapid and repeatable assay for quantifying variation in *in vitro* starch digestion within cultivars of barley, sorghum and wheat.
2. Investigate the variability of selected physical and chemical characteristics of cultivars of barley, sorghum and wheat by adapting several *in vitro* analytical methods; and
3. Use the *in vitro* assay (from objective one) to determine the extent to which these physical and chemical characteristics of barley, sorghum and wheat grains influence starch digestion.

# Chapter 2 Literature review

This literature review will examine the importance of cereal grains to the intensive animal industries. This will be followed by examining the principles of starch digestion and the effects of variation in starch digestibility on animal physiology. The factors that influence starch digestibility and utilisation of cereal grains by monogastric animals will also be addressed.

## 2.1 Cereal grains in the pig and poultry industries

Animal feeds represent the second largest consumer of cereal grains worldwide, and their proportional share is increasing (Meyer Strategy Group, 1995). Cereal grains compose the main source of energy in animal feeds and can make up more than 80% of the diet in conventional intensive pig and poultry production (Meyer Strategy Group, 1995). In Australia, barley, sorghum and wheat are the most common sources of energy in pig and poultry diets (Meyer Strategy Group, 1995). Consequently these three grain types were selected for further investigation in this thesis.

During diet formulation, it is not uncommon to incorporate several different cultivars of the same grain type into animal feeds, and a single value is used to represent the energy content of that grain type. However, several studies indicate that there is a wide range of variation in the available energy of grains belonging to the same grain type when fed to pigs and poultry (Hughes and Choct, 1999; van Barneveld, 1999a) (Chapter 1, Table 1.1). Such variation in the available energy values of grains could lead to inefficient utilisation of dietary energy by pigs and poultry, leading to reduced animal performance and consequently a decrease in industry profitability (van Barneveld and Hughes, 1994; Black, 1997).

Thus, accounting for the variation in the energy availability of grains is likely to be the single most effective way to maximise the profitability of the pig and poultry industries (Edwards, 1997). Since the energy content of cereal grains is considered as one of the most important contributors to the overall nutritional quality, it is imperative that the factors causing variation in grain available energy be identified and characterised.

## 2.2 Factors which may influence the available energy of grains fed to pigs and poultry

Cereal grains such as barley, sorghum and wheat are composed of carbohydrate (55-85%), protein (8-22%), fat (1-4%), minerals (1.7 to 4.2%) and water (10% to 20%) (McDonald *et al.*, 1992b). For pigs and poultry, the energy in cereals is derived from the carbohydrate (mainly starch), protein and fat components (Boisen and Verstegen, 2000). In general, the processes that define digestion and absorption of energy sources in animals are complex, highly integrated and adaptable (Savoie, 1994; Moughan, 1999). The digestion and absorption of energy from cereal grains is therefore likely to be influenced by numerous factors that can be broadly classified into animal-related and grain-related factors (Figure 2.1). Both elements are important to consider, however it is beyond the scope of this thesis to cover all aspects that influence energy availability. This review will discuss characteristics of starch before addressing the main animal-related factors that influence starch digestion. It will then focus in more detail on the grain-related factors that may contribute to variations in the energy availability of grains fed to pigs and poultry.

## 2.3 Starch: the main source of energy in cereal grains

Starch is the major carbohydrate in cereal grains, representing 50 to 80% of total cereal kernel weight (Stone, 1996), and is thus one of the most abundant energy components of feed grains for pigs and poultry (MacGregor and Fincher, 1993). Starch is composed of α-linked polymers of glucose of which there are two types, amylose (predominantly α1-4 linked) and amylopectin (containing both α1-4 and α1-6 linkages) (MacGregor and Fincher, 1993). In cereal grains, starch is formed in the endosperm cells, within membrane-bound organelles known as plastids (Stone, 1996). At the microscopic level, starch in the endosperm appears in the form of discrete granule bodies referred to as starch granules (Stone, 1996).

### *2.3.1 Starch digestion in monogastric animals*

Several endogenous enzymes (such as α-amylase) and other proteins (such as the glucose transporters) are required for the digestion and utilisation of starch by monogastric animals (Drochner, 1991; Thorens, 1993; Goda and Isemura, 2000).

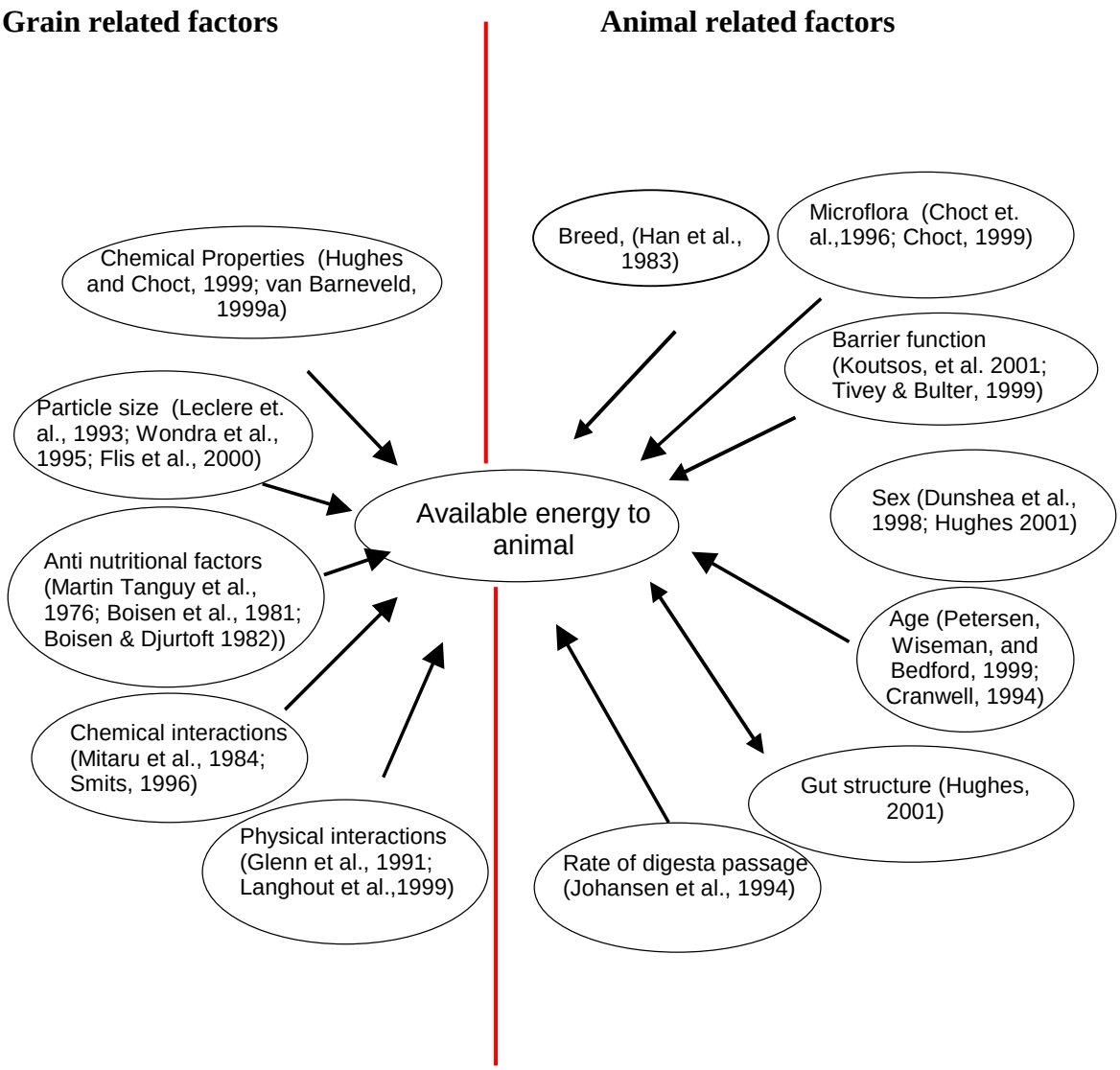



**Figure 2.1 Factors that may influence the available energy values of feed-grains to animals.**

In pigs and poultry (as well as in other monogastric animals), starch can also be digested and utilised by microbial populations present in the digestive tract (Drochner, 1991; Longland, 1991). The final energy available to the animal is therefore influenced by the equilibrium of these two routes of breakdown.

#### *2.3.1.1 Starch digestion by host digestive enzymes*

Starch digestion in pigs (but not poultry) begins in the mouth with α-amylase. This enzyme is secreted by the submaxillary and sublingual salivary glands and acts to convert starch into maltose, maltotriose and dextrins (Walker and Whelan, 1960). The activity of α-amylase is maximum at near neutral pH and requires the presence of calcium (Longland, 1991). Its activity is therefore limited in the stomach due to its low pH. Starch enzymatic hydrolysis is continued in the small intestine, the main site of starch digestion in pigs and poultry (Longland, 1991) by the α-amylase secreted from the pancreas into the duodenal region. The maltose, maltotriose and dextrin products of starch hydrolysis by α-amylase, cannot be directly utilised by pigs or poultry and require further hydrolysis by the complementary action of three integral brush border enzymes at the surface of the small intestine: glucoamylase (maltase-glucoamylase, amyloglucosidase), sucrase (maltase-sucrase) and alpha-dextrinase (isomaltase) (Gray, 1992). These enzymes hydrolyze the maltose, maltotriose and dextrins into their monomeric units (glucose). The activities of these enzymes are maximum at pH 6.0 to 6.5 (Longland, 1991), and in a review by (Cranwell, 1995) it has been summarised that the activities of these enzymes are maximum in the proximal half of the small intestine (jejunum area).

Glucose, the end-product of starch digestion, is then transported into the epithelial cells lining the small intestine by the sodium-dependent symporter SGLT1 (Thorens, 1993), which is located in the apical membrane of enterocytes (intestinal lining cells) (Thorens, 1993). It is then released close to blood capillaries by the activity of the sodium–independent glucose transporter GLUT2, which is located in the baso-lateral membrane of enterocytes, and is then absorbed into the blood vessel system (Thorens, 1993). Similar to brush border enzyme activities, the jejunum region of the small intestine has the highest activity of glucose transport (Pawlak *et al.*, 1971; Puchal and Buddington, 1992). Therefore, the site of starch digestion, and the absorption of its monomeric glucose units, plays an important role in determining the efficiency of starch utilization by pigs and poultry.

#### 2.3.1.2 *Starch digestion by gut microflora enzymes*

Microflora populations present in the stomach and hindgut including the ileal region of the small intestine of pigs and poultry, compete with the host for nutrients such as starch (Bach Knudsen *et al.*, 1991; Ratcliffe, 1991). While a limited breakdown of starch by microbial activity in the stomach of pigs has been reported (Drochner, 1991), the microbial population in the ileal region of poultry and pigs can reach high numbers ($10^9$ per $g^{-1}$ digesta) (Jorgensen and Just, 1988). The most common bacteria in intestinal system are lactic acid bacteria, enterobacteria and streptococci (Conway, 1996) that are competing for energy sources with the host and each other. Therefore, it is speculated that the enzymatic digestion of nutrients such as starch or its products (eg. maltose, maltotriose, dextrins) in the ileal region are more likely to be digested and utilised by microflora because of the inherent actions of these organisms which include: 1) degrading digestive enzymes and bile salts; 2) their attachment to the absorptive surface area and, in some cases, actively damaging the intestinal surface, and; 3) extracting nutrients in competition with the host digestive system (Bedford, 2000b). Microbial digestion of grain components such as starch is known to be 30 - 50% less energy efficient than the enzymatic digestion by host enzymes present in the animal (Just, 1983; van Es, 1987). However, the proportion of starch digested by endogenous (animal) enzymes versus microbial digestion, and the influence this has on energy utilisation by host animals, is yet to be determined (Graham, 1991). The main products of fermentation of bacteria in the small intestine are short chain fatty acids (i.e. lactic acid) that are less effective as an energy source for the host than glucose (Martin *et al.*, 1998).

## 2.4 Variation in starch digestibility

Not all starch can be digested by host digestive enzymes in monogastric animals (Englyst, 1989), and the extent and rate of its digestion can vary significantly depending on its source (Nicol *et al.*, 1993; De Schrijver *et al.*, 1999). For example, ileal starch digestibility in poultry can vary from 33% (potato starch) to 93.9% (wheat starch) (Weurding *et al.*, 2001b). In pigs, the digestibility of starch has been reported to range from 83.7 to 100% as summarised from 38 individual experiments assessing the digestibility of starch by the amount of non-digested product recovered at the end of the small intestinal tract (Bach Knudsen and Jorgensen, 2001).

The extent of starch digestibility for the majority of these studies has been determined by the disappearance of ingested starch during its oral to ileal or faecal transit (Riesenfeld *et al.*, 1980; Just *et al.*, 1985; Graham *et al.*, 1986a; Graham *et al.*, 1986b; Pettersson and Lindberg, 1997; Weurding *et al.*, 2001b). However, this mode of measurement does not provide any insight into the form and site of digestion and absorption of starch along the intestinal system, which can have several significant effects on the physiology of the animal (Low, 1980). It has been suggested that determining the kinetic aspects of the glucose and short chain fatty acid concentrations in animal blood that perfuses the gastrointestinal tracts may provide more accurate information regarding starch digestibility and absorption from diets (Rerat *et al.*, 1984b). The following section will briefly outline some physiological effects of variability in starch digestion.

### *2.4.1 The physiological consequences of variation in starch digestibility*

The bio-available energy value of a diet can be influenced by the degree of starch digestibility, which can depend on the site and form of its digestion and utilisation in animals (Wenk, 2001). In pigs, the degree of starch digestibility can influence the ratio of glucose to organic acid (e.g. lactic acid, short chain fatty acid) absorption from the small intestine (Figure 2.2) (Rerat *et al.*, 1984b; Bach Knudsen *et al.*, 2000). Increased hind gut fermentation can also decrease the available energy values of starch in grains. It has been shown that the relative energy values resulting from microbial fermentation of starch are 0.7 of the energy obtained through enzymatic digestion in the small intestine (Jorgensen *et al.*, 1996).

In poultry, it has also been demonstrated that starch digestibility correlates to apparent metabolisable energy values for cereal grains (Mollah *et al.*, 1983; Rogel *et al.*, 1987; Wiseman *et al.*, 2000) and legumes (Carre *et al.*, 1998).

Other physiological effects that can be caused by variations in the digestion and absorption of starch have been listed as follows:

1) an increase in starch digestibility can increase plasma lipid concentrations in rats (Lerer-Metzger *et al.*, 1996) and humans (Bach Knudsen and Jorgensen, 2001) that can result in increased body fat deposition.
2) the long-term consumption of a diet with a high absorbable glucose content can result in the development of insulin resistance, leading to diabetes (Higgins *et al.*,

1996). Although the above outcomes have been proven only in rats, similar results could be expected for other monogastric animals and humans.

3) It has been speculated that the starch digestion rate could also influence the efficiency of amino acid absorption and deposition (Rerat *et al.*, 1984a; Weurding *et al.*, 2001a) since the supply of energy-giving nutrients at the sites of protein synthesis need be synchronised with the supply of amino acids in order to obtain the maximum positive influence on nitrogen balance (Elman, 1953).

### *2.4.2 Classification of starch digestibility based on its physiological properties*

Glucose is the final product of starch hydrolysis by host digestive enzymes, and is absorbed and transported into the blood stream via the portal vein (Miller and Debarthe, 1974). In monogastric animals, after consuming a carbohydrate-based diet, the concentration of glucose in the portal vein increases over a time period and is referred to as the glycaemic response (Bannister *et al.*, 1975; Jenkins *et al.*, 1981; Leclerc *et al.*, 1993; Englyst *et al.*, 1996; Noah *et al.*, 1999). It has been shown that the rate of starch digestion in food and the post prandial glycaemic response have a significantly positive relationship (Jenkins *et al.*, 1982) indicating that the glycaemic response is a good indicator of starch digestibility.

In order to categorise and compare starch from different dietary sources based on its digestibility properties, the glycaemic index has been introduced. This is measured by the glycaemic response (glucose concentration in blood) during a series of time points after consuming foods containing equal amounts of carbohydrates. The glycaemic response curve for a two hour period following food ingestion is determined, and the glycaemic index is calculated from the area under the glycaemic response curve (Jenkins *et al.*, 1981). The glycaemic index of foodstuffs shows great variation both within and between food types, suggesting that significant variation in starch digestibility within and between dietary sources occurs (Jenkins *et al.*, 1981).

Currently in the human food industries, due to the physiological importance of starch digestibility, carbohydrate-based foods are classified according to the digestibility of carbohydrates, by predicting their glycaemic index using *in vivo* assays. Such a classification has been used in order to regulate diets for diabetics (Jenkins *et al.*, 1981; Wolever *et al.*, 1994), and for sports and appetite research (Brand-Miller, 1999).

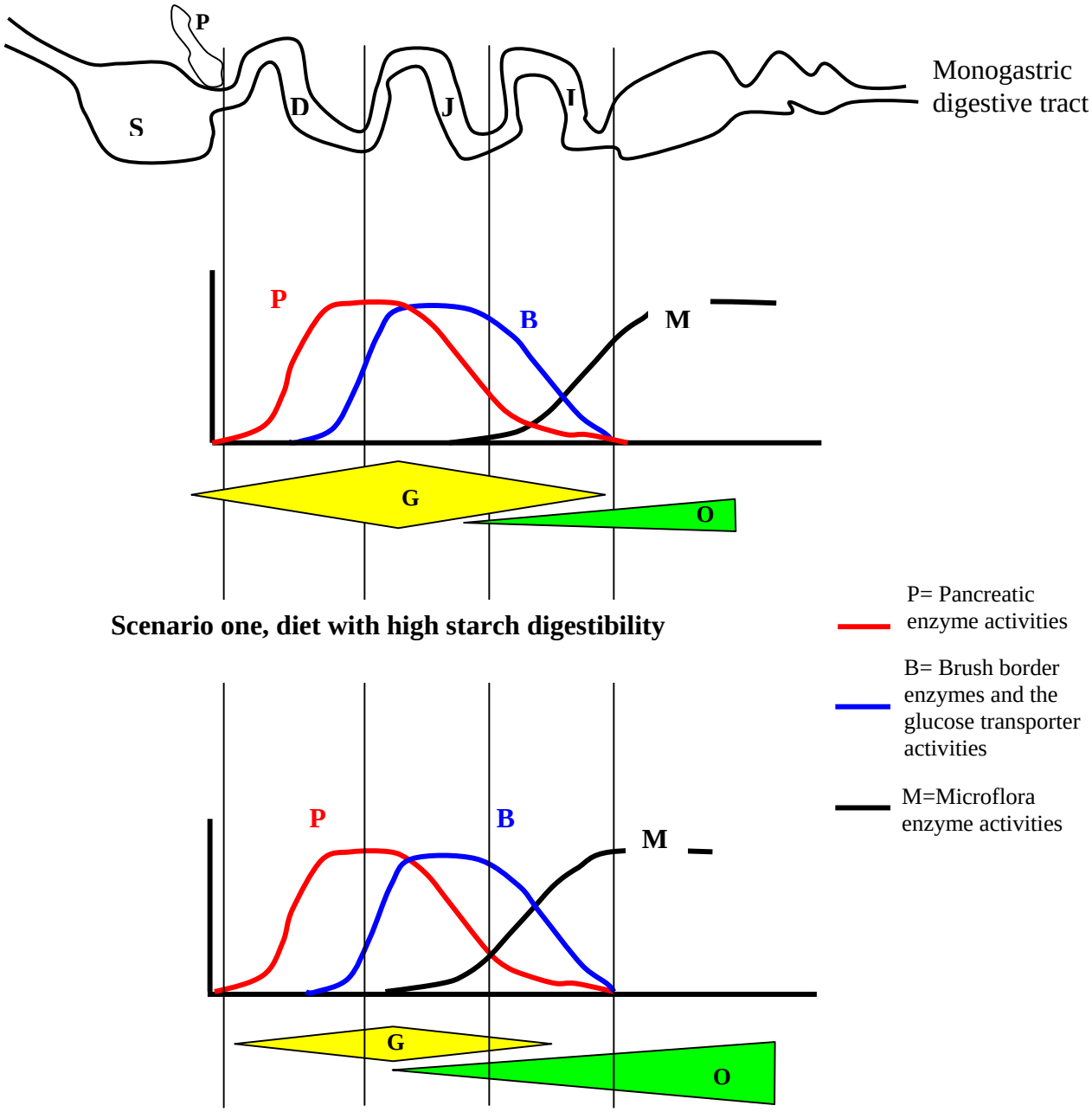



**Figure 2.2 The hypothetical effects of variable starch digestibility on the ratio of animal enzyme to microbial enzyme digestion activities, and the consequence on the ratio of glucose and organic acid production in the small intestine of monogastric animals. S=Stomach, D=Duodenum, J= Jejunum, I=Ileum, G=Glucose absorption by portal vein, O=Organic acid absorption by portal vein, P= Pancreas.**

The determination of the glycaemic index, much like any other *in vivo* assay, has several practical drawbacks, such as being expensive, time consuming, laborious and difficult to conduct due to increasing ethical issues. Consequently several *in vitro* methods have been developed in order to classify the digestibility of starch from different diets (Jenkins *et al.*, 1982; Holm *et al.*, 1988; Englyst *et al.*, 1992). Among these *in vitro* assays, the assay developed by Englyst *et al.* (1992) provides rapid and reproducible values and more importantly shows a strong correlation with the *in vivo* glycaemic index values in humans (Englyst *et al.*, 1996). Englyst *et al.* (1992) introduced the terms "rapidly available glucose" and "starch digestion index" for particular diets, which represent the amount of glucose released following enzyme hydrolysis under a defined set of *in vitro* conditions during a specific time period (commonly 20 minuntes), and the percentage of digested starch to total starch content, respectively.

Based on these *in vitro* assays, starch from different dietary sources including cereal grains has been broadly classified into three major groups according to the extent and rate of its digestion: rapidly digestible starch, slowly digestible starch, and resistant starch (Englyst *et al.*, 1992). Based on this classification, starch from cereal grains belongs to the slowly digestible starch category (Englyst *et al.*, 1992). However, in the pig and poultry feed industries, despite the increasing demand to evaluate the energy availability of feed more accurately, such a classification has not been adopted.

## 2.5 Factors that may influence starch digestibility of grains in pigs and poultry

In pigs and poultry, the digestion and absorption of starch are complex and influenced by several animal and grain related factors.

### *2.5.1 Animal related factors*

In order to hydrolyse starch to glucose *in vivo*, secretion of sufficient enzymes of the appropriate type in the presence of a suitable mineral solution and pH conditions are required. Furthermore the ability of the mucosal cells to remove the glucose from the small intestinal lumen also plays an important role in glucose uptake and utilisation. It has been indicated that 1) animal age, 2) the microbial populations and their activity in the small intestine and 3) the genotype of animals can influence starch digestion and utilisation.

1) Age: in pigs, the efficiency of starch digestion after birth is limited due to low production of α-amylase but its production rapidly increases by the first few days of life (Becker *et al.*, 1954). In contrast, in poultry, it seems that the secretion α-amylase after hatching is more than sufficient for the hydrolysis of starch (Jin *et al.*, 1998). Furthermore, the activity and distribution pattern of the brush border enzymes (maltase, glucoamylase and amyloglucosidase) along the intestinal system can vary depending on the age of pigs (Gray, 1992; Cranwell, 1995). Consequently it can be concluded that the site and form of starch digestion and absorption can vary with the age of pigs.

2) Microbial population and activity: as highlighted in section 2.3.1.2, if the hydrolysis of starch is not complete by the end of the jejunum region, the chance of microbial hydrolysis of starch increases and this would be less efficient than hydrolysis by animal enzymes.

3) Genotype: it has been suggested that glucose absorption from the intestinal system may become rate-limiting for full phenotypic expression of favorable production traits due to the intensive genetic selection for these traits in poultry (Croom *et al.*, 1998). Thus, the manipulation of either the rate, total capacity or efficiency of intestinal glucose absorption may be necessary to sustain maximum growth and performance of animals (Croom *et al.*, 1998).

### *2.5.2 Grain related factors that could influence starch digestibility*

A number of studies have identified several physical and chemical properties of feeds that could influence the rate and extent of their starch digestion by animals (Englyst *et al.*, 1992; Leclere *et al.*, 1993; Johansen *et al.*, 1996; Noah *et al.*, 1999). In cereal grains, Wiseman *et al.* (2000) noted several physical and chemical properties of wheat that could influence its starch digestibility and they classified these factors into two groups, starch related- and non-starch related factors. The starch-related factors include chemical composition of starch, crystallisation/gelatinisation of starch, starch granule size and distribution. The non starch-related factors are cell walls, protein matrix and lipid content. The physical and chemical properties of grains can vary depending upon the agronomic conditions under which the plant is grown, such as irrigation, availability of nutrients from soil and temperature (Evers *et al.*, 1999). Therefore, it could be speculated that such variation in the physical and chemical properties of grains could contribute to the variation in starch digestibility of grains by animals.

In order to better understand the mechanism(s) of grain related factors that may influence starch digestibility of grains and consequently their available energy values for pigs and poultry, the relationship between the physical and chemical properties of grains with their starch digestibility (extent and rate) requires further investigation. In order to achieve this, an understanding of the anatomy of cereal grain kernels is needed.

## 2.6 Cereal grain anatomy

In general the structure of cereal grains can be divided into three major sections, endosperm, hull and germ (Figure 2.3). The endosperm in a well-developed kernel can contribute up to 85% of its weight and is divided into two sections, the starchy endosperm and the aleurone layer. In the mature grain, the starchy endosperm consists of dead cells packed with starch granules and some protein (Egli, 1998). In contrast, the aleurone layer consists of 1 to 3 layers of cells (one in wheat and sorghum and 3 in barley), which are rich in protein. The hull layer can make up to 15% of kernel weight and consists of large empty cells mainly composed of non starch polysaccharides (NSP). These form two layers (seed coat and pericarp) in barley and wheat and only a single layer of cells (pericarp) in sorghum (Evers *et al.*, 1999). The germ consists of primordial roots and shoots with leaf initials that can make up to 2% of total kernel weight. The germ is mainly composed of protein and lipid (Evers *et al.*, 1999).

## 2.7 Characteristics of starch that could influence its digestion

### *2.7.1 Starch granule size*

Starch granules in cereal grains can differ in size and shape depending on their botanical origin (MacGregor and Fincher, 1993). In wheat and barley, starch granules can be classified into two major groups based on their diameter: 1) large starch granules (A-type) which are greater than 10 $\mu$m in diameter; and 2) small starch granules (B-type) which are smaller than 10 $\mu$m in diameter and are more deeply embedded within the endosperm protein matrix (May and Buttrose, 1959; Morrison and Scott, 1986; Churchill *et al.*, 1997). Large starch granules contribute most to the weight of starch (85-90%) but are few in number (10%), while the reverse is true for small starch granules (Fulcher *et al.*, 1997). For sorghum grains however, there is a limited information on starch granule size distribution. .

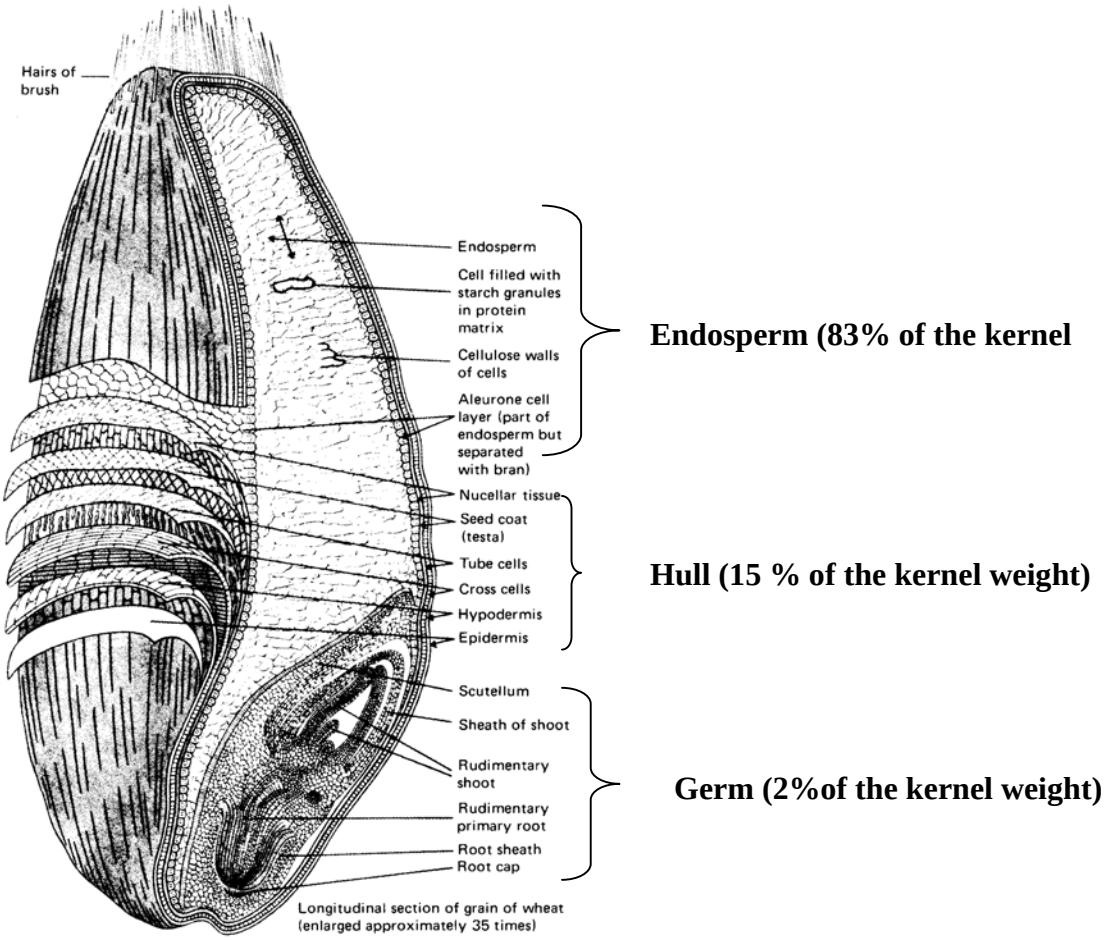



**Figure 2.3 Longitudinal section of a wheat grain. Reproduced from Pomeranz (1987).**

It has also been shown that starch granule size distribution and the ratio of A-type to B-type starch granules varies between cultivars of the same grain type (barley and wheat) (Chojecki *et al.*, 1986; Blumenthal *et al.*, 1994; Psota *et al.*, 2000; Peterson and Fulcher, 2001). The variability of starch granule size and shape within one grain type could result from three factors: starch granule development and growth; physiological/chemical conditions existing during the period of seed growth; and starch granule chemical composition (Evers *et al.*, 1999)Determining starch granule size and distribution has been used for predicting the quality of grains for pasting, malting and milling purposes (Zayas *et al.*, 1993; Dunn *et al.*, 1996; Peterson and Fulcher, 2001). It has been speculated that the surface area to volume ratio influences enzyme accessibility. The higher the ratio of surface area to volume in starch granules (smaller size starch granules) the more accessible the substrate is to enzymes and the greater the starch digestibility (Morrison and Scott, 1986). However, the precise relationship between starch granule size and digestibility in cereal grains needs further investigation

### *2.7.2 Chemical composition of starch granules*

As indicated in section 2.3, starch is mainly composed of amylose and amylopectin. Amylose is a relatively small linear polymer and displays varying degrees of polymerisation ranging between 500 and 6000 glucose units. In contrast, amylopectin is a very large branched molecule with degrees of polymerisation ranging from $3 \times 10^5$ to $3 \times 10^6$ glucose units (Manners, 1985; Zobel, 1988). It has been demonstrated that starch with a higher content of amylose has lower digestibility (Cone and Wolters, 1990; Blakeney, 1993). In solution, amylose molecules form helical structures and can bond with other molecules such as organic acids, alcohols, and more importantly lipids that can depress digestive enzyme accessibility and consequently the digestibility of amylose (Evers *et al.*, 1999). Furthermore, amylose has a relatively low molecular weight and forms into a compact crystal structures that can also inhibit enzyme accessibility and thus digestibility (Siljeström *et al.*, 1989). In contrast, amylopectin has more branch points than amylose and provides a larger and less dense surface area, increasing the exposure of the polysaccharide to digestive enzymes (Rooney and Pflugfelder, 1986; Bedford, 2000a).

The amylose to amylopectin ratio of cereal grains can vary depending on the grain variety (Klassen and Hill, 1971; MacGregor and Fincher, 1993). It has been speculated that the ratio of amylose : amylopectin in grains may influence their available energy

values in monogastric animals (Black, 2000). However, there is conflicting evidence surrounding the effect of the amylose : amylopectin ratio in starch and the available energy values under *in vivo* conditions. For example, in barley grains with a lower amylose content, a higher available energy value was obtained in pigs compared with animals fed barley cultivars with higher amylose contents (Pettersson and Lindberg, 1997). In contrast, in sorghum, variation in the amylose : amylopectin ratio did not influence the available energy and starch digestibility in pigs (van Barneveld *et al.*, 2001). Therefore further investigations into the influence of variations of the amylose : amylopectin ratio on starch digestibility of whole grain (not isolated starch) in comparison with other grain related factors could provide a better understanding of the factors influencing variations in the starch digestibility of grains.

### *2.7.3 Crystalline structure of starch granules and gelatinisation properties*

Starch granules are partially crystalline and amorphous in structure, with a 20-40% degree of crystallinity (Hizukuri, 1996). Wide-angle X-ray scattering has revealed three forms of packing of amylopectin double helices, giving rise to A-, B-, and C-crystal types (Gidley and Bociek, 1985). This classification of crystalline structure of starch does not correspond to the classification of starch granules that was discussed in section 2.7.1. The A-type crystalline structure is more hydrated and because of this is more rapidly digested compared to the B- and C-type crystalline structures of amylopectin (Bedford, 2000a). The linear molecules of amylose are believed to intersperse between amylopectin (Jane *et al.*, 1992) and are mainly located in amorphous layers of the growth rings (Jenkins *et al.*, 1994) (Figure 2.4).

The crystalline structures of starch granules can vary within one type of grain (Nikuni, 1978). Such a variation can influence the gelatinisation properties of starch in grains (Fujita *et al.*, 1998). In human food industries, the gelatinisation properties of starch are commonly used for determining grain quality for baking (Leon *et al.*, 1998), brewing (malting quality) (Holmes, 1995) and cooking (Lai, 2001). During the gelatinisation process, which involves the application of heat in the presence of water, the molecular structure of starch granules is irreversibly destroyed (Jacobs and Delcour, 1998).

It has been indicated that the gelatinisation properties vary between starch from different botanical sources (Jacobs *et al.*, 1995) and within the same botanical source (Wootton *et al.*, 1998), and can result from the variation in the crystal structure of starch

molecules. The alteration in the molecular structure of starch can influence the susceptibility of starch to digestive enzymes under *in vitro* and *in vivo* conditions (Holm *et al.*, 1988; Xiong *et al.*, 1990). Since the crystallisation and gelatinisation properties of starch are interdependent, it could be expected that the gelatinisation properties or molecular structure of starch may affect its digestibility. However, the significance of this relationship in cereal grains needs further investigation.

## 2.8 Non starch-related factors thought to influence starch digestion

### *2.8.1 Cell walls*

Cell walls in cereal grains are extracellular structures overlying the plasma membrane and can act as physical barriers between endogenous digestive enzymes of animals and their substrates (e.g. starch). Cell walls are mainly composed of a rigid cellulose skeleton [β-(1-3) and β-(1-4) linked glucose monomers] which is embedded in a gel-like matrix composed of NSP including arabinoxylans, β-glucans and glycoproteins (Stone, 1996).

The cell walls of the pericarp seed coat contain lignins (phenylpropanoid units associated in a complex cross-linked structure (McDonald *et al.*, 1992a), which are impermeable to water (Stone, 1996) and resistant to enzymatic degradation (Jorgensen *et al.*, 1996). In contrast, the cell walls surrounding the starchy endosperm, embryonic tissues and the scutellum do not contain any lignins and are thus more accessible to depolymerizing enzymes secreted from the aleurone layer (which occurs during germination) (Stone, 1996) or the gut microflora in both monogastric (Bach Knudsen and Canibe, 2000; Bach Knudsen, 2001) and ruminant animals (Engels, 1989).

Monogastric and ruminant animals do not have enzymes to hydrolyse NSP in grains, thus for digestion of these components they must rely on enzymes produced by the microflora present in their intestinal tract. The extent to which grain NSP are digested by gut microflora depends on the chemical composition of the NSP (Jorgensen *et al.*, 1996).

Some of the anti-nutritional properties of NSP and their physiological impact on animal production have been discussed previously (Smits and Annison, 1996; Iji, 1999; de Lange, 2000). The mechanisms of NSP anti-nutritional activity in animals is complex and can result in several direct and indirect consequences on the extent of starch digestibility in grains by animals. In the following sections (2.8.2 - 2.8.5), the anti-nutritional properties of NSP and their mechanisms of action will be reviewed.

### *2.8.2 Particle size*

It has been shown that feed processing, chewing (in pigs) or the grinding action of the crop and gizzard in poultry, can remove or decrease the physical barrier presented by cell walls which are mainly composed of NSP (Black, 2000). In order to break down the physical barriers presented by the cell wall, and in turn increase the accessibility of digestive enzymes to their substrates in grains. In conventional intensive pig and poultry production, cereal grains are processed prior to feeding by hammer-milling, dry-rolling, pelleting, steam-flaking or extrusion. These processes are used to enhance the digestibility and utilization of nutrients in grains by animals (Carter, 1996; Bhatty, 1997; Nielsen and Ingvartsen, 2000).

In pigs, a positive effect of reducing grain particle size by milling on growth and the feed conversion ratio has been observed for cereal grains (Lawrence, 1983; Healy *et al.*, 1994; Wondra *et al.*, 1995b; Wondra *et al.*, 1995c; Wondra *et al.*, 1995d; Albar *et al.*, 2000). In contrast, reducing particle size did not improve the nutritional quality of maize and wheat for laying hens (Ouart *et al.*, 1986; Deaton *et al.*, 1989), and in broilers, smaller particle size of sorghum grains showed a negative effect on nutritional quality (Nir *et al.*, 1990). Such different results in poultry compared to pigs may be explained by the grinding action of the gizzard in poultry, which can effectively grind the grains into fine particles (less than 800μm) (Nir *et al.*, 1994).

It has been demonstrated that the milling quality of grains, which can be defined as the distribution of grain particle sizes following milling, can vary within and between grain types (Berman *et al.*, 1996; Bhatty, 1997; Lempereur *et al.*, 1997; Glitso and Bach Knudsen, 1999). The variation in particle size distribution of ground grains can be expected to influence the available surface area to digestive enzymes and subsequently grain digestibility.

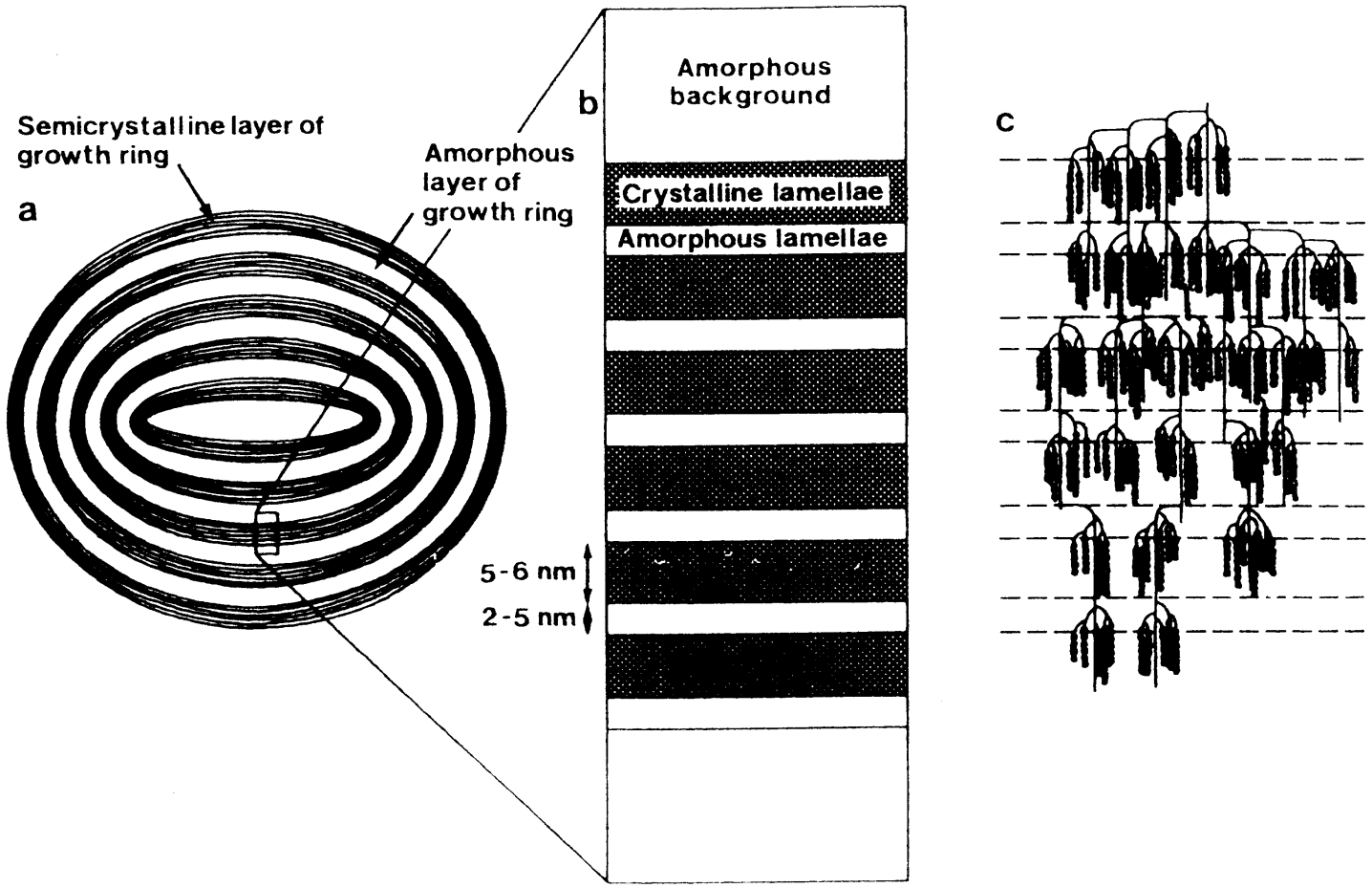



**Figure 2.4 Schematic representation of starch granule structure: (a) a single granule with alternating amorphous and semicrystalline layers, representing growth rings; (b) expanded view of the semicrystalline layer of a growth ring, consisting of alternating crystalline and amorphous lamellae; (c) the cluster of amylopectin within the semicrystalline layer of the growth ring. From Jenkins *et al.*, (1994)**

The integrity of the endosperm cell wall could affect the milling quality of grains (Ellis *et al.*, 1992) and consequently the particle size distribution of milled grains. Grain integrity which is commonly determined by the grain hardness index, has been shown to influence milling quality of grains (Dobraszczyk, 1994; Morris and Rose, 1996). It has also been demonstrated that grain hardness varies within and between grain types (Allison *et al.*, 1979; Glenn and Saunders, 1990). Such variation may influence the accessibility of enzymes to grains which may consequently affect their nutritional quality. For example, Choct (1995) showed an 11% increase in the total area of cell walls (thicker cell walls) in low metabolisable energy (ME) wheat compared to wheat with a higher ME. The influences of grain hardness on particle size distribution, and the extent of starch digestibility in milled grains requires further investigation.

### *2.8.3 Viscosity*

Generally, NSP can be classified into two groups, soluble and insoluble, although the extent of NSP solubility depends on their extraction conditions (Graham *et al.*, 1988). The soluble NSP have a highly viscous nature that can negatively impact digestion and utilisation of feed in poultry (Choct and Annison, 1990; Annison, 1993; Choct, 1993; Hughes *et al.*, 1996; Smits and Annison, 1996; Vetesi *et al.*, 1998). An increase in grain viscosity is considered to be anti-nutritional because as the NSP content of grains increase, the viscosity of digesta also increases (Smits, 1996). The extent of the influence of NSP viscous properties on dietary energy utilisation by pigs has not been firmly established and needs further investigation (Graham, 1991; van Barneveld, 1999a). However, work conducted by van Barneveld and Pluske (2001) indicates that the viscosity of digesta in pigs fed on barley correlates with their available energy values.

Increasing the viscosity of digesta can decrease enzyme accessibility to substrate (such as starch) by impairing their diffusion (Antoniou and Marquardt, 1981). It is known that both the NSP content and their physical and chemical characteristics can vary within and between grains (Hughes and Choct, 1999; van Barneveld, 1999a). Therefore, it can be expected that the variations in viscous properties of NSP in grains influence the accessibility of digestive enzymes to starch and thus its digestibility.

The characterisation of physical and chemical properties of NSP in grains (based on their viscous properties) may be used as an indirect marker to predict starch digestibility and the available energy values of grains fed to pigs and poultry.

### *2.8.4 Transit time*

In monogastric animals, increasing the insoluble NSP content in diets can increase overall feed intake through dilution of available energy content of the feed (de Lange, 2000). This effectively decreases the retention time of chyme in the intestinal tract and thus reduces the exposure of feed ingredients to digestive enzymes (Graham and Aman, 1991). For example, Jorgensen *et al.* (1996) showed that in pigs fed a high-fibre diet, the flow of digesta through the terminal ileum increased by 5-6 times. It has been speculated that insoluble NSP influence the rate of absorption of glucose by increasing the gastric emptying rather than displacement of the site of starch absorption in the small intestine (Johansen and Bach Knudsen, 1994).

Increases in insoluble NSP content do not always result in an increased feed intake, because when the inclusion level of insoluble NSP is high in a diet, digesta retention time may actually increase due to the water holding capacity of NSP (Eastwood *et al.*, 1983; Graham and Aman, 1991). The soluble and to a lesser extent insoluble NSP have a high water holding capacity which could influence the feed intake in pigs (Kyriazakis and Emmans, 1995). It has been speculated that variations in water-holding capacity of NSP could influence the starch digestibility since this property could influence the available water to hydrate starch molecules and thus aiding enzymic digestion (Choct and Cadogan, 2001).

### *2.8.5 Microbial over growth*

Increasing the soluble NSP content in animal diets can cause microbial overgrowth in the intestinal system (Choct and Annison, 1992b; Choct and Annison, 1992a; Smits and Annison, 1996; Langhout, 1998). This can result in increased small intestinal fermentation and is partly responsible for the anti-nutritive activity of NSP in chickens (Choct *et al.*, 1996). Increasing the amount of fermentable substrates entering the ileum and hindgut of pigs (Pluske *et al.*, 1996) or in broiler chicks (Bedford, 1996; Choct *et al.*, 1996) can increase the microflora populations in the intestinal system. The microflora populations in the ileum can compete for the digestion of feed ingredients, such as starch that has escaped duodenal digestion with the endogenous jejunal animal enzymes (as described in section 2.4.1) and can also influence the morphological structure of the intestinal system and its functions (Rijnen *et al.*, 2001).

### *2.8.6 Protein matrix*

The protein component in grains is mainly located in the starchy endosperm area, in the form of a storage protein matrix (Shewry, 1996). However some protein can also be found in the aleurone layer and germ region in the form of enzymes (Shewry, 1996). Starch in grains is embedded to various degrees in the protein matrix (Glenn and Saunders, 1990; Bechtel and Wilson, 1997). The extent of protein association with starch granule surfaces may influence the accessibility of enzymes to starch granules resulting in decreased starch digestibility and thus energy availability (Darlington *et al.*, 2000). For example, the lower feeding value of sorghum compared with maize for ruminants is partially related to the chemical and/or structural composition of the protein matrix (Ackerson *et al.*, 1978; Rooney and Pflugfelder, 1986).

The negative influence of the protein matrix on starch digestion in grains could be magnified in the presence of anti-nutritional factors that are naturally present in grains (Black, 2000). For example, several protease enzyme inhibitors (eg. trypsin, chymotrypsin) have been reported in barley and wheat grains (Boisen *et al.*, 1981; Boisen, 1983; Shewry, 1996). These protease inhibitors may act to reduce the ability of endogenous proteases (present in the stomach and small intestine) to digest the protein matrix surrounding starch granules in grains and consequently indirectly reduce the digestibility of starch. Another example is the formation of tannin/protein complexes such as in some sorghum grains, can depress normal enzymic digestion in pigs and poultry (Mitaru *et al.*, 1984; Myer and Gorbet, 1985; Halley *et al.*, 1986). The poor growth rate and feed conversion ratio in pigs fed sorghum may partially be caused by their low protein digestibility and consequently low starch digestibility (Deshpande, 1986; Nyachoti *et al.*, 1997). Furthermore, differences in grain hardness have been shown to be due to differences in the continuity of the protein matrix in the endosperm and the extent to which it entraps starch granules (Stenvert and Kingswood, 1977; Ellis *et al.*, 1992). However, the effect of the variation in protein matrix quality and quantity on starch digestion and available energy values in barley, wheat and sorghum for pigs and poultry is unknown.

### *2.8.7 Lipids*

It is believed that the hydrophobic nature of lipids inhibits the accessibility of amylase enzyme to starch (Asp *et al.*, 1996). However, it could be expected that the

influence of lipid-amylose complexes on starch digestibility is not significant since cereal grains contain only a small proportion of lipid (1 to 2% of kernel weight), although, the effect of lipids on starch digestibility could become significant in presence of a lipid rich diet.

## 2.9 Summary

This review has highlighted the need to determine variation in the available energy content of cereal grains for pigs and poultry. Furthermore, the potential for specific physical and chemical characteristics of grains to influence starch digestibility in pigs and poultry has been addressed. This has led to the primary hypothesis that physical and chemical characteristics of grains significantly influence starch digestibility, and an understanding of these mechanisms may facilitate improved utilisation of energy from cereal grains by pigs and poultry.

To address this hypothesis, the following was undertaken:

1. Development of a rapid, reproducible *in vitro* assay that can detect differences in the starch digestibility of selected samples of barley, sorghum and wheat grains.
2. Identification of the physical and chemical characteristics starch of grains that vary significantly within and between grain types.
3. Investigation of the relationship between physical and chemical characteristics of grains and starch digestibility.

# Chapter 3 The glucose release index as a predictor of starch digestibility within and between cereal grain types.

## 3.1 Introduction

Starch is a major energy component of feed grains (Stone, 1996). In pigs and poultry, the most efficient use of starch is by hydrolysis into monomeric glucose units. This occurs through the combined action of pancreatic α-amylase and a number of carbohydrases (e.g. amyloglucosidase, maltase) that are present along the small intestinal tract especially in the brush border of the jejunum region (Longland, 1991). The action of these enzymes on starch, results in the release glucose that is readily available for absorption through the small intestinal mucosa.

Starch digestibility in cereal grains is widely viewed as an important factor contributing to energy availability to monogastric animals, and studies with wheat have shown that *in vitro* starch digestibility correlates to AME in poultry (Wiseman *et al.*, 2000). Thus variations in starch digestibility, which may occur within and between cereal grains, are of major concern to the pig and poultry industry with respect to formulating a cost-effective diet.

Numerous physical and chemical grain characteristics have been implicated in affecting starch digestibility including the degree of gelatinisation, cell wall composition and structure, particle size of the milled sample, amylose : amylopectin content, protein encapsulation, amylose-lipid complexes, retrogradation during processing (e.g., extrusion), and the presence of inhibitors (e.g., lignins and tannins) (Blakeney, 1993). However, there is a paucity of information with respect to their influence on starch digestibility between and particularly within species of cereal grains. In order to further define such factors and rank cereal grains for their nutritional quality, there is a need to develop an appropriate and preferably rapid procedure for measuring starch digestibility in cereal grains that has application to the pig and poultry industries.

Several *in vitro* starch digestibility assays have been developed and used across a variety of species including humans, ruminants and poultry (Englyst *et al.*, 1992; Bird *et al.*, 1999; Weurding *et al.*, 2001a). In pigs, Boisen and Fernández (1997) developed an *in vitro* assay for assessing organic matter digestibility and these values were highly correlated with their corresponding *in vivo* values. The basic principle applied to most *in vitro* starch digestibility assays is similar and aims to partially mimic the digestive conditions present in the small intestine by using similar starch digestion enzymes and pH

conditions. For example, Wiseman *et al.* (2000) assessed the extent of starch digestibility in different wheat samples fed to poultry by *in vitro* digestion with α-amylase, whereas Weurding *et al.* (2001a) assessed starch digestibility in different poultry feedstuffs by pepsin and HCl pre-treatment followed by digestion with a mixture of enzymes (amyloglucosidase, invertase, pullulanase, heat stable α-amylase).

Englyst *et al.* (1992) used a more rapid approach to measure the digestibility of starch and other carbohydrates such as fructose, by designing a single time point multi-enzyme assay. This assay has been successfully used for rapidly assessing variations in starch digestibility between different foodstuffs (Englyst *et al.*, 1996). A "starch digestion index" was utilised and defined as the percentage of rapidly digested starch (following a 20 minute incubation) to total starch (Englyst *et al.*, 1992). The aim of the present work was to develop methodology specifically suited to cereal grains. Only two digestive enzymes (heat stable α-amylase and amyloglucosidase) out of the 6 enzymes originally used by Englyst *et al.* (1992) were considered necessary to investigate the variation in starch digestibility between cereal grains, since almost all of the digestible carbohydrate in cereal grains is starch. Data from this modified procedure of determining the ratio of the released glucose in the initial stage of starch digestion to the released glucose at the completion of starch digestion is termed in this thesis as the glucose release index (GRI). Therefore, the hypothesis of the current work was that GRI values within and between cultivars of barley, sorghum and wheat vary significantly. The main aims of this experiment were to:

1. Develop an *in vitro* starch digestibility assay that is repeatable and rapid for determining the GRI of starch in barley, sorghum and wheat samples based on the principle of the assay developed by Englyst *et al.* (1992).
2. Measure and test the significance of the differences of GRI values within and between barley, sorghum, and wheat grains.

## 3.2 Materials and Methods

### *3.2.1 Selection and preparation of grain samples for GRI analysis*

Samples of Australian barley (n=18), sorghum (n=15) and wheat (n=10) differing in strain, location and cultivar were selected to investigate whether differences in the GRI occur within and across grain types fed to pigs and poultry (these grains were selected based on their availability, for the first phase of the Premium Grains for Livestock Program). Different cultivar growing locations were used to help insure a range of nutritional quality of grains for pigs and poultry existed (refer to Appendix 3.1).

Throughout the thesis, the term cultivar is used to distinguish between the individual samples of each grain type. In some instances, the same cultivar was grown at more than one geographic site, as listed in Appendix 3.1, and in these cases, "cultivar" also refers to the different sources of the same cultivar.

Predicting the GRI in grains requires enzymatic hydrolysis of starch, and in order to minimise the physical barrier presented by the cell wall of the starchy endosperm in grains towards substrate accessibility (Section 2.8.1), approximately 100g of each grain was milled through a 0.5 mm screen by an ultra centrifugal miller (ZM1-Retschand, Haan, Germany) and then the dry matter content determined using the method outlined in AOAC (1995) method 4.1.06.

### *3.2.2 Determining the total and digestible starch content in barley, sorghum and wheat cereal grains*

The total starch and digestible starch content analysis was conducted by the School of Rural Sciences and Agriculture at the University of New England for the Premium Grains for Livestock Program.

The Megazyme™ assay, based on the methods outlined in AOAC (1995) method 996.11 and AACC (1995) method 76.13, was used to measure total starch and total digestible starch in barley, sorghum and wheat samples following α-amylase and amyloglucosidase starch hydrolysis to its monomeric glucose units. To determine the total starch (resistant plus digestible), the original Megazyme™ starch assay was used with an extra step involving pre-incubation of samples with 2ml of dimethyl sulphoxide (DMSO) (Sigma Chemical Co. USA) solution in a boiling water bath for 5 minutes prior to adding the thermostable α-amylase (Sigma Chemical Co. USA) and amyloglucosidase (Sigma Chemical Co. USA). This modification was based on the method of McCleary *et al.* (1997).

### *3.2.3 Development and optimisation of an* in vitro *method for evaluating the GRI as a measure of starch digestibility in cereal grains*

#### *3.2.3.1 Justification of methodology*

In a non-limiting system, the substrate utilisation by an enzyme over time usually displays a curve such as that shown in Figure 3.1. The conversion of substrate (such as starch) to product (glucose) is initially rapid and linear before the progression of the reaction slows down and gradually reaches a plateau (Figure 3.1).

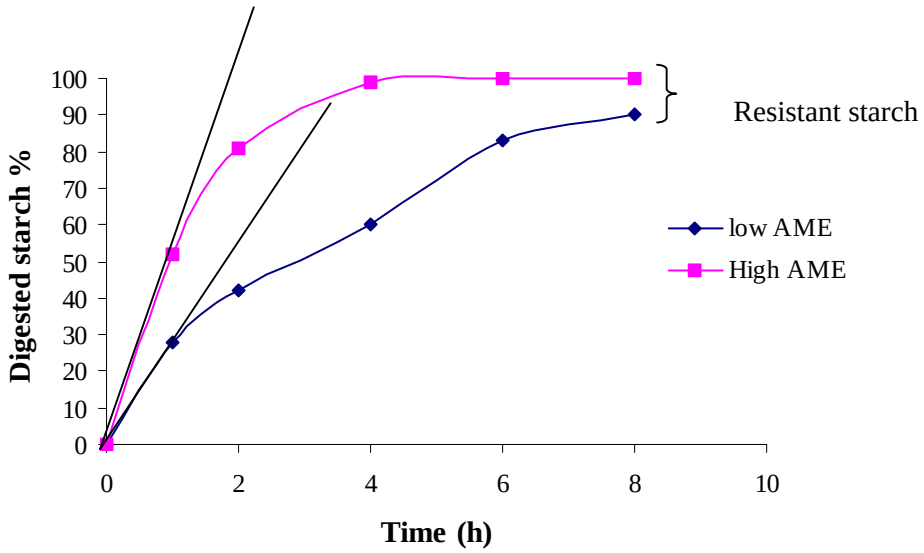



**Figure 3.1 The rate of starch hydrolysis *in vitro* from high and low AME wheat samples. Adapted from (Wiseman, *et.al* 2000).**

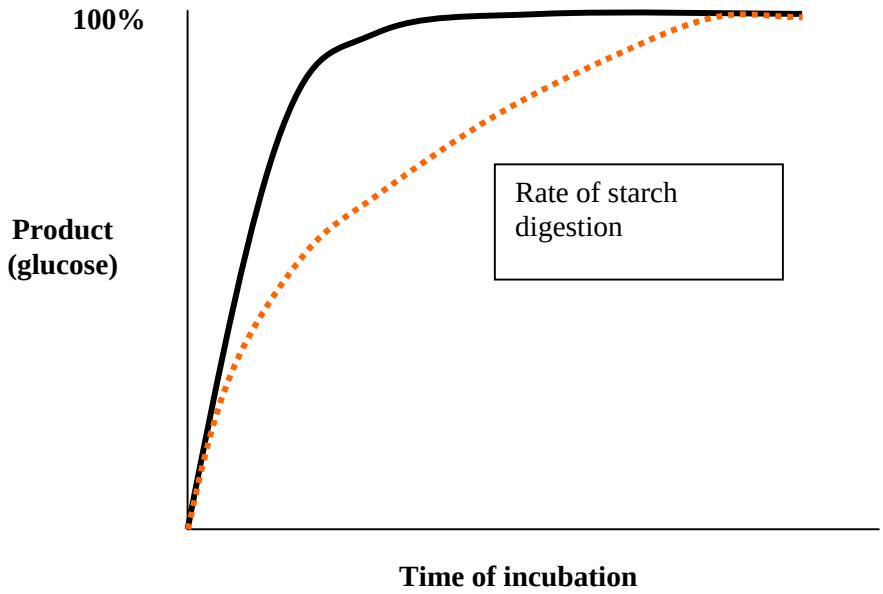



**Figure 3.2 A representative diagram showing a comparison of the rate of *in vitro* starch digestion that is typical when using the Megazyme™ total starch assay (▬) and the desirable rate for determining differences in starch digestibility of grains (····).**

Consequently, differences in the amount of glucose released from the digestion of starch in grains would be maximal during the initial rapid phase of starch digestion. It has been widely accepted that restricting measurements of the progression of an enzymic reaction to a period where less than 20% of total substrate consumption has occurred, will provide a value that reflects the conversion rate of a substrate (starch) to its product (glucose), when the initial substrate concentration is in excess of the Km value of the enzyme (Tipton, 1992). In practice however, determining the amount of glucose released from digestible starch when less than 20% of the total starch content of grains is digested would be difficult, due to the lack of sensitivity of standard analytical instruments (spectrophotometer). Consequently it was considered that an assay condition that allows the release of 20% to 50% of glucose from the total starch content of grains would be desirable since it would reflect the conversion rate of starch digestion with minimising the artefacts (errors) associated with using the spectrophotometer. This would generate absorption values within a range of 0.15 to 0.85.

### *3.2.3.2* In vitro *GRI assay methodology*

Representative samples from two wheat cultivars with known ileal DE differences of more than 1MJ/kg in pigs (van Barneveld *et al.*, 2001) were selected for the development of a quantitative *in vitro* GRI assay. The grain samples were hammer-milled (particle size ≤0.5mm) and duplicates of 0.1g were accurately weighed into McCartney bottles (AdeLab, South Australia).

To measure the GRI, the principle procedure of the Megazyme™ total starch assay, based on the procedures outlined in AOAC (1995) method 996.11 and AACC (1995) method 76.13, was employed. The original Megazyme™ assay has been designed to determine total digestible starch of grain samples by maximising the amount of starch digestion in a minimum time period. Although the conditions in which the Megazyme™ assay is carried out allow a rapid determination of total digestible starch content, the potential differences in the rate of starch digestion that may occur between different cultivars of grains cannot be easily determined due to the rapid rates of digestion (Figure 3.2). Thus in order to detect differences in starch digestibility between grain samples (as measured by glucose release), the rate of starch digestion should ideally be reduced and terminated at the point where 20 to 50% of the total starch is digested to glucose (Section 3.2.3.1). To achieve this, the assay conditions of incubation time and temperature, and enzyme concentration were modified from the original conditions defined in the

Megazyme™ assay, and a single time point measurement was used as previously described by Englyst *et al.* (1992) (Figure 3.3).

A 3 x 3 x 3 factorial experiment was designed with the main variables being incubation time, enzyme concentration and temperature. The incubation times were three minutes, two minutes or one minute for the α-amylase step and 18 min, 15 min, or 12 min for the amyloglucosidase digestion step respectively (a1=3/18 min, a2=2/15 min, a3=1/12 min). The enzyme concentrations for both α-amylase and amyloglucosidase were b1=70%, b2=50% or b3=30% of the standard enzyme concentration and the temperature set to 65°C, 50°C or 35°C for α-amylase step and 50°C, 40°C or 35°C for amyloglucosidase respectively (c1=65/50°C, c2=50/40°C, c3=35/35°C) (Figure 3.3). This resulted in 27 different treatments (Table 3.1).

Samples were assayed in duplicate for each treatment. In each experimental run, glucose (G-7528, Sigma Chemical Co. USA) and pure starch (102713R, BDH Limited England) were used as positive controls, and a substrate blank was used as a negative control. For each treatment, the GRI of the samples during the initial phase of the assay were determined (Table 3.1). This was calculated by the percentage of glucose released under the experimental conditions to the total glucose released from complete starch digestion (as determined by the Megazyme™ total starch assay, see section 3.2.2). The resulting GRI was calculated as follows:

$$\text{GRI (\%)} = \frac{\text{100 x glucose released by modified Megazyme™ method}}{\text{total glucose released by complete starch digestion}}$$

The optimal condition for the *in vitro* GRI assay (the condition displaying the largest significant difference in GRI between the two selected samples) was then chosen to determine the GRI values of all barley, sorghum and wheat grain samples in duplicate.

### *3.2.4 Statistical analysis*

#### *3.2.4.1 Development of the* in vitro *assay*

Differences in the GRI values obtained with the 27 treatments were analysed by ANOVA and least significant differences (LSD) (Genstat 4.2).

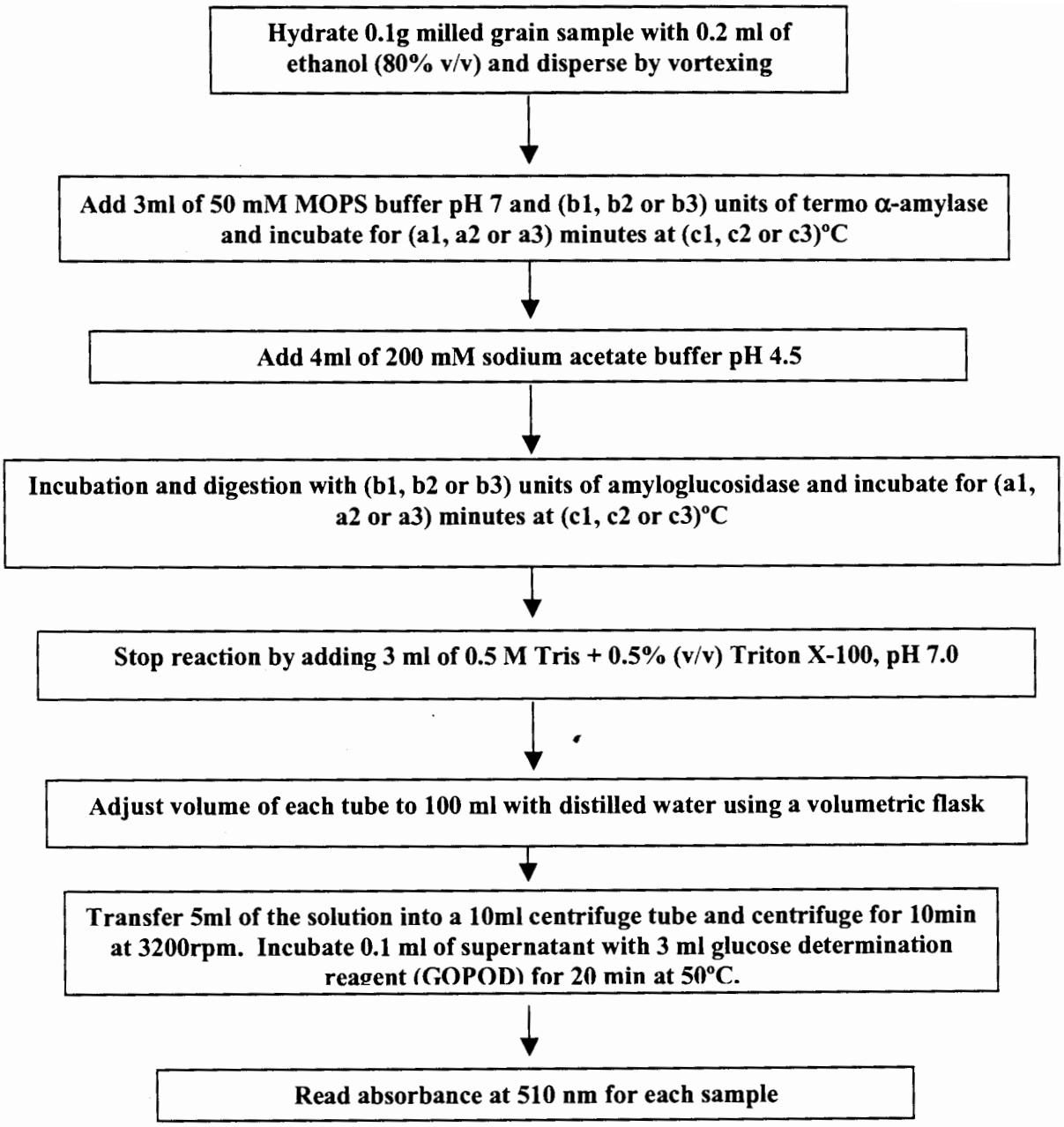



**Figure 3.3 Flow diagram of the rapid digestible starch assay for cereal grains based on the modified Megazyme™ total digestible starch assay.**

- MOPS buffer=50 mM, pH=7 plus calcium chloride (5 mM) and sodium azide (0.02%).
- GOPOD= glucose determination reagent = Glucose oxidase + Peroxidase + 4-Aminoantipyrine.
- (a1=3/18 min, a2=2/15 min, a3=1/12 min) = 3 min, 2 min or 1 min for the α-amylase step and 18 min, 15 min, or 12 min for the amyloglucosidase digestion step.
- ( b1=70%, b2=50% and b3=30%) = 70%, 50% or 30% of the enzyme concentration used by Megazyme total starch kit for both α-amylase and amyloglucosidase.
- (c1=65/50°C, c2=50/40°C, c3=35/35°C) = the temperature set to 65°C, 50°C or 35°C for α-amylase step and 50°C, 40°C or 35°C for amyloglucosidase respectively.

**Table 3.1. The 27 treatments used for designing the glucose release index *in vitro* assay for cereal grains.**

| Temperature (c)[1] | Enzyme concentration (b)[2] | Time (a)[3] | | |
|---|---|---|---|---|
| | | a1=3/18 min | a2=2/15 min | a3=1/12 min |
| c1=65/50°C | b1=70% | a1b1c1 | a2b1c1 | a3b1c1 |
| | b2=50% | a1b2c1 | a2b2c1 | a3b2c1 |
| | b3=30% | a1b3c1 | a2b3c1 | a3b3c1 |
| c2=50/40°C | b1=70% | a1b1c2 | a2b1c2 | a3b1c2 |
| | b2=50% | a1b2c2 | a2b2c2 | a3b2c2 |
| | b3=30% | a1b3c2 | a2b3c2 | a3b3c2 |
| c3=35/35°C | b1=70% | a1b1c3 | a2b1c3 | a3b1c3 |
| | b2=50% | a1b2c3 | a2b2c3 | a3b2c3 |
| | b3=30% | a1b3c3 | a2b3c3 | a3b3c3 |

[1] the temperature set to 65°C, 50°C or 35°C for α-amylase and 50°C, 40°C or 35°C for amyloglucosidase.
[2] 70%, 50% or 30% of the enzyme concentration used in the Megazyme total starch kit for both α-amylase and amyloglucosidase.
[3] 3 min, 2 min or 1 min for the α-amylase step and 18 min, 15 min, or 12 min for the amyloglucosidase digestion step.

### 3.2.4.2 *Testing significance of difference in the GRI values between grain types and cultivars*

The variation in the average values of GRI between the three grain types was tested using ANOVA and LSD (Genstat 4.2). Similarly, ANOVA and LSD were used to analyse the GRI values between cultivars of barley, sorghum and wheat.

### 3.2.4.3 *Regression analysis of the GRI in grains with their corresponding digestible and total starch values*

The quartile range and skewness of the GRI values obtained for the grain samples was calculated to identify any possible outlier values. Following this, the relationship between the GRI values in grains to their 1) total starch content (digestible and resistant) and 2) digestible starch content, was assessed by single and multiple linear regression analysis (Genstat 4.2).

## 3.3 Results

### 3.3.1 *Development and optimisation of an* in vitro *method for determining the variation in the GRI from starch in cereal grains*

The analysis of data showed that each main factor of the *in vitro* assay (time, enzyme concentration and temperature) displayed a statistically significant impact on the GRI ($P<0.001$, Table 3.2). Furthermore (with the exception of the interactions between sample *vs* incubation time and sample *vs* enzyme concentration), a two-, three- and four-way interaction between factors was also statistically significant (Table 3.2). Of all main factors, temperature had the largest significant impact on the GRI values; is was over 46 times higher than the next largest sum of squares, followed by incubation time and then enzyme concentration (Table 3.2). Thus in order to simplify the interpretation of analysis and to investigate the influence of the enzyme concentration and time of incubation on the GRI values of the samples, the data was reanalysed for each set of temperatures individually.

In the treatments with the highest temperature setting (c1=65/50°C) over 80% of total glucose was released from the total starch content, thus this temperature was not selected to predict the variation in starch digestibility between grains, based on the criteria described in Section 3.2.3. Further analysis showed significant differences in GRI values of the two samples at temperature c2=50/40°C but not c3=35/35°C ($P<0.001$) (Table 3.3).

Consequently, temperature c2 (50/40°C) was used to determine the influence of incubation time and enzyme concentration on the GRI values between the two wheat samples (Table 3.4 a, b & c). Results in Table 3.4a show that at temperature c2, the largest measurable difference in the GRI between the two wheat samples was apparent at an incubation time of a1=3/18 min in combination with an enzyme concentration of b1=70%. These conditions (a1b1c2) were therefore selected for subsequent analysis of the GRI in wheat, barley and sorghum samples forming the second part of the study.

### *3.3.2 Comparison of the GRI within barley, sorghum and wheat*

Percentage of glucose released during the enzyme digestion under the condition described in Section 3.2.3 and total glucose release from total starch digestion for each cultivar are listed in Appendix 3.2. Within barley, sorghum and wheat grains, the GRI values varied significantly between cultivars ($P<0.001$; Figures 3.4, 3.5 and 3.6 respectively).

In barley, the GRI values showed a 1.6-fold difference between the minimum and maximum values (ranged from 27.2% to 44.8%). Similarly in wheat, the GRI values displayed a 1.7-fold difference between the minimum and maximum GRI values (ranged from 31.9% to 52.9 %). The GRI values of sorghum cultivars exhibited a 2.2-fold difference between the minimum and maximum values (range from 24.4% to 54.5%). It was further shown that the GRI values for two of the sorghum cultivars, Normal Isoline and Mr Maxi cultivars, fell outside the quartile range of all other sorghum grains analysed.

### *3.3.3 Comparison of the GRI between barley, sorghum and wheat*

The average GRI value for wheat was 23.7% and 17.5% higher than sorghum and barley respectively ($P<0.001$). In contrast, no significant differences in GRI values were observed between sorghum and barley (Figure 3.7).

### *3.3.4 The relationship of the GRI to total starch and total digestible starch in barley, sorghum and wheat*

The GRI values (falling within the quartile range) in barley, sorghum or wheat were not significantly related to their corresponding total starch or digestible starch values (Appendix 3.3) ($P>0.05$, Figures 3.8 a, b & c).

**Table 3.2 Analysis of variance of the glucose release index in two randomly selected wheat samples determined by varying temperature, incubation time and enzyme concentration.**

| Source of variation | d.f. | Sum of squares | P |
|---|---|---|---|
| Time of incubation (a) | 2 | 1826.3 | <0.001 |
| Enzyme concentration (b) | 2 | 407.7 | <0.001 |
| Temperature (c) | 2 | 86265.2 | <0.001 |
| Sample (d) | 1 | 128.8 | <0.001 |
| Interaction of a & b | 4 | 74.8 | 0.003 |
| Interaction of a & c | 4 | 597.3 | <0.001 |
| Interaction of b & c | 4 | 148.23 | <0.001 |
| Interaction of a & d | 2 | 8.2 | 0.376 |
| Interaction of b & d | 2 | 7.6 | 0.400 |
| Interaction of c & d | 2 | 76.3 | <0.001 |
| Interaction of a & b & c | 8 | 193.2 | <0.001 |
| Interaction of a & b & d | 4 | 104.7 | <0.001 |
| Interaction of a & c & d | 4 | 105.4 | <0.001 |
| Interaction of b & c & d | 4 | 76.1 | 0.003 |
| Interaction of a & b & c & d | 8 | 121.7 | 0.002 |

**Table 3.3 Analysis of variance of the glucose release index in two randomly selected wheat samples, assayed at temperature c2 =50/40°C with varying enzyme concentrations and incubation times.**

| Source of variation | d.f. | Sum of squares | P |
|---|---|---|---|
| Time of incubation (a) | 2 | 1290.7 | <0.001 |
| Enzyme concentration (b) | 2 | 201.6 | <0.001 |
| Sample (d) | 1 | 172.1 | <0.001 |
| Interaction of a & b | 4 | 123.8 | <0.001 |
| Interaction of a & d | 2 | 14.85 | 0.184 |
| Interaction of b & d | 2 | 68.7 | 0.002 |
| Interaction of a & b & d | 4 | 168.7 | <0.001 |

**Table 3.4 Differences in the glucose release index between the two randomly selected wheat samples (sample 1 and 2) determined for incubation times (a1, a2, a3)[1] and enzyme concentrations (b1, b2, b3)[2], at a constant temperature level of c2 (50/40 °C)[3].**

a)

| [enzyme]= 70% of standard assay concentration | | | | |
|---|---|---|---|---|
| **Time** | **Sample 1** | **Sample 2** | **sed** | **P** |
| **a1**[4] | 53.98 | 40.59 | 1.99 | 0.001 |
| **a2** | 39.98 | 34.63 | 1.99 | 0.001 |
| **a3** | 31.93 | 29.37 | 1.99 | NS[5] |

b)

| [enzyme]= 50% of standard assay concentration | | | | |
|---|---|---|---|---|
| **Time** | **Sample 1** | **Sample 2** | **sed** | **P** |
| **a1** | 52.96 | 45.15 | 1.99 | 0.001 |
| **a2** | 38.28 | 33.77 | 1.99 | 0.001 |
| **a3** | 32.78 | 28.81 | 1.99 | NS |

c)

| [enzyme]= 30% of standard assay concentration | | | | |
|---|---|---|---|---|
| **Time** | **Sample 1** | **Sample 2** | **sed** | **P** |
| **a1** | 34.33 | 41.64 | 1.99 | 0.001 |
| **a2** | 36.82 | 29.27 | 1.99 | 0.001 |
| **a3** | 30.24 | 28.71 | 1.99 | NS |

[1] 3 min, 2 min or 1 min for the α-amylase step and 18 min, 15 min, or 12 min for the amyloglucosidase digestion step.
[2] 70%, 50% or 30% of the enzyme concentration used by Megazyme total starch kit for both α-amylase and amyloglucosidase.
[3] the temperature was set to 50°C for the α-amylase step and 40°C for the amyloglucosidase step respectively
[4] The selected condition displaying the largest significant difference between the two wheat samples.
[5] not significant (P>0.05).

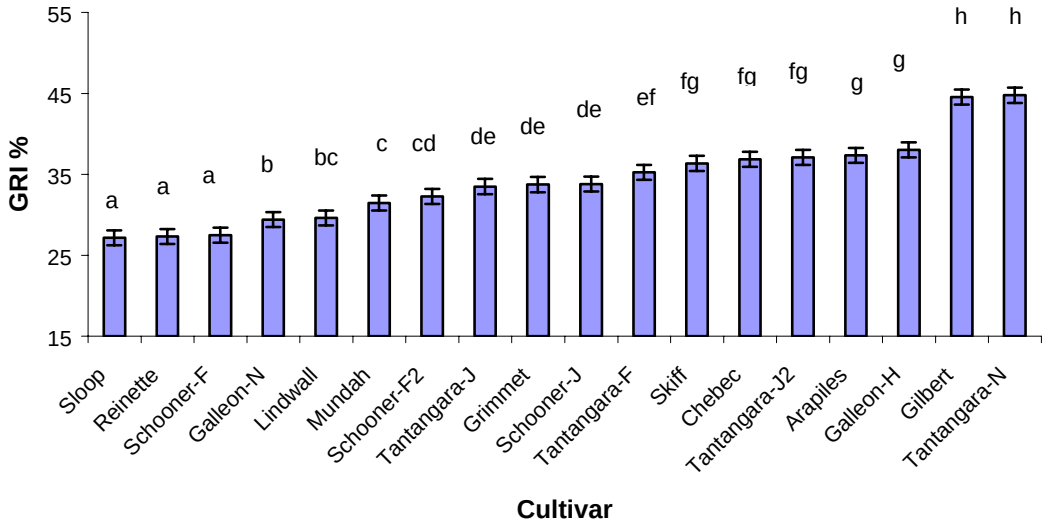



**Figure 3.4 The distribution of the glucose release index (GRI) within the barley samples, bars with different superscripts differ significantly (P<0.001), error bars indicate standard error.**

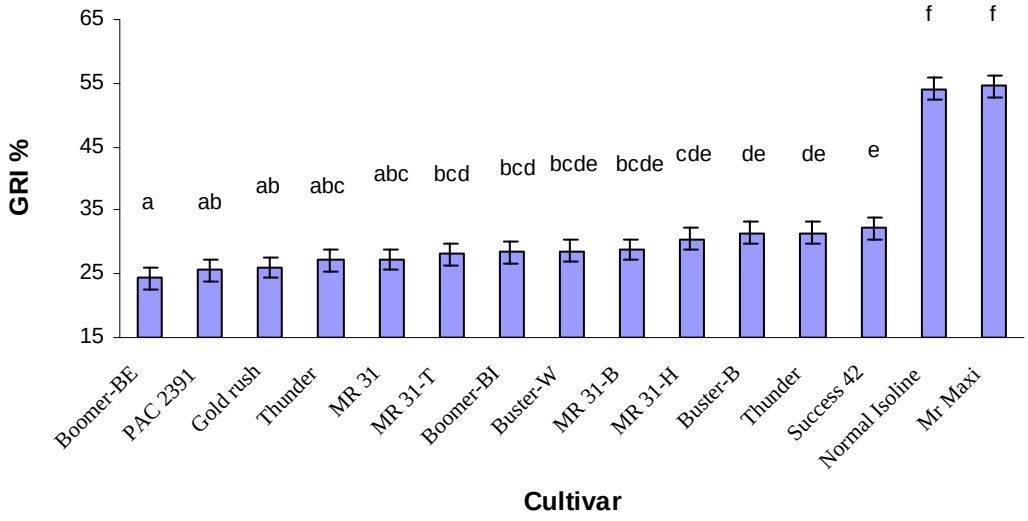



**Figure 3.5 The distribution of the glucose release index (GRI) within the sorghum samples, bars with different superscripts differ significantly (P<0.001), error bars indicate standard error.**

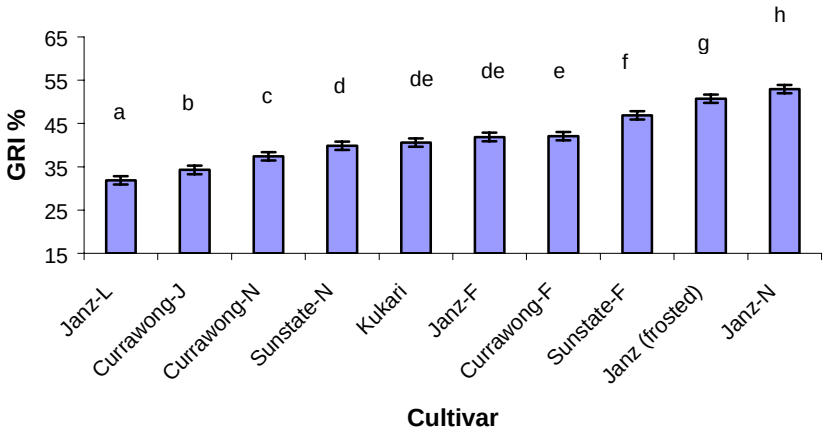



**Figure 3.6 The distribution of the glucose release index (GRI) within the wheat samples, bars with different superscripts differ significantly (P<0.001), error bars indicate standard error.**

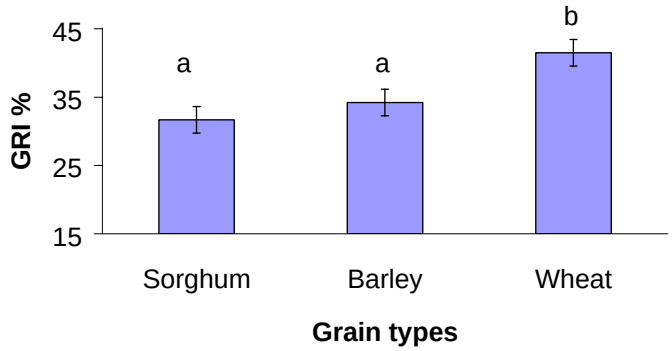
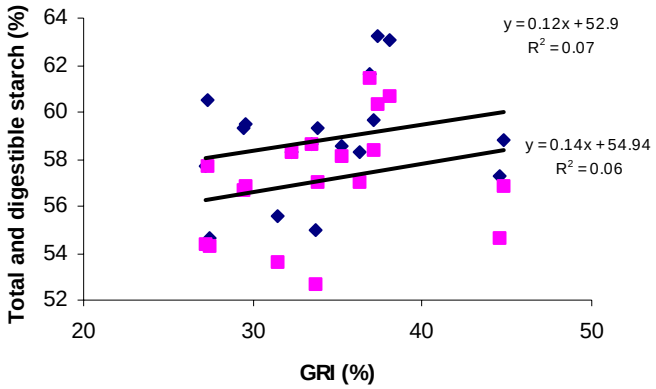
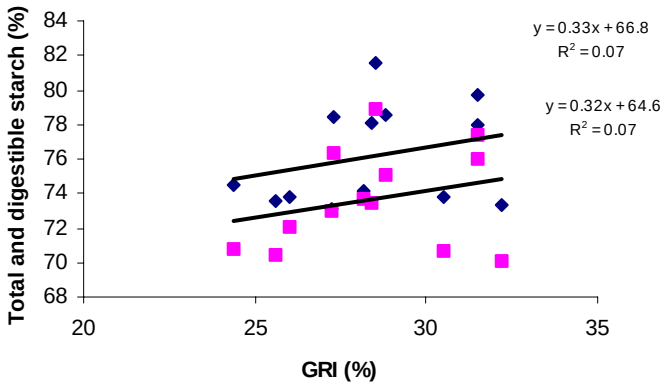



**Figure 3.7 The distribution of the glucose release index (GRI) across sorghum, barley and wheat samples (bars with different superscripts differ significantly, barley n=36, sorghum n=32, and wheat n=21, P<0.001), error bars indicate standard error.**

a) Barley




b) Sorghum




c) Wheat

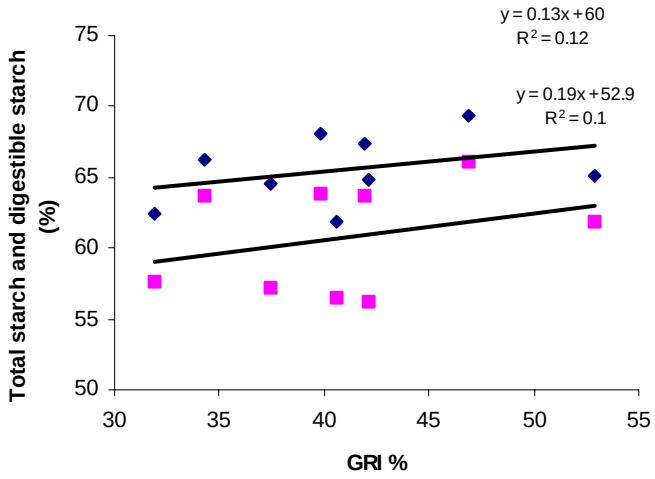



**Figure 3.8 The relationship between the glucose release index (GRI) values of barley, sorghum and wheat with their corresponding total and digestible starch content, (P>0.05). (♦=Total starch ■=Digestible starch)**

## 3.4 Discussion

Starch is a major component of pig and poultry diets. It has been hypothesised that starch digestibility is an important determinant of the feed energy value in poultry (Wiseman *et al.*, 2000). As well as providing a major source of energy to animals, the digestion of starch to glucose may influence the availability of other nutrients such as amino acids, by affecting, for example, plasma insulin levels (Englyst *et al.*, 1996; Weurding *et al.*, 2001a). The need to further define characteristics in cereal grains that influence starch digestibility, prompted the development of a rapid and reproducible assay designed to measure starch digestibility as defined by the GRI within and between sorghum, barley and wheat cereal grains.

The results outlined in this chapter support the hypothesis that the GRI of cereal grains varied significantly between and within their cultivars. Of 27 combinations of incubation time, temperature and enzyme concentration, temperature had the greatest influences on the *in vitro* GRI from starch in grains, followed by incubation time and then enzyme concentration. This was not an unexpected result, as temperature has been shown to influence the swelling and gelatinisation status of starch granules, which in turn is important for the accessibility of the amylolytic enzymes to starch polymers, ultimately resulting in their hydrolysis to glucose units (Moran, 1982; Vasanthan *et al.*, 1995; Carter, 1996). Lengthening incubation time would also be expected to increase the *in vitro* GRI from starch in grains as it would lead to an increase in the chance of enzyme-substrate interactions resulting in elevated starch hydrolysis.

Following selection of optimum GRI assay conditions, results indicated that differences existed in GRI values between cultivars of barley, sorghum and wheat samples. However there was no relationship between the GRI values and either the total starch or the digestible starch content for any of the three grain types, indicating that starch digestibility of grains is not influenced by the total starch content. The results shown in this chapter are partially in line with those reported by Weurding *et al.* (2001b) and Bird *et al.* (1999) that showed that starch digestibility in barley and wheat is higher than in sorghum samples. Such findings strengthen the potential use of the GRI as an indicator of starch digestibility in feed grains.

In the current study, the two outlier sorghum cultivars elevated the average GRI value in sorghum. An increase in the number of sorghum cultivars analysed may be necessary to provide a more representative average GRI value for this grain type.

Although predicting the energy availability of cereal grains remains a cumbersome task and is most accurately represented by *in vivo* assays, the described *in vitro* GRI assay could be applied to the development of a rapid and objective test for grain quality for monogastric animals. In support of this, Wiseman *et al.* (2000) and Zarrinkalam *et al.* (2001) showed that variation of *in vitro* starch digestion is associated with AME of wheat and the DE values of barley for poultry and pigs, respectively. Furthermore, the application of the GRI assay can be used to investigate which intrinsic (starch related) and extrinsic (non-starch related) grain characteristics may influence the starch digestibility of grains in animals.

Cummings and Englyst (1987) and Blakeney (1993) have highlighted several factors that could influence starch digestibility. These factors include the degree of starch gelatinisation; grain cell wall composition and structure; size of the starch granule; amylose : amylopectin content; protein encapsulation of starch granules; amylose-lipid complexes; retrogradation during processing (e.g., extrusion), and the presence of enzyme inhibitors (e.g., lignins and tannins). Physical and chemical characteristics of grain cell walls and starch granules, as well as the concentration of starch digestive enzymes (e.g. α-amylase) are also important determining factors in the starch digestibility (Gray, 1992). Furthermore, the retention time of feed, age of the animal and microbial overgrowth in hindgut, are also thought to influence the degree of starch digestion by the animal (Rogel *et al.*, 1987; Drochner *et al.*, 1993; Choct, 1999).

An understanding of the physical and chemical characteristics of grains that influence starch utilisation would be valuable, since it could be used to assess which processing methods might hold the greatest potential for improving the grain digestibility by animals (e.g., grinding or extruding grains may enhance their starch digestibility). Furthermore, plant breeders may also benefit from using GRI values to aid in the selection of a desired trait in selection programs.

The work reported in the following chapters characterises the influence of some of the above factors in cereal grains on *in vitro* starch digestion, by using the GRI values.

# Chapter 4 Physical and chemical characteristics of starch granules and their relationship to the glucose release index in barley, sorghum and wheat.

## 4.1 Introduction

During the second and third phases of seed development, starch is formed in the endosperm cells, within membrane-bound organelles known as plastids (Stone, 1996). At the microscopic level, starch is found in the form of discrete granule bodies, which can differ in size and shape (MacGregor and Fincher, 1993). Several studies have investigated sizes of starch granules in barley, wheat and sorghum (Rooney and Pflugfelder, 1986; Bechtel *et al.*, 1993; Blumenthal *et al.*, 1994; Borem *et al.*, 1997; Fulcher *et al.*, 1997; Fujita *et al.*, 1998). In barley and wheat, starch granules have been classified into two groups based on their diameter as follows: A-type starch granules (larger than 10$\mu$m) and B-type starch granules (smaller than 10$\mu$m). A-type starch granules make up about 10% of the total number and approximately 85% of total starch content by weight. For sorghum starch granules, a range of 2 to 30$\mu$m in diameter has been reported (Rooney and Pflugfelder, 1986), but there are no published data on the size distribution of starch granules.

Starch granules in grains are composed mainly of amylose and amylopectin (>98%), but also contain lipids, proteins, phosphorus, and other minerals (Jacobs and Delcour, 1998). There is evidence to suggest that starch granule chemical composition plays a major role in influencing its digestibility. For instance, it is known that the rate of digestion and the digestibility of amylose and amylopectin differ, with amylose in general displaying a slower rate of digestion compared to amylopectin (Evers *et al.*, 1999; Bedford, 2000a). Amylose in solution may form a helical structure and bond with organic acids, alcohols and, more importantly lipids, that are believed to display resistance to enzymic digestion (Holm *et al.*, 1983). It has also been suggested that the helical structure of amylose itself could depress enzymic digestion (Evers *et al.*, 1999). In comparison, amylopectin has more branch points (Evers *et al.*, 1999), and this branched structure is thought to provide a larger area for exposing the polysaccharide to digestives enzymes compared to the helical structure of amylose (Bedford, 2000a). Several studies also indicate that gelatinisation properties of starch influence the rate and digestibility of starch under *in vitro* and *in vivo* conditions (Snow and O' Dea, 1981; Aman and Hesselman, 1984; Holm *et al.*, 1988; Xiong *et al.*, 1990). The gelatinisation properties of starch are

influenced by changes in the crystalline structure of starch during the heating period (Holm *et al.*, 1988; Erdogdu *et al.*, 1995; Vasanthan *et al.*, 1995; Fujita *et al.*, 1998; Jacobs and Delcour, 1998).

Starch granule size varies among grains of the same type (Palmer, 1972; Nikuni, 1978) and even among the same cultivars of grains (Evers *et al.*, 1999). The variation in starch granule size would affect the surface area : volume ratio of starch granules; e.g. doubling the surface area of a sphere would decrease the ratio of surface area : volume by 12.5%, and consequently reduce the accessibility of digestive enzymes to starch and decrease the digestibility of grains. In support, Bathgate and Palmer (1973) indicated that during malting, small starch granules of barley hydrolysed at a faster rate than larger starch granules. However, Fulcher *et al.* (1997) speculated that in barley and wheat, B-type starch granules are less susceptible to enzymatic digestion compared to A-type starch granules since they tend to be embedded within the endosperm protein matrix and gelatinise over a wider temperature range.

The variability of starch granule size and shape could be the result of three factors: starch granule development and growth; physiological/chemical conditions during the period of seed growth and; starch granule chemical composition (Evers *et al.*, 1999). There is evidence to suggest that starch granule chemical composition plays a major role in influencing the size of starch granules. For example, in barley it has been shown that starch granule size is related to its amylose content (Palmer, 1972). Other investigations have reported that the molecular architecture and composition of amylopectin but not amylose, influence starch granule size and its crystalline structure (MacGregor and Fincher, 1993). Hence, starch granule sizes in grains may be indirectly related to starch digestibility through physical and chemical properties of starch granules. It is hypothesised that:

1. Starch granule size is related to viscoelasticity and the amylose : amylopectin ratio of starch in barley, sorghum and wheat, and
2. The variation in the amylose : amylopectin ratio, viscoelasticity and surface area of starch granules influences GRI values of grains

The aims of this study were to:

1. Determine starch granule sizes in barley, sorghum and wheat samples by developing a rapid and accurate method for quantifying the surface area of starch granules.
2. Investigate the variation of the amylose : amylopectin ratio between the sample types.

3. Investigate the variation in the gelatinisation characteristics of starch in barley, sorghum and wheat by measuring viscoelasticity.
4. Investigate the relationship between the physical and chemical characteristics of starch granules in barley, sorghum and wheat grains and their GRI values, as reported in Chapter 3.

## 4.2 Materials and Methods

In this study, starch granule surface area and viscoelasticity were selected to represent starch granule physical properties, whereas the amylose : amylopectin ratio was chosen to represent starch granule chemical characteristics.

### *4.2.1 Sample selection and preparation*

Barley, sorghum and wheat samples were selected and prepared as described in Chapter 3, Section 3.2.1.

### *4.2.2 Histomorphometric determination of starch granule surface area in barley, sorghum and wheat*

#### *4.2.2.1 Sample preparation and staining*

Duplicates of 0.5mg milled samples (particle size $\leq$ 0.5mm) were weighed into 2ml eppendorf tubes (AdeLab, South Australia). For barley and wheat, a modified method based on the original developed by Fulcher *et al.* (1997) was used to isolate and stain starch granules. This modification involved increasing the amount of milled sample from 0.1mg to 0.5mg. The protein matrix surrounding the starch granules in barley and wheat was denatured and dissolved by the addition of 1ml of 2% (w/v) sodium dodecyl (lauryl) sulphate (SDS) (Sigma Chemical Co, USA) and 25µl of 2mM Dithiothreitol (DTT) (Sigma Chemical Co, USA) to each sample. The solubilized proteinaceous material was separated from the starch granules by centrifugation at 5000*g* for 10 minutes (Megafuge 1.0R, Heraeus Instruments, Germany) and subsequent removal of the supernatant. The pelleted starch granules in each sample were then stained by the addition of 100µl saturated sucrose (Sigma Chemical Co. USA) and 100µl of a solution containing 5% (w/v) potassium iodide (AJAX Chemicals, Australia) with 0.5% (w/v) iodide (BDH Australia). All the above solutions were prepared in distilled water.

In sorghum grains, the protein matrix surrounding starch granules was removed by treatment with 1ml of 0.5% (w/v) protease type XXIII (Sigma Chemical Co. USA) dissolved in phosphate buffered saline (Sigma Chemical Co. USA) at 40°C for 30 minutes. The sorghum samples were then centrifuged at 5000*g* for 10 minutes and the solubilized proteinaceous material contained in the supernatant discarded. Starch granules in the remaining pellet were then stained as outlined for barley and wheat.

Staining of all samples was complete within 30 minutes and starch granule morphometric analysis was carried out within 24 hours of staining.

#### *4.2.2.2 Morphometric analysis*

Starch granule surface area was the parameter selected to best describe their physical characteristics (as opposed to diameter), since the shape of starch granules in barley and wheat grains is elliptical and, in sorghum, polyhedral. The stained starch granules were briefly vortexed and a 100µl sub-aliquot of the mixture was placed onto a microscope slide (Livingstone Int., Australia) and covered with a cover-slip. A light microscope (Olympus model BH-2, Japan) set to a final magnification of X100, with an attached colour video camera (Panasonic model WV-GL760, Japan) was used to visualise the starch granules. For each sample slide, the surface area of 2000 granules was determined by an image analyser program (Video Pro 32, Leading-Edge PLC. Ltd. Australia).

Starch granules of all grain types were divided into two groups, based on the classification of Fulcher *et al.* (1997): those with surface area larger than 100µm$^2$ (A-type granules) and those with a surface area smaller or equal to 100µm$^2$ (B-type granules). For each sample, the mean, median and total surface area for A type and B type starch granules was calculated. In addition, the ratio of the number of B-type to A-type starch granules in each sample was also determined.

### *4.2.3 Analysis of starch viscoelasticity in barley, sorghum and wheat*

The Bread Research Institute, Australia Limited, conducted this assay for the Premium Grains for Livestock Program. A rapid visco analyser (RVA) was used to measure viscoelasticity of starch isolated from barley, sorghum and wheat, to determine the gelatinisation and pasting properties of starch in cereal grains according to Allen *et al.* (1998), DesRochers and Walker (1998) and Wootton *et al.* (1998).

Starch was isolated and purified by a modification to a method originally developed by Welsh (1990). Starch was isolated from 25g of each milled grain (particle size ≤ 0.5

mm) by a series of steps involving washing the grain three times with 0.2M ammonia (UNIVAR Australia) extracting solution, a wash in distilled water, and blending the milled sample with 0.2M acetic acid (UNIVAR Australia) for 30 seconds. The isolated starch was then washed twice each with ethanol (UNIVAR Australia) and then acetone (UNIVAR Australia), using centrifugation in between the washes to remove the washing solution.

Starch sub-samples of 2g (±0.05) were weighed and prepared for viscoelasticity analysis as outlined in AACC (1995) method 76.21. An aqueous suspension of the starch was heated and stirred in the instrument, causing the starch to gelatinise and form a paste. Paste viscosity (viscoelasticity), was continually monitored during the period of increasing temperature to 95°C and then decreasing temperature to 50°C (Bason, 1996). Maximum viscosity before the onset of cooling (peak viscosity), minimum viscosity after the peak viscosity (holding viscosity), and final viscosity commonly determine as an indication of the pasting properties of grain and hence its processing value for baking and other purposes (Bason, 1996). A RVA model 3D (Newport Scientific, Australia) was used to measure the peak, holding and final viscosity of starch from each cultivar as representative points for characterising the pasting quality of starch for grains (Batey and Curtin, 1996). The results were recorded and reported in rapid visco analyser units (RVU).

### *4.2.4 Determining the amylose content and the amylose : amylopectin ratio in starch isolated from barley, sorghum and wheat*

The Bread Research Institute, Australia Limited, conducted this assay for the Premium Grains for Livestock Program. Approximately 1 to 2g of starch (isolated as described in section 4.2.3) was weighed and defatted using 100ml of 85% (v/v) methanol (UNIVAR Australia) for 16 hours. The starch samples were dried at room temperature for two days and then ground again by an ultra centrifugal miller (ZM1-Retschand, Haan, Germany). The amlyose content of the isolated starch was determined using the method as outlined in AACC (1995) method 61-03, and values were subsequently converted to a dry matter (DM) basis by using their corresponding moisture content (as measured in Section 3.2.1). The amylose content was subsequently used to calculate the ratio of amylose : amylopectin.

### *4.2.5 Statistical analysis*

#### *4.2.5.1 Analysis of variance for starch granule surface area parameters in cereal grains*

Significant differences between grain types and cultivars in the mean, median and total surface area for A type and B type starch granules and in the ratio of the number of B-type to A-type starch granules were determined by ANOVA and LSD (Genstat 4.2).

Significant variations in the viscoelasticity (as described in section 4.2.3) and amylose: amylopectin ratio (as described in section 4.2.4) between grain types were determined by ANOVA and LSD (Genstat 4.2).

#### *4.2.5.2 Regression analysis between the physical and chemical properties of starch granules and their GRI values*

In order to test the first hypothesis, grain cultivars showing significant differences in starch granule surface area were selected to investigate the relationship between starch granule surface area with viscoelasticity and the amylose : amylopectin ratio values. Stepwise linear regression analysis (Genstat 4.2) was used to conduct this analysis. The quartile range and skewness for all variables was calculated to identify any possible outlier values, which could influence the regression analysis.

In order to examine the second hypothesis of the present work, the chemical and physical characteristics of the starch granules (amylose : amylopectin ratio, viscoelasticity and starch surface area) were related to GRI values using stepwise linear regression analysis (Genstat 4.2).

## 4.3 Results

### *4.3.1 Physical characteristics of starch granules*

The physical characteristics of starch granules displayed wide variation between the cultivars and between the grain types (Appendices 4.1, 4.2 and 4.3). The maximum and minimum values for total starch granule area, the mean and median area for A-type and B-type starch granules, as well as the ratio of B-type : A-type starch granules for the different grain types is displayed in Table 4.1. The coefficient of variation (CV%) within each grain type was less than 15% for the mean areas of starch granules, 55% for the ratio of B-type to A-type starch granules and 39% for the total area of starch granules.

The total area of A-type starch granules for sorghum were 3.2- and 4.7-fold higher than for barley and wheat respectively (Table 4.1). Wheat had the highest total area of B-

type starch granules (1.3- and 2.4-fold higher) compared to barley and sorghum respectively.

#### *4.3.1.1 Differences in starch granules between barley cultivars*

The distribution patterns for the surface area of A-type starch granules in barley were skewed, with the majority of starch granules covering an area between 100 and 600$\mu m^2$ (Figure 4.1). The distribution in the surface area of B-type granules was similarly skewed, with the majority displaying an area between 2 and 20 $\mu m^2$ (Figure 4.2).

A significant difference in the total surface area of A-type starch granules between the different barley cultivars was observed ($P<0.01$; Figure 4.3). In contrast, no statistical difference was observed in the total surface area of B-type starch between the barley samples. Statistical analysis of the mean area for A-type starch granules between barley samples showed significant differences ($P<0.001$; Figure 4.4), whereas the mean area for B-type starch granules did not differ.

In barley, the average number of B-type starch granules was 11 times higher than the number of A-type starch granules (1833 B-type starch granules to 167 A-type starch granules), and the ratio of B:A type starch granules varied significantly between the barley cultivars despite the high standard error from variations in the duplicate values ($P<0.05$; Figure 4.5).

**Table 4.1. Maximum and minimum values of starch granule physical characteristics in barley, sorghum and wheat**

| | Barley *(n=18)* | | Sorghum *(n=15)* | | Wheat *(n=10)* | |
|---|---|---|---|---|---|---|
| | ***Range*** | **CV%**[1] | ***Range*** | **CV%**[1] | ***Range*** | **CV%**[1] |
| Mean area for A type SGs[2] ($\mu m^2$) | 211 – 355 | 6.4 | 213 - 301 | 6.6 | 241 - 384 | 10.9 |
| Mean area for B type SGs [3]($\mu m^2$) | 6.7 - 10.3 | 14.1 | 24.3 - 34.3 | 12.7 | 13.4 - 18.1 | 12.0 |
| Ratio of number of B-type : A-type SGs | 3.8 - 21.9 | 7.3 | 0.7 - 3.3 | 55.2 | 9.0 - 12.4 | 31.4 |
| Median areas for A type SGs ($\mu m^2$) | 189 - 340 | 16.5 | 192 - 282 | 8.3 | 204 - 302 | 16.9 |
| Median areas for B type SGs ($\mu m^2$) | 3.5 – 6.8 | 39.5 | 12.6 - 26.4 | 23.2 | 8.6 – 18.9 | 13.4 |
| Total area measured for A type SGs ($\mu m^2$) | 29,357 - 159,179 | 39.5 | 138,951 - 344,050 | 25.4 | 24,931 - 79,287 | 34.2 |
| Total area measured for B type SGs ($\mu m^2$) | 15,649 - 22,237 | 12.2 | 28,365 - 405,050 | 13.5 | 25,544 - 165,543 | 14.1 |

[1] coefficient of variation
[2] A-type starch granules>100$\mu m^2$
[3] B-type starch granules≤100$\mu m^2$

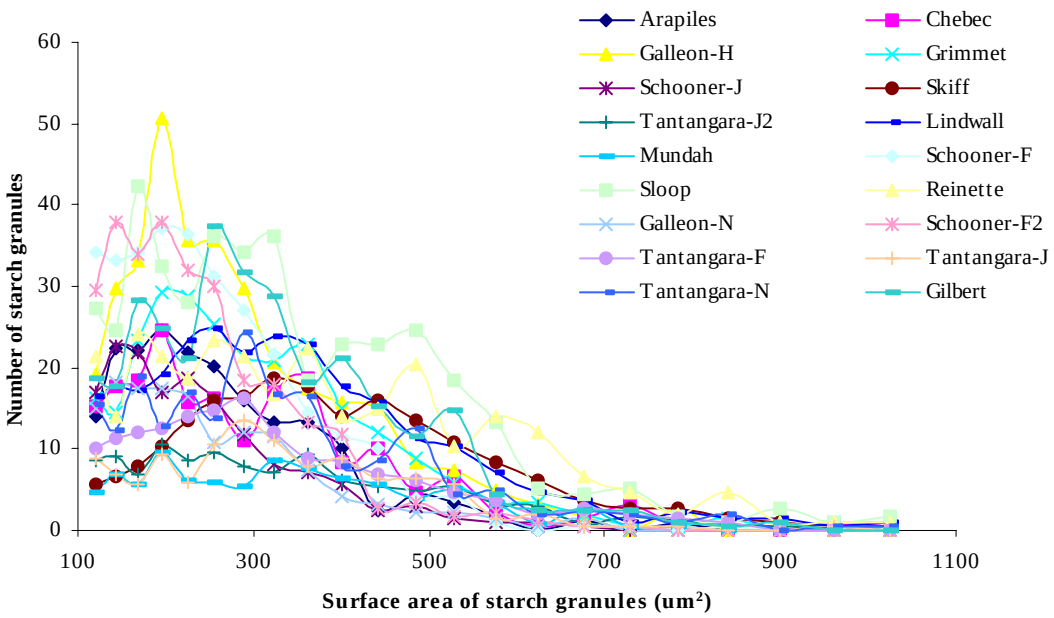



**Figure 4.1 Surface area distribution pattern of A-type starch granules (µm2) in the barley samples.**

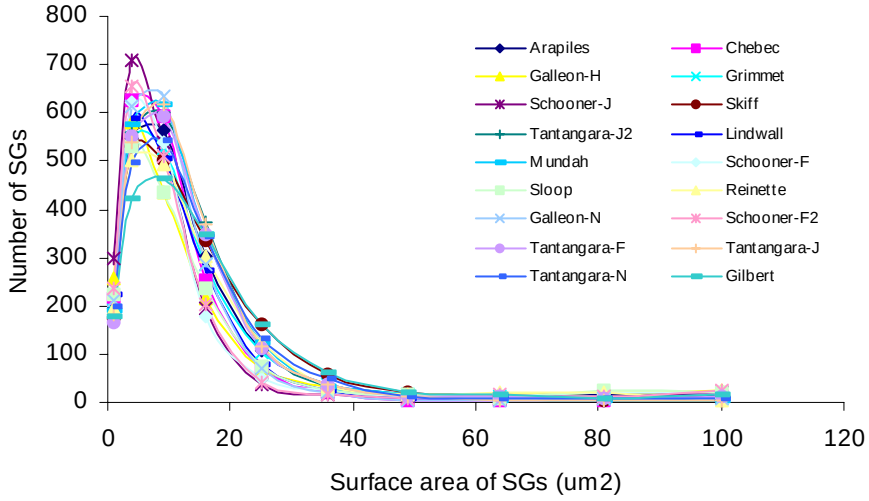



**Figure 4.2 Surface area distribution pattern of B-type starch granules (µm2) in the barley samples.**

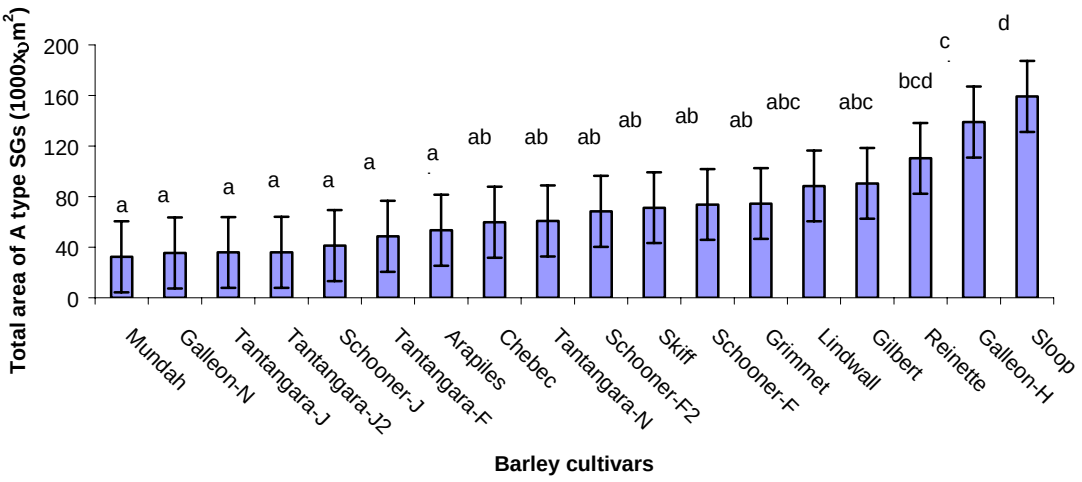



**Figure 4.3 The variation of the total area of A-type starch granules (1000 x μm$^2$) between the barley samples. Bars with different superscripts differ significantly (P<0.008), error bars indicate standard error.**

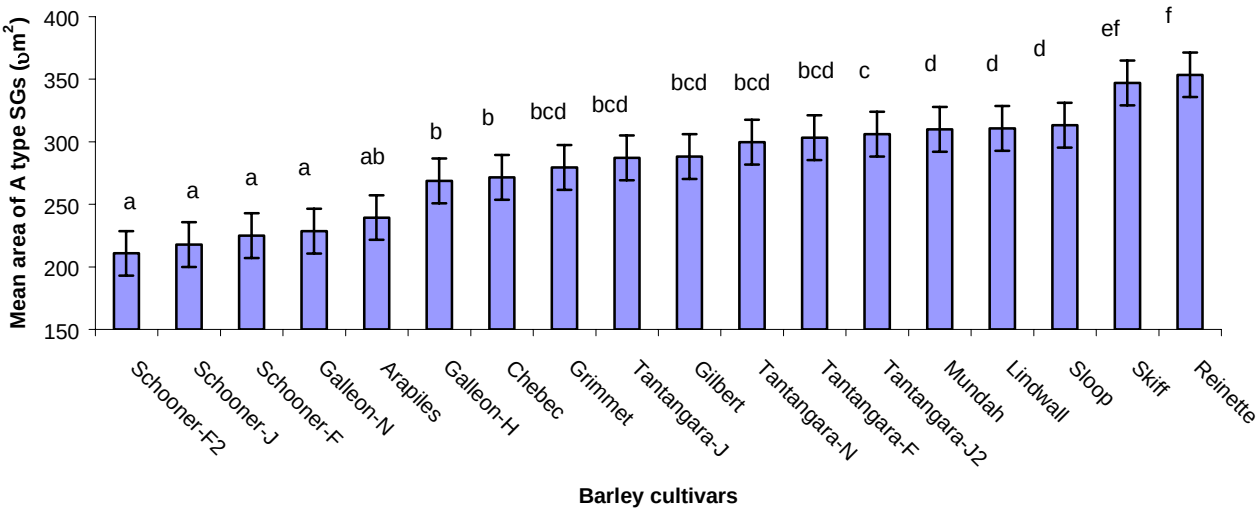



**Figure 4.4 The variation of the mean area of A type starch granules (μm$^2$) between the barley samples. Bars with different superscripts differ significantly (P<0.001), error bars indicate standard error.**

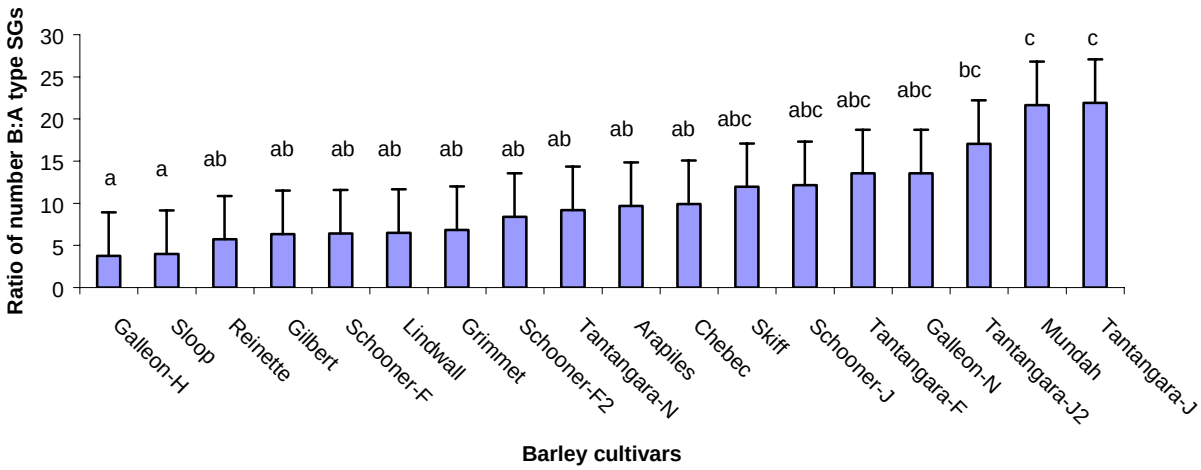



**Figure 4.5 The variation in the number of B type : A type starch granules between the barley samples. Bars with different superscripts differ significantly (P=0.05), error bars indicate standard error.**

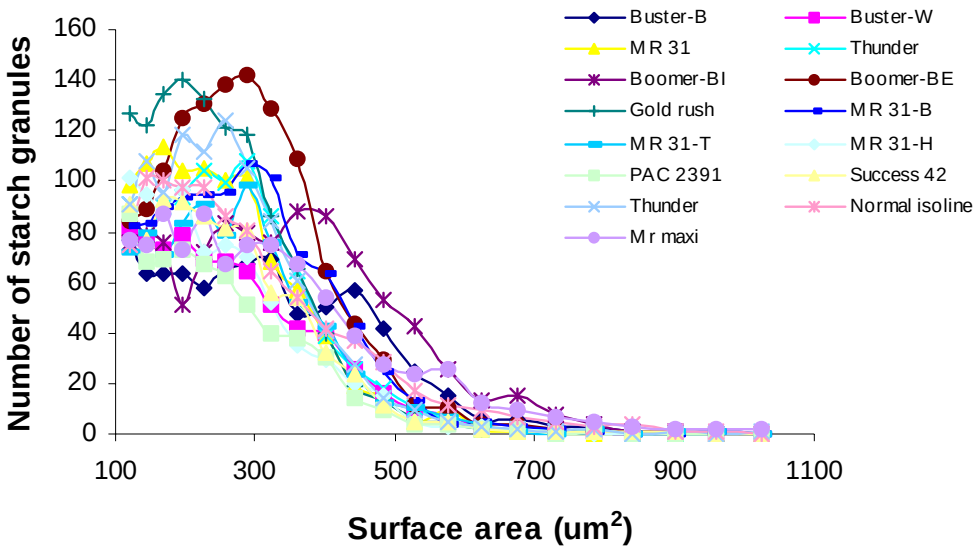



**Figure 4.6 Surface area distribution pattern of A-type starch granules ($\mu m^2$) in the sorghum samples.**

#### 4.3.1.2 *Differences in starch granules between sorghum cultivars*

In sorghum, the distribution of the surface area for A-type starch granules was skewed, with the majority of granules displaying an area between 100 and 500$\mu m^2$ (Figure 4.6). The distribution pattern for B-type starch granule surface area was also skewed, with most values falling between 0.1 and 20$\mu m^2$ (Figure 4.7). The skewness however, was not as pronounced as for the barley samples.

The total surface area of A-type starch granules did not differ significantly between the 15 sorghum samples. However, the mean area of A-type starch granules varied significantly between the samples ($P<0.05$; Figure 4.8). The total area and mean area of B-type sorghum starch granules, as for barley, was not significantly different. The average number of B-type starch granules was 1.5 times higher than the number of A-type starch granules in sorghum (1200 B-type starch granules : 800 A-type starch granules). The ratio of B-type : A-type starch granules did not differ significantly between the samples.

#### 4.3.1.3 *Differences in starch granules between wheat cultivars*

The distribution patterns for the surface area of A-type starch granules in wheat were skewed, with the majority of granules displaying an area between 100 and 300$\mu m^2$ (Figure 4.9). Similarly, the distribution patterns for the surface area of B-type starch granules were also skewed, with most values falling between 1 and 35 $\mu m^2$ (Figure 4.10).

The total area of A-type and B-type starch granules did not differ significantly between the 10 wheat samples. However, the mean area of A-type (but not B-type) starch granules differed significantly between the wheat samples ($P<0.05$; Figure 4.11).

The average number of B-type starch granules was approximately 14 times higher than that of A-type starch granules in the wheat samples (1870 B-type starch granules : 130 A-type starch granules). The ratio of B-type : A-type starch granules did not significantly differ between the samples.

#### 4.3.1.4 *Differences in starch granule characteristics between barley, sorghum and wheat*

The mean area and total area of A-type starch granules differed significantly between barley, sorghum and wheat samples ($P<0.05$, Table 4.2). In sorghum the mean area of A-type starch granules was smaller than in barley and wheat ($P<0.05$, Table 4.2). However the total surface area of A-type starch granules in sorghum was significantly greater than

barley (3.3 fold) and wheat (4.7 fold) ($P<0.05$, Table 4.2) respectively, reflecting the large number of A-type starch granules in sorghum.

In contrast to A-type starch granules, the mean surface area of B-type starch granules in sorghum was larger than in barley (3.6 fold) and wheat (1.9 fold) ($P<0.05$, Table 4.2), however the total surface area of B-type starch granules in wheat samples was 2.4 and 1.3-fold higher than in barley and sorghum, respectively ($P<0.05$, Table 4.2).

The average ratio of B-type to A-type starch granules in barley, sorghum and wheat also showed significant differences ($P<0.05$, Table 4.2). In barley and wheat, the number of B-type starch granules was greater than the number of A-type starch granules. In contrast, the average numbers of B-type and A-type starch granules were almost equal in sorghum.

### *4.3.2 Viscoelasticity of starch isolated from barley, sorghum and wheat*

The peak, holding and final viscosity of starch isolated from barley, sorghum and wheat displayed a wide range both between and within the grains (Appendix 4.4). The differences between the grains types were significant ($P<0.05$). Table 4.3 shows the maximum and minimum values in the ranges of viscosity values obtained for each grain type. Barley cultivars displayed the widest range in peak and final viscosity, whereas the largest range in holding viscosity values occurred with the wheat cultivars.

The average peak and holding viscosity of starch isolated from sorghum samples was significantly higher than for barley (1.4 and 1.5 fold respectively) and wheat (1.3 and 1.4 fold, respectively) cultivars (Figures 4.12 and 4.13, $P<0.001$). Furthermore, the average final viscosity of starch isolated from both sorghum and wheat was significantly higher than for barley (Figure 4.14, $P<0.001$).

### *4.3.3 Amylose: amylopectin ratio of barley, sorghum and wheat*

The amylose: amylopectin ratio between the grain types did not vary significantly ($P>0.5$) (reported in Appendix 4.4).

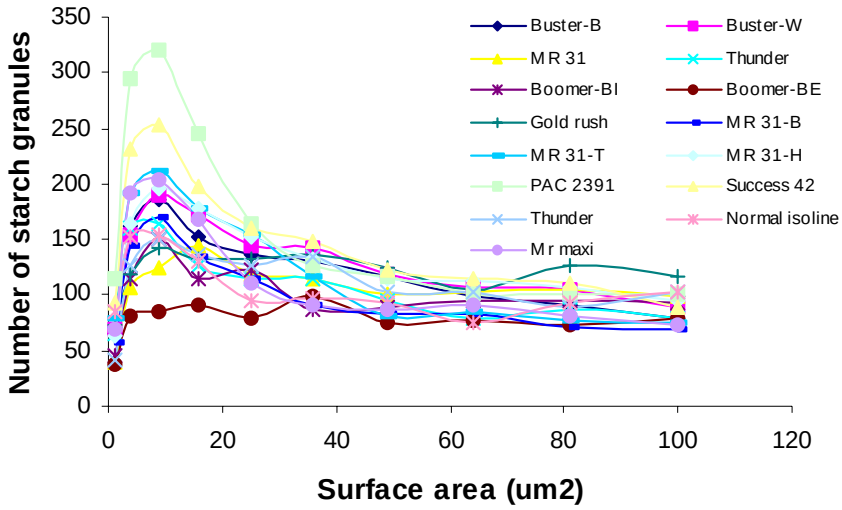



**Figure 4.7 Surface area distribution pattern of B-type starch granules (μm$^2$) in the sorghum samples.**

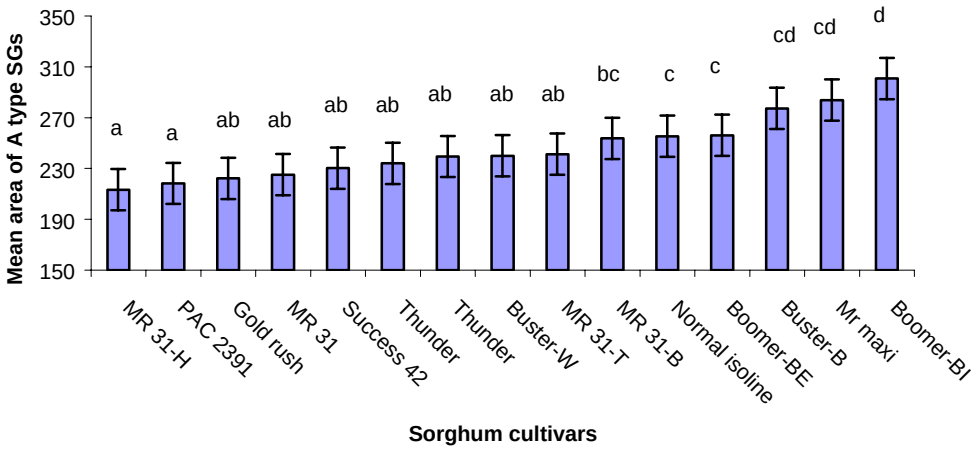



**Figure 4.8 The variation of the mean area of A-type starch granules (μm$^2$) between the sorghum samples. Bars with different superscripts differ significantly ($p<0.005$), error bars indicate standard error.**

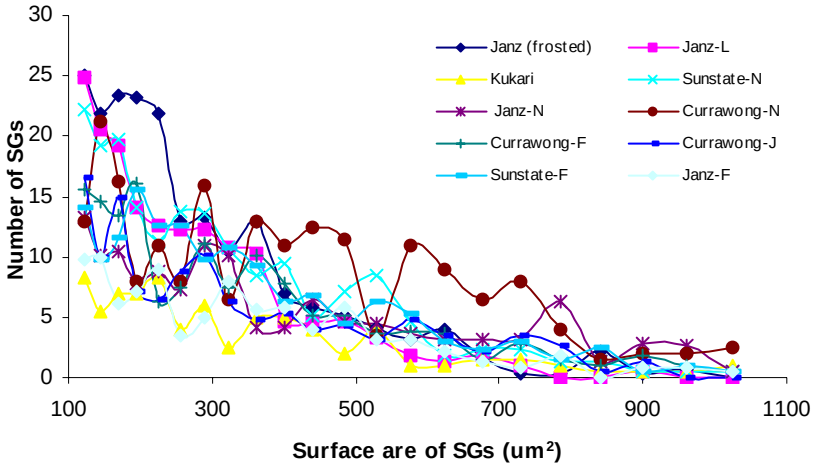



**Figure 4.9 Surface area distribution patterns of A-type starch granules (μm$^2$) in the wheat samples.**

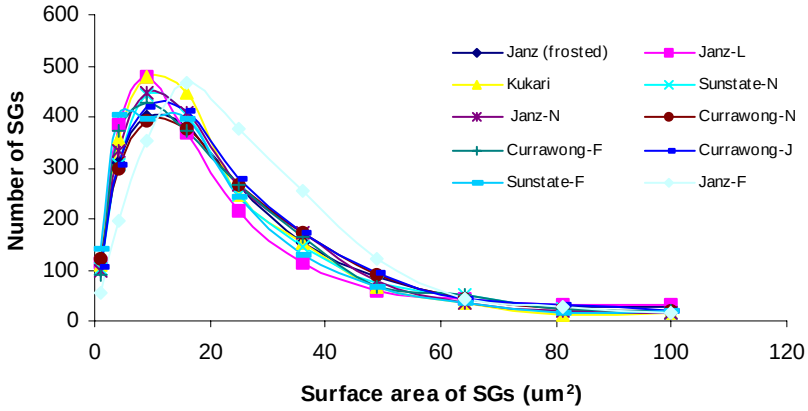



**Figure 4.10 Surface area distribution patterns of B-type starch granules (μm$^2$) in the wheat samples.**

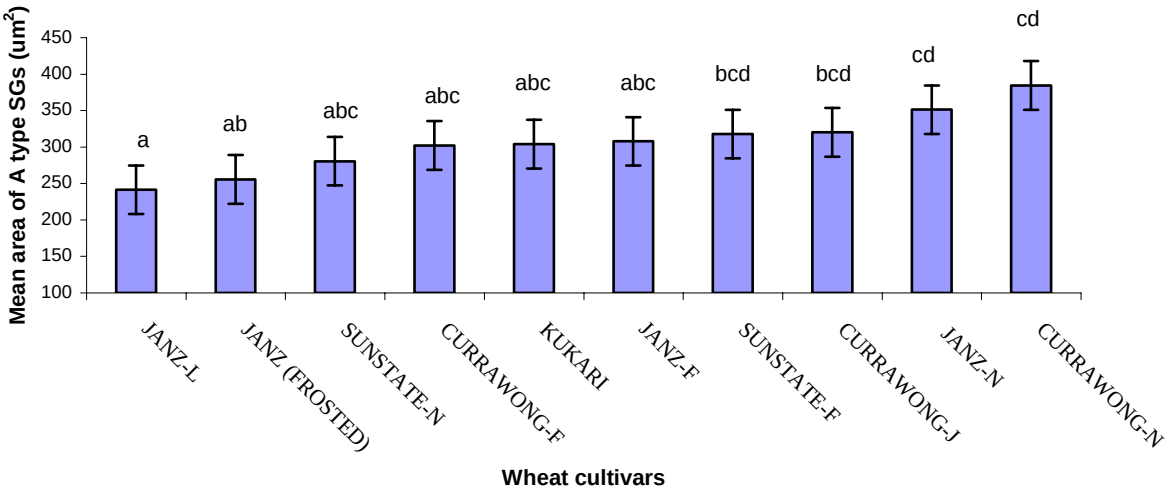



**Figure 4.11 The mean area of A type starch granules ($\mu m^2$) between wheat samples. Bars with different superscripts differ significantly (P<0.05), error bars indicate standard error.**

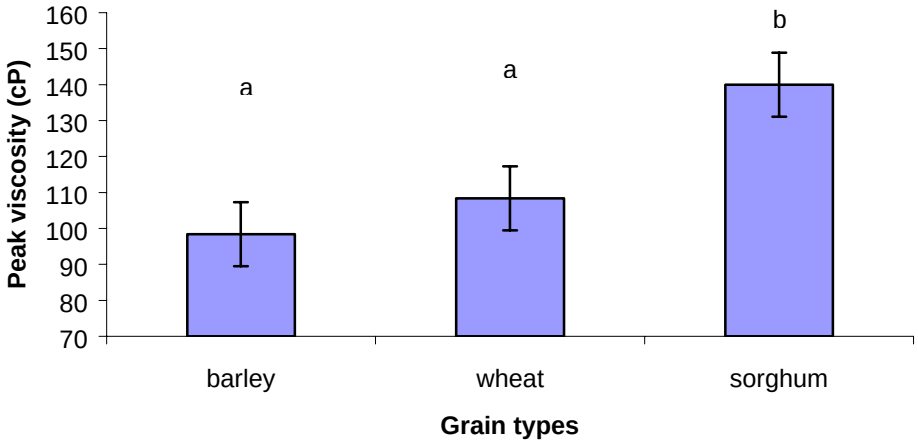



**Figure 4.12 The variation in peak viscosity between barley, sorghum and wheat cereal grains. Bars with different superscripts differ significantly (P<0.001), error bars indicate the standard error.**

**Table 4.2 Comparison of the physical characteristics for the measured 2000 starch granules from each meal sample of barley, sorghum and wheat (values with different superscripts differ significantly ($p<0.05$)).**

| Physical properties | Grain types | | | |
|---|---|---|---|---|
| | Barley (n=18) | Sorghum (n=15) | Wheat (n=10) | SED[2] |
| Mean area of A type SGs[1] ($\mu m^2$) | 281.1 [b] | 246.1 [a] | 306.5 [c] | 10.6 |
| Mean area of B type SGs ($\mu m^2$) | 8.1 [a] | 29.4 [c] | 15.2 [b] | 0.5 |
| Total area for A type SGs ($\mu m^2 \times 10^{-3}$) | 69 [b] | 230 [c] | 48.6 [a] | 10.3 |
| Total area of B type of SGs ($\mu m^2 \times 10^{-3}$) | 18.7 [a] | 35 [b] | 44.3 [c] | 8.3 |
| Ratio of number of B-type : A-type SGs | 10.5 [b] | 1.5 [a] | 14.4 [c] | 1.2 |

[1] Starch granules
[2] Standard error of the difference between means

**Table 4.3 Maximum and minimum values of starch viscoelasticity in barley, sorghum and wheat.**

| Viscoelasticity measurements | Barley | Sorghum | Wheat |
|---|---|---|---|
| Range of the peak viscosity (RVU)[1] | 51-139 | 127-160 | 78-160 |
| Range of the holding viscosity (RVU) | 45-102 | 103-123 | 62-119 |
| Range of the final viscosity (RVU) | 70-165 | 138-179 | 108-201 |

[1] Rapid visco analyser units is equal to 10 mPa.s

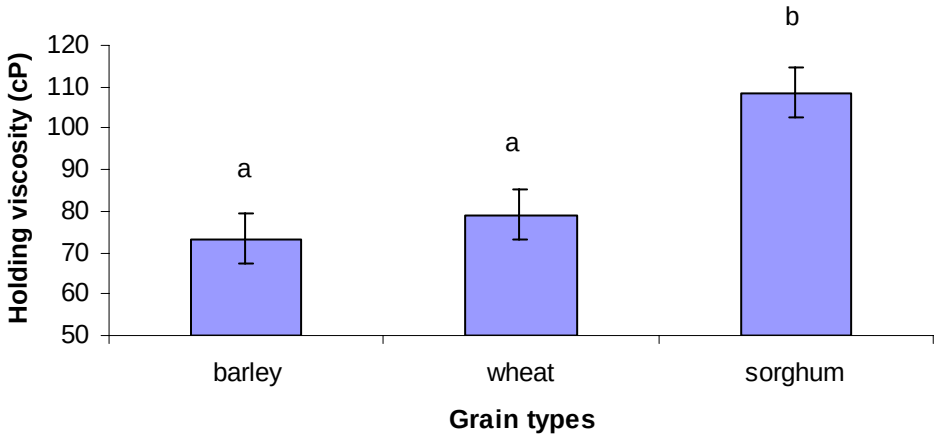



**Figure 4.13 The variation in holding viscosity between barley, sorghum and wheat cereal grains. Bars with different superscripts differ significantly (P<0.001), error bars indicate the standard error.**

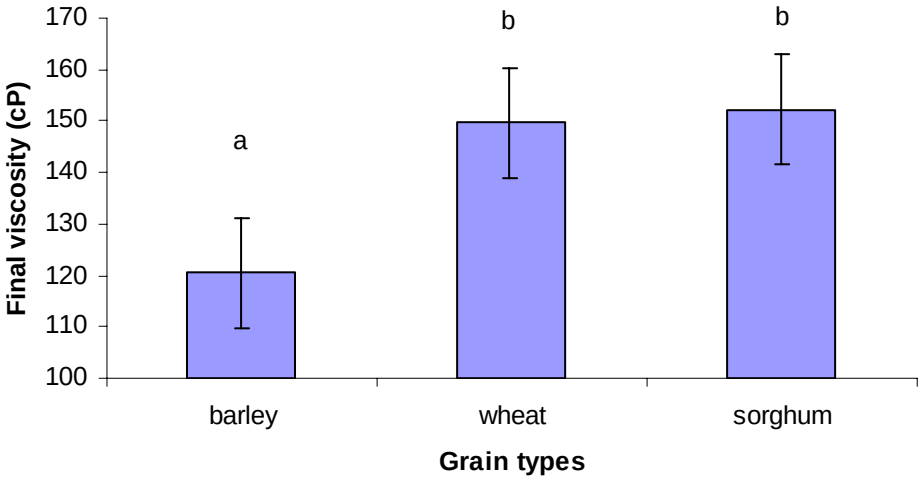



**Figure 4.14 The variation in final viscosity between barley, sorghum and wheat cereal grains. Bars with different superscripts differ significantly (P<0.001), error bars indicate the standard error.**

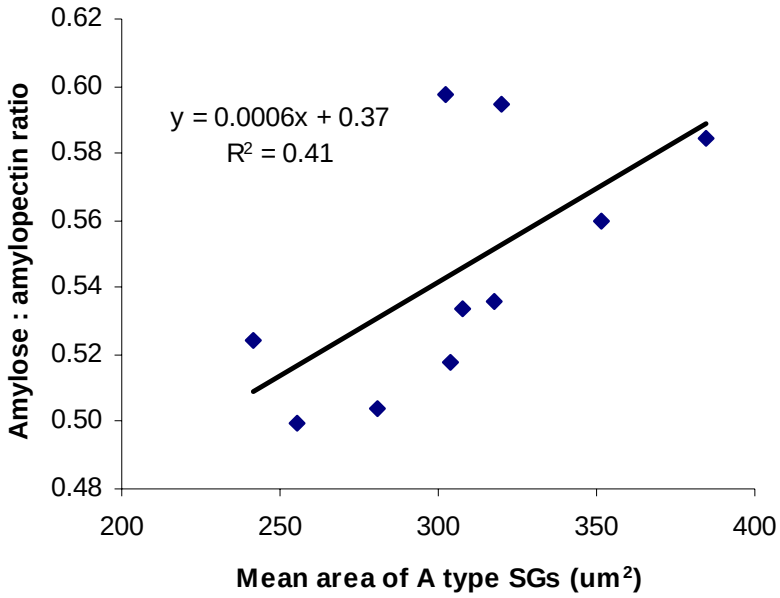



**Figure 4.15 The positive relationship between the mean area of A-type starch granules (SGs) and the percentage of amylose content for the wheat samples (dry matter basis =DM), ($P<0.05$).**

**Table 4.4 Linear regression analysis of the glucose release index values with the chemical and physical characteristics of starch granules in (a) barley, (b) sorghum and (c) wheat.**

a) Barley

| | P | $R^2$ | Equation |
|---|---|---|---|
| Amylose:amylopectin ratio | P<0.05 | 0.35 | Y=-82.03x+79.4 |
| Peak viscosity | P<0.05 | 0.22 | Y=0.09x+25.2 |
| Holding viscosity | P<0.05 | 0.25 | Y=0.14x+23.6 |
| Final viscosity | NS[1] | - | - |
| Total surface area of A-type SGs[2] | NS | - | - |
| Mean surface area of A-type SGs | NS | - | - |
| Ratio of number of A-type : B-type SGs | NS | - | - |

b) Sorghum

| | P | $R^2$ | Equation |
|---|---|---|---|
| Amylose : amylopectin ratio | P<0.05 | 0.39 | Y=-52.6x+56.3 |
| Peak viscosity | NS | - | - |
| Holding viscosity | NS | - | - |
| Final viscosity | NS | - | - |
| Mean surface area of A-type SGs | NS | - | - |

c) Wheat

| | P | $R^2$ | Equation |
|---|---|---|---|
| Amylose : amylopectin ratio | NS | - | - |
| Peak viscosity | NS | - | - |
| Holding viscosity | NS | - | - |
| Final viscosity | NS | - | - |
| Mean surface area of A-type SGs | NS | - | - |

[1]Not significant; P>0.05
[2] Starch granules

### 4.3.4 *Relationship of starch granule size and distribution to the viscoelasticity and amylose :amylopectin ratio in barley, sorghum and wheat*

The relationships between of starch granule size and distribution and their physical (viscoelasticity) or chemical (amylose : amylopectin) characteristics of starch in barley and sorghum were not significant. In contrast, the mean area of A-type starch granules in wheat cultivars displayed a positive linear relationship with the ratio of amylose : amylopectin ($P<0.05$, Figures 4.15) but not to viscoelasticity parameters.

### 4.3.5 *Relationship between the viscoelasticity, starch granules sizes and amylopectin ratio of starch with GRI of barley, sorghum and wheat*

In barley, the ratio of amylose : amylopectin, peak viscosity and holding viscosity all displayed a significant relationship with the GRI (Table 4.4a). There was an inverse relationship between GRI and amylose : amylopectin ratio, whereas the peak and holding viscosity of the samples displayed a positive relationship with the GRI.

In sorghum, the amylose : amylopectin ratio displayed an inverse relationship with the GRI (Table 4.4b), similar to barley, while there was no relationship with viscoelasticity (Table 4.4). Two sorghum samples, Normal Isoline and Mr Maxi, displayed GRI values that were higher than the limit of the quartile range and displayed peak viscosities that were 7 and 15 RVU higher than the upper limit of the quartile range respectively (see Appendices 4.4 for the raw values).

In wheat, no chemical or physical properties of starch were significantly related to the GRI ($P>0.05$; Table 4.4c).

## 4.4 Discussion

These experiments were designed to compare starch granule sizes, amylose : amylopectin ratio and viscoelasticity of starch granules in different cultivars of the same grain type and those between different grain types, with their GRI values, in order to provide insight into the relative importance of each factor towards starch digestibility. The results of this work are discussed in two sections; (1) between cultivars within the same grain type, and (2) between the different grain types.

### *4.4.1 Within grain type*

There was significant variation in the surface area of A-type starch granules within cultivars of barley, sorghum and wheat. This was not however, related to the measured chemical and physical properties of starch in the barley and sorghum samples and is in contrast to results reported by Palmer (1972) who showed a negative relationship between the amylose content and the size of A-type starch granules in barley. It has been suggested that the molecular architecture and composition of amylopectin and not the ratio of amylose to amylopectin may influence starch granule size (MacGregor and Fincher, 1993). Furthermore, the current study showed that the ratio of B type : A type starch granules varied significantly in barley, but did not relate to the chemical and physical properties of starch. Collectively, these results imply that the surface area measurements of starch granules in these barley and sorghum samples are not useful predictors of other physical and chemical properties of starch.

In wheat, regression analysis indicated that A-type starch granule surface area was positively correlated to the ratio of amylose : amylopectin. This finding was similar to the results reported by Gaines *et al.* (2000) who showed that wheat samples with a higher amylose content had larger starch granules. The results reported for wheat in this study lend support to the first hypothesis, that starch granule size can be used to predict its amylose and amylopectin content. Therefore it could be suggested that wheat grains with larger starch granules are digested more slowly than cultivars with smaller granules due to their higher amylose content and smaller surface area to volume ratio. However, the lack of a relationship between starch granule size and GRI values is the current study does not support this conclusion. Similarly in barley and sorghum the starch granule surface area did not show any relationship with GRI values. Therefore, in rejection of the second hypothesis of this study, the variation in the ratio of surface area to volume within grains did significantly not influence their starch digestion.

The highly variable nature of starch granule size, the limitation in the number of duplicate measurements and finally, possible technical error can explain the large CV% and standard error values for some replicates of starch granule size measurements. Therefore in order to improve the accuracy of the current assay, a greater number of sample replicates, improvement of the current histomorphometric technique, or the use of alternative methods such as a Coulter counter (Morrison and Scott, 1986) may be required to strengthen or reject the current results.

In barley and sorghum, results revealed that the GRI values were inversely related to their amylose : amylopectin ratios. This inverse relationship can be explained by considering the structure of amylose. As Bedford (2000a) suggests, amylose displays a tight helical structure and can readily bind to compounds such as lipids. These properties displayed by amylose may depress enzyme accessibility and thus digestion. As a consequence, barley and sorghum samples with a larger amylose : amylopectin ratio have lower starch digestibility.

Viscoelasticity (RVA) and GRI values were positively correlated in barley, but not in wheat or sorghum. Thus, results in this experiment indicate that the variation in starch gelatinisation properties (characterised by the RVA assay) influence GRI in barley but not sorghum or wheat. Furthermore, due to the relationship between the gelatinisation properties and the crystalline structure of starch (Holm *et al.*, 1988; Vasanthan *et al.*, 1995; Fujita *et al.*, 1998; Jacobs and Delcour, 1998), it could also be speculated that differences in the crystalline structure of starch in barley partially determines starch digestibility. Determining the variation in gelatinisation properties of grains could be utilized in order to select an optimal temperature for heat treatment of grains to maximize gelatinisation without subsequent pasting. It is known that when the starch paste cools, starch molecules re-associate in a new ordered structure. This process is referred to as retrogradation of starch (Atwell *et al.*, 1988) and results in the formation of resistant starch (resistant to digestive enzymes such as α-amylase).

### *4.4.2 Between the grain types*

Morphometric data from this work demonstrated that starch granule sizes varied significantly between barley, sorghum and wheat grains. Since the amylose : amylopectin ratio of the different grain types did not vary significantly, this suggests that differences in the size of starch granules between the different grain types may be influenced by variation in starch molecular size and/or its crystalline structure. In support of this view, Nikuni, (1978) and Zobel (1988) indicated that the crystallisation of amylopectin, but not its ratio to amylose, influences the physical characteristics of starch granules. In addition, the significant variation in viscosity between the grain types also supports this notion, since the variation in RVA values is mainly influenced by the crystalline properties of starch molecules (Holmes, 1995; Farhat *et al.*, 1999; Shim and Mulvaney, 1999; Sekine and Horiuchi, 2001).

The surface area to volume ratio of starch granules is an important determinant of enzyme accessibility, and thus starch digestibility. In general the microscopy study indicated that sorghum had a larger number of A-type (large) starch granules compared to barley and wheat. Furthermore, the average surface area of B-type starch granules in sorghum was larger than in barley and wheat. As a consequence, the surface area : volume ratio in sorghum was significantly lower than the other grain types. This could be expected to result in a lower accessibility of digestive enzymes to their starch substrate and thus a decrease in digestibility. The lower GRI in sorghum reported in chapter 3 (section 3.3.3) could be partially due to the lower surface area to volume ratio of starch granules. In contrast, wheat displayed the highest number of B type starch granules, and thus the highest ratio of surface area to volume for its starch granules. Wheat was found to display the highest GRI. These findings are consistent with the second hypothesis of this study, and suggest that the physical characteristics of starch granules influence starch digestibility, at least when comparing between different grain types.

### *4.4.3 Summary*

The current work demonstrates that in barley and sorghum, GRI is related to the amylose : amylopectin ratio of starch, and for barley only, GRI is related to the gelatinisation properties of starch. In wheat, the chemical and physical properties of starch did not show any relationship to their GRI. Thus in wheat, non-starch related factors may influence starch digestibility. The variation of starch granule sizes between but not within grain types influences their starch digestibility. In general, the GRI values in barley and sorghum showed a weak relationship ($R^2 < 0.4$) with their corresponding physical and chemical properties of starch. Therefore similar to wheat, it can be speculated that the non-starch related properties of these grains may play a role in starch digestion.

With regard to non-starch related factors of grains, several studies have demonstrated that NSP composition (cell walls of grains) as well as the protein matrix could influence starch digestibility (refer to sections 2.8.1 to 2.8.6). The NSP in cereal grains have several anti-nutritional properties in the digestive tract of monogastric animals (Iji, 1999; de Lange, 2000). The negative effects of NSP on starch or protein digestion in monogastric animals (particularly in poultry) has been demonstrated (Antoniou and Marquardt, 1981; Campbell *et al.*, 1989; Choct and Annison, 1992c; Choct, 1993; Choct, 1995; Choct *et al.*, 1995; Fuente *et al.*, 1998). The negative influence of the protein matrix surrounding starch

granules on starch digestion in ruminants, but not in monogastric animals, has been documented (Owens *et al.*, 1986; McAllister *et al.*, 1993).

In the following chapters, the physical and chemical properties of the NSP and the protein matrix in cereal grains were determined and then their relationship to the GRI was investigated.

# Chapter 5 Extract viscosity as a predictor of anti-nutritional properties of non-starch polysaccharides in barley, sorghum and wheat for pigs and poultry.

## 5.1 Introduction

Non-starch polysaccharides (NSP) represent a group of heterogeneous compounds that cannot be hydrolysed by the digestive enzymes of monogastric animals (de Lange, 2000). NSP are broadly classified into two groups, soluble and insoluble, although the extent of NSP solubility depends on their extraction conditions such as the pH and temperature (Graham *et al.*, 1988). NSP are mainly located in the cell walls of cereal grains, where they represent one of the major constituents (Fincher and Stone, 1986). The insoluble NSP such as cellulose are located mainly in the hull layer of cereal grains (Section 2.8.1), whereas the soluble NSP such as β-glucan and arabinoxylan are located in the endosperm region (Fincher and Stone, 1986).

The NSP in cereal grains exert several anti-nutritional properties in the digestive tract of monogastric animals (Iji, 1999; de Lange, 2000). Soluble NSP is the more important NSP fraction because it can reduce the digestibility of starch, fat and protein in cereal grains (Choct and Annison, 1992c; Choct *et al.*, 1992; Smits, 1996). However, the extent to which this occurs in pigs has not been firmly established (Graham, 1991; van Barneveld, 1999a). Insoluble NSP may also have a negative impact on the digestion and absorption of feed grain nutrients in both pigs (Taverner and Farrell, 1981; Vervaeke *et al.*, 1989; Baidoo and Liu, 1998; van Barneveld, 1999b; Bach Knudsen and Canibe, 2000; de Lange, 2000) and poultry (Mraz *et al.*, 1957).

The mechanisms by which cereal grain soluble and insoluble NSP exert their anti-nutritional properties are complex, but mainly result in reducing or inhibiting substrate breakdown by endogenous digestive enzymes (Smits and Annison, 1996; de Lange, 2000). Their proposed mechanisms of action can be summarised as follows:

I. Insoluble NSP such as cellulose present in cell walls of grains can act as a physical barrier to the digestive enzymes, inhibiting their accessibility to substrates (Black, 2000). Insoluble NSP can also decrease the retention time of chyme in intestinal tract thereby reducing the exposure time of feed to digestive enzymes (Mraz *et al.*, 1957; Owen and

Ridgman, 1967). Furthermore, the high water holding capacity of insoluble NSP can increase the bulk density of the digesta, and thus reduce voluntary feed intake by animals (Brouns *et al.*, 1991; Choct and Cadogan, 2001; Partridge, 2001).

II. Soluble NSP can increase the viscosity of digesta, and in turn, decrease enzyme accessibility by impairing diffusion of the digestive enzymes to their substrates (Antoniou and Marquardt, 1981). It has also been shown that increasing the soluble NSP content in animal diets could mediate microbial overgrowth in the intestinal system (Choct *et al.*, 1996; Smits and Annison, 1996; Langhout, 1998), leading to a possible negative influence on digestion and utilisation of grains. NSP, especially the soluble type, may also form molecular complexes with digestive enzymes that could inhibit the degree of enzyme activity (Story and Kritchevsky, 1976; Story, 1986; Coles *et al.*, 1996).

It has been hypothesised that variations in both chemical (e.g. chemical composition) and physical (eg. viscosity) characteristics of NSP within grains, can contribute to differences in cereal grain nutritional quality (Hughes and Choct, 1999; van Barneveld, 1999a). It can also be expected that the GRI of grains may be influenced by the soluble or insoluble NSP content since NSP can affect enzyme accessibility and thus function. The analytical methods that are currently used to study NSP chemical composition such as crude fibre, acid detergent fibre and neutral detergent fibre, are basic and inadequate for within and between grain analysis (de Lange, 2000). Therefore, it has been suggested that using a more precise analytical method such as analysis of NSP chemical composition using the alditol assay (Englyst and Cummings, 1988), in combination with other analytical procedures such as determining the extract viscosity or water holding capacity of NSP, should be considered (Smits and Annison, 1996; de Lange, 2000). This type of information could provide a better understanding of the anti-nutritional properties of NSP in grains, and lead to the development of an assay for assessing grain nutritional quality.

Extract viscosity is a qualitative rather than quantitative measurement and provides some information on the molecular structure of the NSP. Indeed it has been demonstrated that the extract viscosity values of grains could be used as predictors of anti-nutritional properties of NSP in cereals for pigs and poultry (Rotter *et al.*, 1989; Choct and Annison, 1992a; Bedford and Classen, 1993; van Barneveld *et al.*, 2001). The viscous properties of NSP depend on several factors, including their level and chemical composition (especially the soluble NSP content), NSP molecular size, degree of NSP branching, the presence of charged groups and the composition of the extraction media (i.e. pH) (Smits and Annison, 1996). The hypotheses tested in the following experiments were that the:

1. Increase in the NSP contents of grains negatively correlates to their GRI values.
2. Grain extract viscosity increases with increasing soluble NSP content.

In order to test these hypotheses the following objectives were addressed to:

1. Investigate the relationship of NSP chemical composition (determined by using the standard alditol acetate method) in barley, sorghum and wheat with their GRI values.
2. Develop a rapid extract viscosity method in order to assess the variation in the molecular structure of NSP in grains.
3. Investigate the relationship between extract viscosity in barley, sorghum and wheat with the different types of chemical composition content of NSP.

## 5.2 Materials and Methods

### *5.2.1 Sample selection and preparation*

Wheat, barley and sorghum samples were selected and prepared as described in Section 3.2.1. Approximately 20g of each grain was milled by an ultra centrifugal grinder (Retsch, ZM1, Germany) fitted with a sieve size of 0.5mm. Samples were milled within 24 hours of commencing experiments to minimise the degradation of NSP by endogenous enzymes.

### *5.2.2 Determining the composition of NSP in barley, sorghum and wheat*

The NSP compositional analysis was conducted by the School of Rural Sciences and Agriculture at the University of New England, for the Premium Grains for Livestock Project. The soluble and insoluble NSP profile of milled barley, sorghum and wheat grains (as prepared in section 5.2.1) was determined using the standard alditol acetate gas chromatography method as described by Englyst and Hudson (1993) and Theander and Westerlund (1993). The NSP values were expressed as a percentage of total grain mass (air-dried) and then converted to percentage DM by using their known moisture content (Section 3.2.1).

### *5.2.3 Determining extract viscosity in barley, sorghum and wheat*

As it indicated in the introduction (section 5.1) the viscosity of NSP depends on several factors such as the pH condition of media. Along the gastrointestinal tract the pH

condition varies approximately from pH 1 to 7. In the following sections, two sets of pH conditions (pH 1.5 and 7) were used to determine the grain extract viscosity.

#### *5.2.3.1 Determining viscosity in barley, sorghum and wheat acid extracts*

Duplicates of 300mg freshly-ground grains (Section 5.2.1) were weighed into 100ml Pyrex tubes (AdeLab, Australia). In each tube, 5ml of 80% (v/v) ethanol (UNIVAR Australia) was added and then tubes were briefly vortexed. The samples were incubated in a shaking water bath at 80°C for 15 min, in order to deactivate the endogenous NSP-degrading enzymes. Following this, the tubes were centrifuged at 2500*g* for 10min (Megafuge 1.0R, Heraeus Instruments, Germany) and the supernatants discarded.

An acidic extract buffer with a pH of 1.5 was made with 83 ml concentrated HCl (UNIVAR Australia) and 7.4g of KCl (Ajax Chemicals Australia), and the total volume adjusted to 1000ml with distilled water. To each grain sample, 2ml of the acid extract buffer was added. Following a brief vortex, the grains were incubated in a 40°C shaking waterbath for 15min followed by centrifugation at 2500*g* for 10min at 25°C. Immediately a 0.5ml aliquot of supernatant from each sample was pipetted into a sample cup of a cone/plate viscometer (Brookfield DV-III, Cone CP-40- USA) and the viscosity of the acidic extract was measured at 25°C. The viscosity values are reported in the units of milli Pascal seconds (mPa.s).

#### *5.2.3.2 Determining viscosity in barley, sorghum and wheat water extracts*

Duplicates of 2g freshly-ground grains (Section 5.2.1) were weighed into 100ml Pyrex tubes and 10ml of 80% (v/v) ethanol was added to each sample. The tubes were briefly vortexed and the samples were incubated in a shaking waterbath at 80°C for 15 min in order to deactivate the endogenous NSP-degrading enzymes.

Following centrifugation at 2500*g* for 10min, the supernatant was discarded and 3ml of distilled water were added to each grain followed by a brief vortex. The mixture was incubated in a 40°C shaking waterbath for 2 hours with intermittent vortexing (approximately every 15min). The samples were centrifuged once again at 2500*g* for 10min and 0.5ml aliquots of supernatant were immediately pipetted from each tube into a sample cup of a cone/plate viscometer. The viscosity of the water extracts were measured at 25°C and the values reported in the units of mPa.s.

### *5.2.4 Determining the composition of NSP in selected barley acid extract residues*

Five barley samples displaying a broad range in their viscosity values for the acid extracts, were chosen for this investigation. The samples Grimmet, Tantangara-N and Galleon-N cultivars displayed the highest viscosity whereas Sloop and Gilbert displayed the lowest viscosity.

Acid extracts of milled barley grains were prepared as outlined in Section 5.2.3 using 3g of milled barley sample, 10ml of ethanol and 10ml of the acidic extraction buffer. The supernatant from the acid extract (approximately 10ml from each barley sample) was transferred into a 50ml plastic container (AdeLab, Australia), frozen overnight at -18°C and then freeze-dried.

The resulting residues of these barley acid extracts were termed "acid extract residues", and their NSP composition was analysed as described in Section 5.2.2.

### *5.2.5 Statistical analysis*

The relationship of the NSP chemical composition with their corresponding GRI values (as reported in Section 3.3.2 of chapter 3) was investigated by stepwise linear regression analysis (Genstat 4.2).

The variation in viscosity values of acid and water extracts between cultivars within barley, sorghum and wheat was analysed by ANOVA and LSD (Genstat 4.2). The relationship between the acid and water extract viscosity values for each grain type was investigated by simple linear regression analysis (Genstat 4.2).

The relationship of viscosity values from the acid or water extracts (which showed significant variation for each grain type) to their corresponding soluble and insoluble NSP values was investigated by stepwise linear regression analysis (Genstat 4.2).

## 5.3 Results

### *5.3.1 The chemical composition of soluble and insoluble NSP in barley, sorghum and wheat*

The soluble and insoluble NSP composition data for each barley, sorghum and wheat cultivar analysed are presented in Appendices 5.1, 5.2 and 5.3, respectively. Table 5.1

displays the minimum, maximum and average values for insoluble and soluble NSP composition for each grain type.

A wide range in the composition of insoluble and soluble NSP within and between each grain type was observed (Table 5.1). The largest range in the total insoluble NSP values was displayed by wheat (7.2 to 13.1% of grain DM), followed by barley (9.5 to 14.2% of grain DM), and then sorghum (4.0 to 7.7% of grain DM). For the soluble NSP, the largest range in values was displayed by barley (0.8 to 6.6% of grain DM), followed by wheat and sorghum, (1.5 to 1.9% and 0.3 to 0.6% of grain DM respectively). The sum of the total soluble NSP components was highest in barley, being 11.8-fold higher than sorghum and 2.9-fold higher than wheat. Sorghum displayed the largest ratio of insoluble to soluble NSP content (13.3:1), followed by wheat (5.8:1) and barley (2.4:1).

Analysis of the average NSP values between the grain types showed that insoluble arabinoxylan accounted for the majority of insoluble NSP in barley, sorghum and wheat, followed by cellulose and other minor insoluble NSP components. The sum of the insoluble NSP components was highest in barley samples, being 2.3-fold higher than in sorghum and 1.3-fold higher than in wheat. The β-glucan accounted for the majority of soluble NSP in barley, sorghum and wheat, followed by soluble arabinoxylan and other minor soluble NSP components.

### *5.3.2 Relationship of the GRI with the soluble and insoluble NSP composition in barley, sorghum and wheat*

The GRI values of barley did not correlate to NSP composition (Table 5.2). In sorghum, Mr Maxi and Normal Isoline cultivars were removed prior to the analysis as these samples displayed high GRI values that fell outside the quartile range when plotted against soluble and insoluble NSP in sorghum. A representative example of the relationship between the GRI and total insoluble NSP in sorghum is shown (Figure 5.1). Removal of the outlier samples in sorghum showed that, similar to barley, the GRI did not show any correlation with the NSP composition (Table 5.2).

In wheat samples, the GRI showed a positive linear relationship with the cellulose content ($P=0.052$, $r^2=0.39$ & Table 5.2), however, the significant correlation was due to the presence of one sample (Janz -frosted), which displayed the highest cellulose content and a large GRI value (Figure 5.2). No other significant correlations were observed between the GRI in wheat and other NSP (Table 5.2).

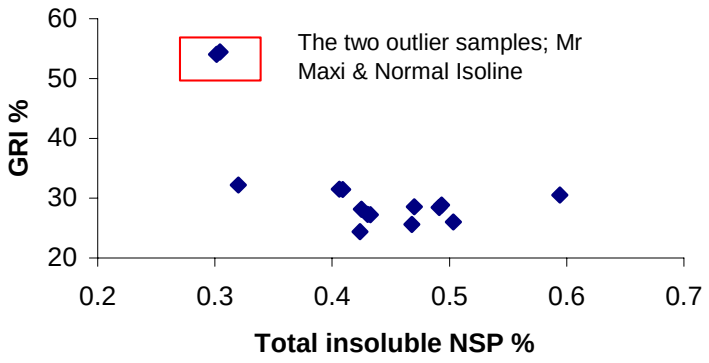



**Figure 5.1 The relationship between total insoluble non-starch polysaccharides (NSP) (determined by using the standard acetate alditol method) and their glucose release index (GRI) values in sorghum. The two outlier samples (boxed in red) were removed prior to stepwise linear regression analysis.**

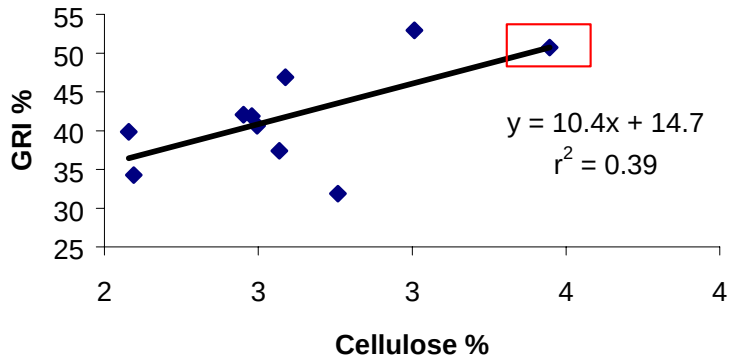



**Figure 5.2 The positive linear relationship between the cellulose content and the glucose release index (GRI) in wheat. The relationship between cellulose and GRI (P=0.052) was significant only when to the Janz-frosted sample (boxed in red) was included in the linear regression analysis.**

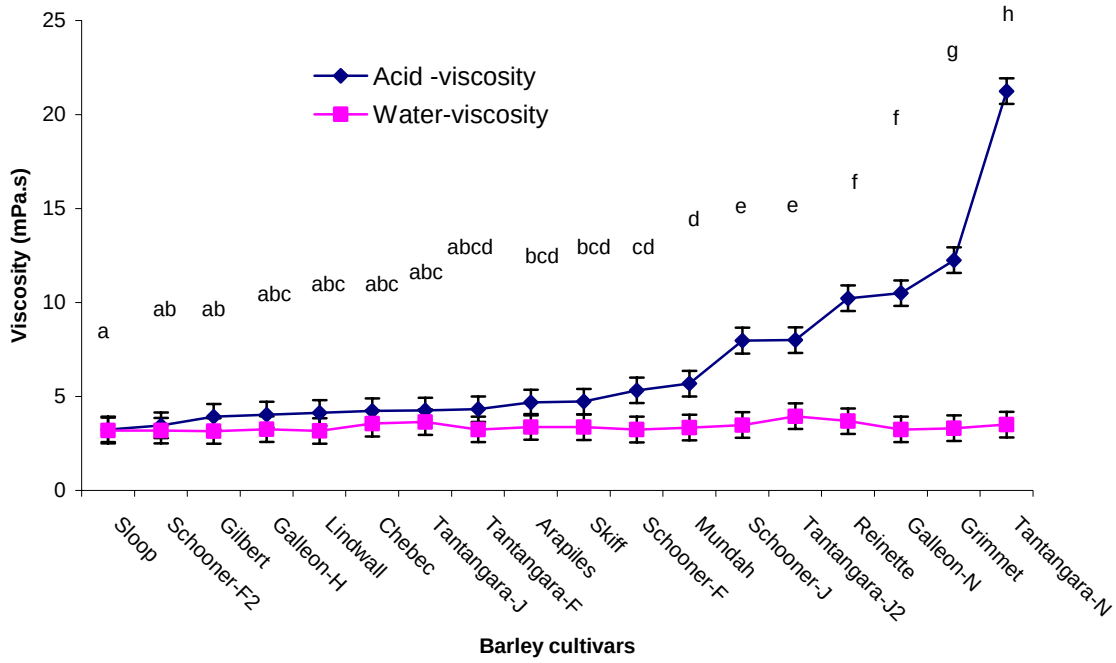



**Figure 5.3 The range in viscosity values for water and acid extracts in barley grains. The acid extract viscosity values with different superscripts vary significantly (P<0.001), error bars indicate standard error.**

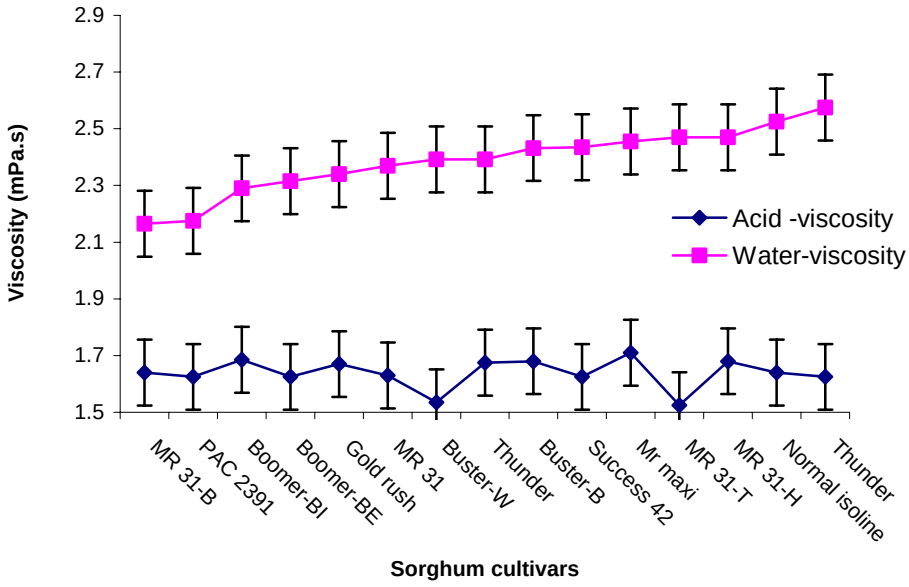



**Figure 5.4 The range in viscosity values for water and acid extracts in sorghum. Error bars indicate standard error.**

**Table 5.1 The range in insoluble and soluble non-starch polysaccharide (NSP) composition, in barley, sorghum and wheat samples.**

| NSP classification | NSP composition [1] (DM)[3] | Barley | | | Sorghum | | | Wheat | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Min | Max | Average | Min | Max | Average | Min | Max | Average |
| Insoluble NSP | Arabinoxylan % | 5.5 | 8.1 | 7.0 | 1.5 | 3.4 | 2.5 | 4.7 | 8.9 | 6.3 |
| | Cellulose % | 3.2 | 5.7 | 4.3 | 1.5 | 3.8 | 2.4 | 2.1 | 3.4 | 2.6 |
| | Other [2] % | 0.5 | 0.8 | 0.6 | 0.3 | 0.8 | 0.4 | 0.4 | 0.7 | 0.4 |
| | Total insoluble % | 9.5 | 14.2 | 12.0 | 4.0 | 7.7 | 5.3 | 7.2 | 13.1 | 9.3 |
| Soluble NSP | Arabinoxylan % | 0.1 | 0.6 | 0.4 | 0.0 | 0.1 | 0.1 | 0.5 | 0.8 | 0.6 |
| | β-Glucan % | 2.9 | 5.7 | 4.3 | 0.1 | 0.3 | 0.2 | 0.7 | 1.0 | 0.8 |
| | Other % | 0.1 | 0.3 | 0.2 | 0.1 | 0.2 | 0.1 | 0.3 | 0.3 | 0.3 |
| | Total soluble % | 0.8 | 6.6 | 4.7 | 0.3 | 0.6 | 0.4 | 1.5 | 1.9 | 1.6 |
| | **Total NSP%** | **12.0** | **19.6** | **16.6** | **4.4** | **8.2** | **5.7** | **9.0** | **15.0** | **11.0** |

[1]Values are presented as a percentage of total weight of grain samples (dry matter)
[2]NSP components exist as minor heteropolyers like xyloglucans, arabinogalactans etc.
[3]Dry matter

**Table 5.2 The relationship between the non-starch polysaccharide (NSP) composition of barley, sorghum and wheat with their glucose release index values (GRI).**

| NSP classification | NSP composition | NSP vs GRI in barley | NSP vs GRI in sorghum | NSP vs GRI in wheat |
|---|---|---|---|---|
| | | P value, n=18 | P value, n=13 | P value, n=10 |
| Insoluble NSP | Arabinoxylan | 0.33 NS | 0.70 NS [2] | 0.28 NS |
| | Cellulose | 0.11 NS | 0.64 NS | **0.05*** |
| | Other[1] | 0.39 NS | 0.42 NS | 0.86 NS |
| | Total insoluble | 0.16 NS | 0.45 NS | 0.12 NS |
| Soluble NSP | Arabinoxylan | 0.19 NS | 0.48 NS | 0.19 NS |
| | β-Glucan | 0.81 NS | 0.45 NS | 0.9 NS |
| | Other | 0.31 NS | 0.6 NS | 0.1 NS |
| | Total soluble | 0.69 NS | 0.43 NS | 0.38 NS |

[1]NSP components exist as minor heteropolyers like xyluglocans, arabinogalactans etc.
[2] Non significant; P>0.05
* Significant (P<0.05)

### 5.3.3 *Extract viscosity and its relationship to the NSP composition in barley, sorghum and wheat*

In barley, the viscosity values of the acid extract differed significantly between the sample cultivars, ranging from 3.2 to 21.2 $mPa.s^{-1}$ ($P<0.001$; Figure 5.3). In contrast, no significant difference was observed in the viscosity values of barley water extracts (Figure 5.3).

In sorghum, there were no differences between cultivars in the viscosity values of either the acid or water extracts (Figure 5.4). In wheat, however, the viscosity values within the water and acid extracts of samples differed significantly ($P<0.001$, Figure 5.5). The water extract viscosity values ranged from 4.6 to 8.0 mPa.s, whereas for the acid extracts, the viscosity of only one sample (Sunstate-F) was responsible for the observed variation.

The acid and water extract viscosity values did not correlate with each other in barley, sorghum or wheat ($P>0.05$).

The average viscosity value for the acid extracts in barley was 2-fold higher than the corresponding average viscosity values of water extracts ($P<0.001$; Figure 5.6). In contrast, the average viscosity values of water extracts in sorghum and wheat were approximately 2-fold higher than the acid extracts ($P<0.001$, Figure 5.6). The average viscosity of the acid extracts in barley was 4.1- and 2.4-fold higher than sorghum and wheat, respectively, while the average viscosity value of water extracts in wheat was approximately 2-fold higher than sorghum and barley ($P<0.001$, Figure 5.6).

Regression analysis of the NSP content within barley and wheat (data reported in Appendixes 5.1 to 5.3) and their extract viscosity values (data presented in Figures 5.3 and 5.5) did not show any significant correlation (Table 5.3). In sorghum, regression analysis was not conducted due to the absence of significant variation in the viscosity values of water and acid extracts between samples (Figure 5.4). Similarly in barley, regression analysis was not conducted for the viscosity values of water extracts due to the absence of significant variation between barley samples (Figure 5.3)

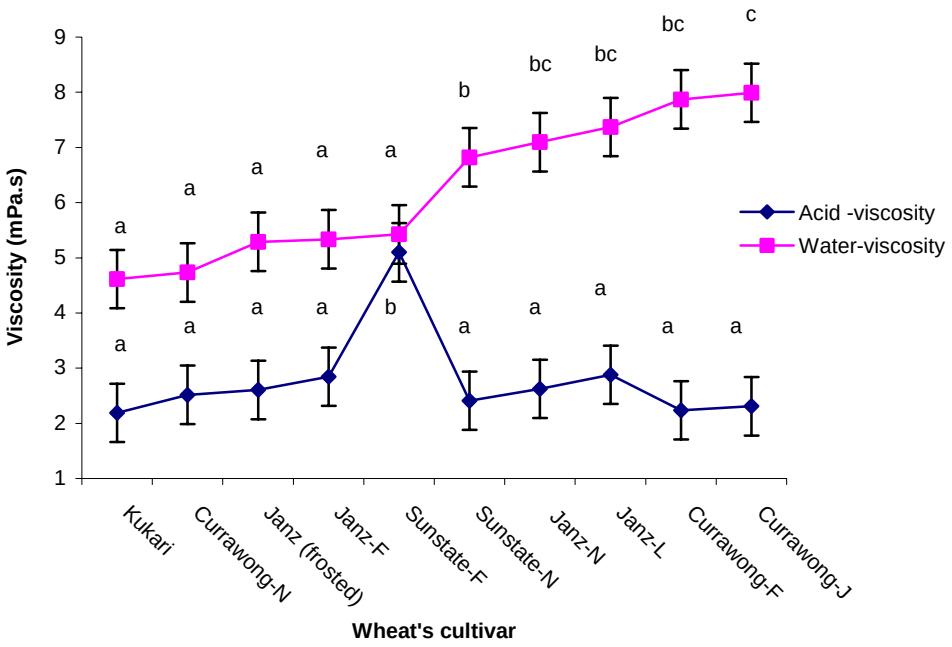



**Figure 5.5 The range in the viscosity values for water and acid extracts in wheat. The acid and water extract viscosity values with different superscripts vary significantly (P<0.001), error bars indicate standard error.**

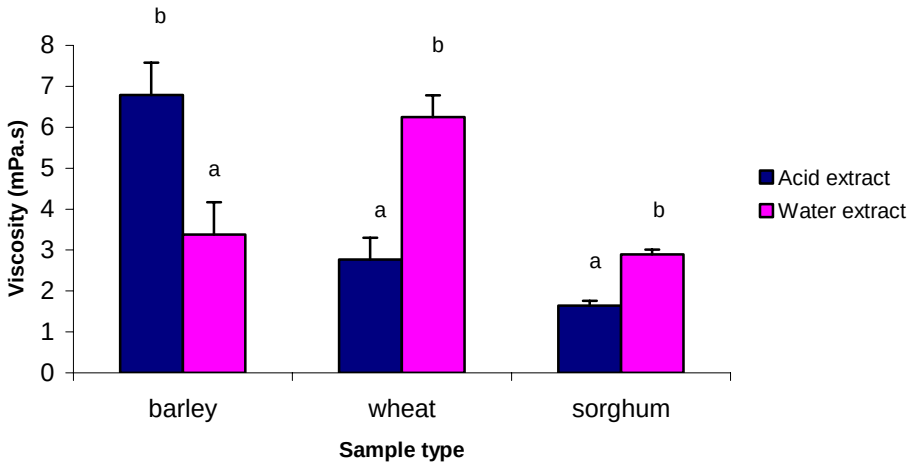



**Figure 5.6 The average viscosity values of water and acid extracts in barley, sorghum and wheat. For each grain type, the acid and water extract viscosity values with different superscripts vary significantly (P<0.001), error bars indicate standard error.**

**Table 5.3 The relationship between the non-starch polysaccharide (NSP) composition in barley and wheat and their viscosity values.**

| NSP classification | NSP composition | NSP vs viscosity of acid extract in barley | NSP vs viscosity of acid extract in wheat | NSP vs viscosity of water extract in wheat |
|---|---|---|---|---|
| | | P value, n=18 | P value, n=13 | P value, n=10 |
| Insoluble NSP | Arabinoxylan | 0.18 NS[2] | 0.68 NS | 0.85 NS |
| | Cellulose | 0.50 NS | 0.76 NS | 0.42 NS |
| | Other [1] | 0.50 NS | 0.52 NS | 0.63 NS |
| | Total insoluble | 0.19 NS | 0.92 NS | 0.48 NS |
| Soluble NSP | Arabinoxylan | 0.64 NS | 0.58 NS | 0.99 NS |
| | β-Glucan | 0.91NS | 0.93 NS | 0.62 NS |
| | Other | 0.92 NS | 0.20 NS | 0.79 NS |
| | Total soluble | 0.46 NS | 0.78 NS | 0.82 NS |

[1] NSP components exist as minor heteropolyers like xyluglucans, arabinogalactans etc.
[2] Non significant; P>0.05

**Table 5.4 Composition of non-starch polysaccharides (NSP) in the acid extract residues of barley.**

| NSP classification | Barley cultivars | %Rhamnose | %Fucose | %Ribose | %Arabinose | %Xylose | %Mannose | %Galactose | %Glucose | %Total NSP |
|---|---|---|---|---|---|---|---|---|---|---|
| Insoluble NSP | Grimmet | - | - | - | - | - | - | - | 0.4 | 0.4 |
| | Sloop | - | - | - | - | - | - | - | 0.1 | 0.1 |
| | Galleon-N | - | - | - | - | - | - | - | 0.5 | 0.5 |
| | Tantangara-N | - | - | - | - | - | - | - | 1.0 | 0.9 |
| | Gilbert | - | - | - | - | - | - | - | 0.2 | 0.1 |
| Soluble NSP | Grimmet | 0.0 | 0.0 | 0.0 | 0.5 | 0.3 | 0.1 | 0.2 | 4.8 | 5.3 |
| | Sloop | - | - | 0.0 | 0.4 | 0.3 | 0.1 | 0.2 | 2.2 | 2.8 |
| | Galleon-N | 0.0 | - | 0.0 | 0.5 | 0.4 | 0.1 | 0.2 | 4.2 | 4.8 |
| | Tantangara-N | - | - | 0.0 | 0.5 | 0.4 | 0.1 | 0.2 | 5.3 | 5.9 |
| | Gilbert | 0.0 | 0.0 | 0.0 | 0.5 | 0.4 | 0.0 | 0.2 | 4.3 | 5.0 |
| Total NSP | Grimmet | 0.0 | 0.0 | 0.0 | 0.5 | 0.3 | 0.1 | 0.2 | 5.2 | 5.7 |
| | Sloop | - | - | 0.0 | 0.4 | 0.3 | 0.1 | 0.2 | 2.3 | 2.9 |
| | Galleon-N | 0.0 | - | 0.0 | 0.5 | 0.4 | 0.1 | 0.2 | 4.7 | 5.2 |
| | Tantangara-N | - | - | 0.0 | 0.5 | 0.4 | 0.1 | 0.2 | 6.4 | 6.9 |
| | Gilbert | 0.0 | 0.0 | 0.0 | 0.5 | 0.4 | 0.0 | 0.2 | 4.5 | 5.1 |

NB. NSP values are presented as a proportion of total acid extract residue weight

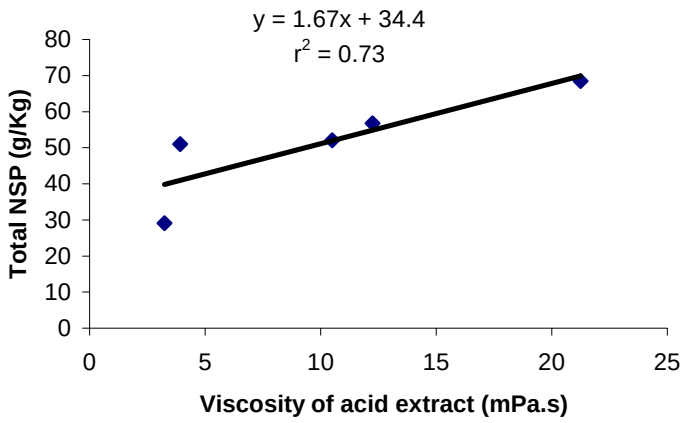



**Figure 5.7 The positive trend between the viscosity values of the acid extract residue and the total non-starch polysaccharides (NSP) in barley (P=0.065).**

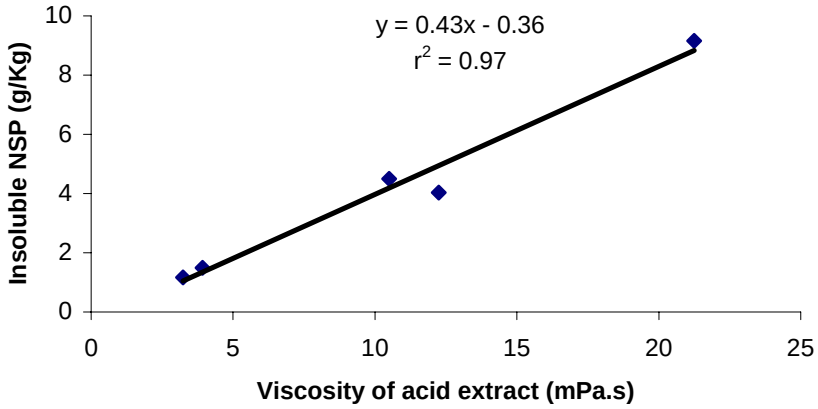



**Figure 5.8 The positive relationship between the viscosity values of the acid extract and the total insoluble non-starch polysaccharides (NSP) in barley (P=0.002).**

### 5.3.5 *The composition of soluble and insoluble NSP in the residues of acid extracts from milled barley, and their relationship to viscosity*

NSP analysis of barley grain acid extract residues revealed that soluble NSP constituted between 2.8 to 5.9% of the total weight of the acid extract residue and insoluble NSP between 0.1 to 1.0% (Table 5.4). The total NSP content showed a positive trend with the viscosity values of the acid extract (P=0.065, $r^2$=0.75, Figure 5.7). This relationship was entirely due to a strong positive linear relationship between the total insoluble NSP content of the acid extract residues and viscosity values of the acid extract (P=0.002, $r^2$=0.97, Figure 5.8). No such relationship was found with the soluble NSP content of the acid extract residue.

## 5.4 Discussion

It is well accepted that NSP in cereal grains have some anti-nutritive properties especially in monogastric animals (Black, 2000). The mechanisms by which NSP exert their anti-nutritional properties in animals are complex and likely to involve the interaction of their physicochemical characteristics with the digestive physiology in animals (Smits, 1996; de Lange, 2000). There is a clear need in the industry to rapidly assess NSP in grains by a method that accurately reflects their anti-nutritional properties. Thus this work was conducted to investigate whether the chemical composition of NSP in barley, sorghum and wheat could influence starch digestibility as assessed by the GRI values, and also whether grain extract viscosity could be used to predict the chemical and physical properties of NSP in grains.

The variation in the soluble and insoluble NSP content and chemical composition within each grain type (barley, sorghum and wheat) did not correlate to grain GRI values, leading to the rejection of the first hypothesis in this study. The lack of a relationship suggests that the NSP content and chemical composition did not restrict enzyme accessibility to starch in grains under the particular *in vitro* condition of the GRI test. The above result may be due a reduction in the barrier function of the cell walls (NSP) by the milling process on grains used in the GRI assay. Furthermore, the concentration of grain samples and consequently NSP content was low in the buffer solution of the GRI assay (~0.1g milled grain / 10 ml buffer solution) in order to enhance the hydrolysis of starch to glucoses by digestive enzymes. The relatively low concentration of NSP in the GRI assay may have been below the detection limit for identifying the negative influence of NSP on

starch digestibility. Interestingly, in the wheat samples, the cellulose content and the GRI displayed a positive linear relationship, however this relationship was mainly due to the presence of Janz frosted cultivar. Several studies have indicated that frosting can have a severe impact on grain nutritional quality (Meredith, 1977; Allen *et al.*, 2001; Richardson *et al.*, 2001). Frost damage of Janz wheat cultivars has been reported to increase the endogenous (grain) α-amylase activity (Allen *et al.*, 2001). This would likely result in an increased GRI value. Frost damaged wheat grains have also been shown to have a higher percentage of cellulose content than normal grains (Allen *et al.*, 2001). These results highlight the important contribution made by environmental influences (such as temperature) on altering NSP chemical properties in grains, during their final maturation stages. Such influences would therefore be expected to alter the nutritional quality of grains and thus should to be considered during nutritional evaluation procedures.

The wide variations in the chemical composition of NSP (both insoluble and soluble components) and in the ratio of insoluble to soluble NSP within grain types did not correlate to viscosity of the acid extracts in barley and both acid and water extracts in wheat. This result rejects the second hypothesis of the present work and suggests that the viscous properties of NSP as indicated by Bedford and Classen (1992) and Saulnier *et al.* (1995) may depend on their molecular size and degree of branching, rather than their chemical composition. In pigs and poultry, it has been shown that the viscous properties of NSP rather than their chemical composition play a more important role in influencing the digestibility of nutrients (Austin and Chesson, 1996; van Barneveld and Pluske, 2001). Bach Knudsen (2001) indicated that chemical composition analysis of NSP provides important information about the degradability of NSP in the large intestine, but not in the small intestine. Therefore, it could be suggested that the current chemical composition analysis of NSP does not reflect the anti-nutritional quality of NSP in grains for monogastric animals.

The presence of insoluble NSP in barley acid extract residues indicates that NSP solubility is influenced by the pH of the extraction condition. The solubilization of NSP could occur by cleaving ester linkages in the insoluble NSP molecules under different pH conditions as reported by Fincher and Stone (1986). Interestingly the content of total insoluble NSP present in the barley acid extract residues displayed a strong relationship with the viscosity of barley acid extracts. Consequently, it can be hypothesised that increasing the solubility of the insoluble NSP could increase the viscosity properties of grains.

The significant differences between the viscosity of acid and water extracts for barley, sorghum and wheat samples is consistent with the work of Rotter *et al.* (1989), who indicated that the *in vitro* solubility and viscosity properties of NSP are highly dependent on the pH of the extract, as well as grain particle size, extraction time and extract temperature. In addition, based on the lack of a significant correlation between the acid and water extract viscosity within the grain types in the current study, it could be concluded that the viscous properties of NSP in different cultivars of the same grain type are influenced to different extents by changes in pH conditions. In barley, it appears that the NSP are released rapidly when the pH is lowered below pH 5 (in the current study, the pH was 1.5 for the extraction buffer), whereas those in wheat are more soluble in near neutral conditions. It is also possible that the arabinose side chain on the arabinoxylan, the main NSP in wheat, may be cleaved off when the pH of the extraction buffer is highly acidic, making the polysaccharide unable to form a viscous solution.

In the present study, significant variation in the viscosity of the acid extract within barley and the viscosity of both the acid and water extracts of wheat was found. This suggests that a simple extract viscosity assay could potentially be used for identifying variation in the molecular structure of NSP within barley and wheat samples. The lack of a significant variation in the viscosity from the water and acid extracts of sorghum may be due to its relatively lower content of NSP compared to barley and wheat. This suggests that the current assay for viscosity may not be sensitive enough to predict subtle differences in grain viscous properties.

Based on the above discussion, determining the viscous properties of NSP under different pH conditions could be valuable for the animal industry since the pH varies along the gastro-intestinal tract and depends on animal age and diet (McDonald *et al.*, 1992c; Nir *et al.*, 1994). Gullion *et al.* (1993) indicated that physical and chemical characteristics of NSP are modified during their passage through the gastro-intestinal tract in animals. Consequently, it could be expected that the variation in chemical and physical properties of NSP as they pass through the gastro-intestinal tract might alter their anti-nutritional activity. Therefore a possible hypothesis may be that determining the viscosity of grain extracts under different pH conditions as opposed to a single *in vitro* condition, may provide a better insight into the anti-nutritional properties of NSP.

As indicated in Section 2.8.1, one of mechanisms by which insoluble NSP could influence the digestibility of starch in cereal grains fed to animals is by acting as a physical barrier, preventing digestive enzymes penetrating the cell walls of endosperm cells. It has

been speculated that the milling, chewing and grinding action of the gizzard (in poultry) could mechanically rupture the cell walls of grain and consequently improve the digestibility (Black, 2000). The extent and type of processing (eg. grinding) of grains influences the extent of the NSP physical barrier function in the cell walls of grains. It has been demonstrated that particle sizes of a milled grain are a consequence of both the type of milling process and the physical and chemical characteristics of the grain (Ellis *et al.*, 1992; Morris and Rose, 1996; Gaines *et al.*, 2000). Therefore, it could be speculated that variations in physical and chemical properties of NSP could influence grain integrity and may contribute to variations in particle size distribution of milled grain. The larger particles have a smaller surface area to volume ratio reducing the accessibility of digestive enzymes to their substrates. This issue is considered in the following chapter.

# Chapter 6 Influence of milling process and kernel integrity of barley, sorghum and wheat on their glucose release index.

## 6.1 Introduction

Different milling processes (e.g. hammer milling and roller milling) are commonly used in the pig and poultry feed industry to produce feed with different particle sizes. The milling process physically breaks apart the grain cell walls that contain NSP (e.g. cellulose, arabinoxylans and β-glucans), which are resistant to host animal digestive enzymes (Jorgensen *et al.*, 1996; de Lange, 2000), in order to expose the digestible components (e.g., starch and protein) to the digestive enzymes. Furthermore, milling increases the surface area to volume ratio, and consequently enhances the exposure of the nutritional elements (such as starch) to the animals' digestive enzymes (Wondra *et al.*, 1995c; Carter, 1996). Milling can also improve the fluidity of digesta and consequently improve the mixability of digestive enzymes with dietary components and accordingly improve the digestion and utilisation of grains by animals (Ohh *et al.*, 1983).

In the pig industry it has been reported that growth performance, apparent digestibility of nutrients (e.g. energy, protein), pathology of the stomach (e.g. gastric ulcer) and the intestinal system, as well as milk production from sows are influenced by the particle size of milled grains (Healy *et al.*, 1994; Wondra *et al.*, 1995d; Wondra *et al.*, 1995e; Ayles *et al.*, 1999; Nielsen and Ingvartsen, 2000). In the review by Guillou and Landeau (2000), it was summarised that in growing pigs, increasing particle sizes of milled grains reduced faecal digestibility of energy and nitrogen respectively. However, fine grinding of grains is not always beneficial for pigs since it may result in stomach and intestinal ulcers, especially in growing-finishing pigs (Wondra *et al.*, 1995d; Wondra *et al.*, 1995e; Monticelli *et al.*, 1996; Nielsen, 1998; Nielsen and Ingvartsen, 2000). Furthermore, the finer the particle size, the higher the energy required for processing, which increases the time and production costs of diet manufacture (Healy *et al.*, 1994). In contrast to pigs, in poultry the particle size of milled grains does not appear to play a major role in bird performance, due to the grinding action of gizzards (Ouart *et al.*, 1986; Deaton *et al.*, 1989). It has been demonstrated that the addition of whole grains of wheat to broiler diets can even enhance feed efficiency, possibly since it can increase the grinding action of gizzards (Plavnik *et al.*, 2002).

During grain milling, variation in particle size distribution of the milled product has been reported (Dobraszczyk, 1994; Bhatty, 1997). The known variation in the physical and chemical properties within and between grains can influence the hardness and the strength of grains (Stenvert and Kingswood, 1977; Glenn and Saunders, 1990; Ellis *et al.*, 1992; Chandrashekar and Mazhar, 1999). Therefore, it is thought that variation in particle sizes is a consequence of both the type of milling process and the physical and chemical characteristics of grains (Ellis *et al.*, 1992; Kavitha and Chandrashekar, 1993; Kavitha and Chandrashekar, 1997).

In the food industries, the grain hardness index is used for predicting the particle size distribution of milled grains. Kernels with a higher hardness index produce more uniform particle sizes than grains with lower hardness index following the milling process (Osborne *et al.*, 2001). Williams *et al.*, (1987) demonstrated a significant negative relationship between the grain hardness and particle size index ($r^2$=0.86). The variation in the distribution of particle sizes of a milled product is an important consideration, since it can influence the amount of surface area of ground grains available to digestive enzymes and as a result could influence the digestion and utilisation of grains by animals (Wondra *et al.*, 1995c). Furthermore, the variation of particle size distribution of milled grains can also affect the stability of mixed feeds (e.g. tendency to segregate) and the quality of the resulting pellet (e.g. variation in particle size distribution can influence the compressibility of a diet mixed that can result in variation in the integrity of pellets) (Kearns, 1989; Traylor *et al.*, 1996; Dirkzwager *et al.*, 1998).

Based on the above information, the current study was conducted to investigate the effects of different milling process on enzyme accessibility under the GRI assay conditions, thus the hypothesis for this experiment were that:

1. Different milling processes effect the GRI of barley, sorghum and wheat, and
2. The hardness index of grains is related to the GRI of ground samples.

The aims of the experiments were to:

1. Determine the influence of the type of milling process on the GRI.
2. Investigate the relationship between grain hardness and GRI.

## 6.2 Materials and Methods

### *6.2.1 Sample selection and preparation*

Wheat, barley and sorghum samples were selected as described in Section 3.2.1 of Chapter 3, (see Appendix 3.1). For each grain sample, 100g sub-samples were weighed in duplicate and processed either by 1): hammer milling through a 2 mm screen using an ultra centrifugal miller which is a type of laboratory hammer mill (ZM1-Retschand, Haan, Germany) or 2): roller-milling by a Quadrumat Junior roller-miller with a 0.4mm gap between the two rollers (Brabender®, OHG Duisburg, Germany).

### *6.2.2 Determining the GRI*

The GRI values for 2 mm-milled and roller-milled grains were determined according to the *in vitro* GRI assay developed in Chapter 3, Section 3.2.3.

To investigate the effect of the type of milling process on the GRI, the GRI values from 2 mm- and roller- milled samples were subtracted from the corresponding GRI values of 0.5 mm-milled samples determined in Chapter 3, Section 3.3.2 & 3.3.3, and the difference (Δ) expressed as a proportion of the corresponding 0.5 mm-hammer milled GRI value as shown below:

$$\Delta GRI\ \% = \frac{GRI\ (0.5\ mm\text{-milled grain}) - GRI\ (2\ mm\text{- or roller-milled grain})}{GRI\ (0.5\ mm\text{-milled grain})} \times 100$$

### *6.2.3 Determining the grain hardness index*

This procedure was conducted by the Bread Research Institute, Australia Ltd. NSW, Australia, for the Premium Grains for Livestock Program.

The hardness of barley, sorghum and wheat grain samples was determined by a Single-Kernel Characterisation System (SKCS 4100) instrument (Perten Instruments, Springfiled, IL, USA). Briefly, the grain hardness index was determined by measuring the crush force of individual grain kernels according to the method reported by Osborne *et al.* (1997).

### *6.2.4 Statistical analysis*

Variation in the ΔGRI% values of 2mm- and roller-milled samples was analysed by ANOVA and LSD (Genstat, 4.2). Similarly, the variation in the hardness index between barley, sorghum and wheat samples was tested by ANOVA and LSD. Finally, the relationship between the grain hardness index and their corresponding ΔGRI% values was investigated using single linear regression analysis.

## 6.3 Results

### *6.3.1 Affect of the type of milling process on the GRI*

#### *6.3.1.1 2mm-milling*

Processing of barley, sorghum and wheat grains by 2mm-milling resulted in significantly reduced GRI values ($P<0.05$) within each grain type, when compared to the GRI of 0.5mm-milled grains (Appendix 6.1). On average the GRI values of 2mm-milled barley, sorghum and wheat samples were reduced by 45%, 55% and 55% respectively compared to their 0.5mm GRI values ($P<0.05$).

There were significant differences in ΔGRI values between grain types and cultivars of barley, sorghum and wheat grains ($P<0.001$, Figure 6.1a, b and c). Barley displayed the widest range of ΔGRI ranging from 20.8 to 65.4% ($P<0.001$, Figure 6.1a). In contrast, wheat displayed the smallest ΔGRI ranging from 42.7 to 55.1% ($P<0.001$, Figure 6.1c).

#### *6.3.1.2 Roller-milling*

The GRI values obtained within each grain type following roller-milling were significantly reduced in wheat ($P<0.01$) but not in barley and sorghum samples when compared to their 0.5mm-milled GRI values (Appendix 6.1). On average, the GRI values of roller-milled barley, sorghum and wheat samples were reduced by 13.1%, 2.7% and 18.8% compared to their 0.5mm GRI values.

As for the 2mm-milled samples, the ΔGRI of roller-milled barley, sorghum and wheat samples varied significantly between grain types and cultivars ($P<0.01$, Figure 6.2a, b and c).

Sorghum showed the largest range in ΔGRI ranging from -53.84 to 50.7% ($P<0.001$, Figure 6.2b), followed by barley (–16.74 to 47.93% ΔGRI, $P<0.001$, Figure 6.2a) and then wheat (5.8 to 31.8% ΔGRI, $P<0.001$, Figure 6.2c).

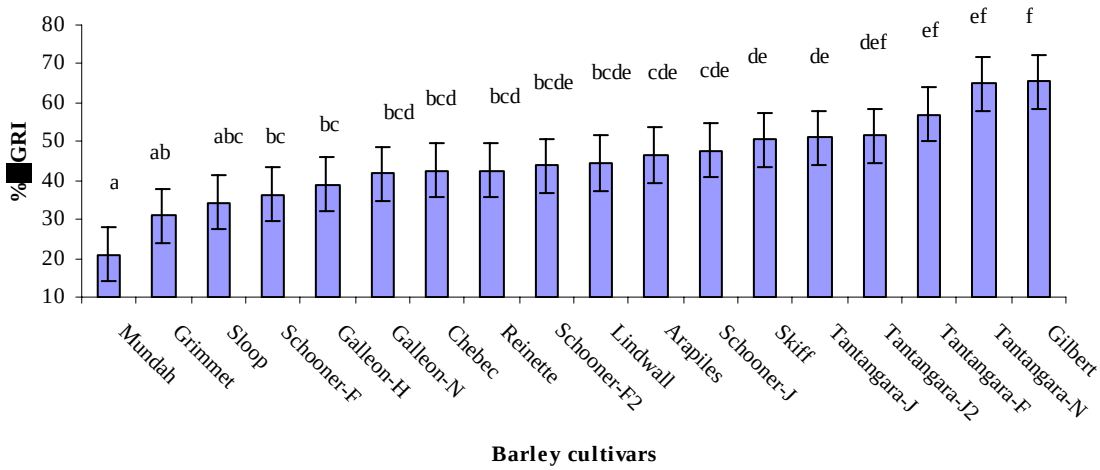
### *6.3.2 Grain hardness index of barley, sorghum and wheat and their relationship to ΔGRI values.*

The grain hardness index values of grains varied between grain types and cultivars (Appendix 6.2). Comparison of the average grain hardness index values between different grains revealed that barley was 1.5- and 1.4- fold lower ($P<0.05$) than in sorghum and wheat respectively. The average grain hardness index of sorghum and wheat were statistically similar to each other (Figure 6.3).

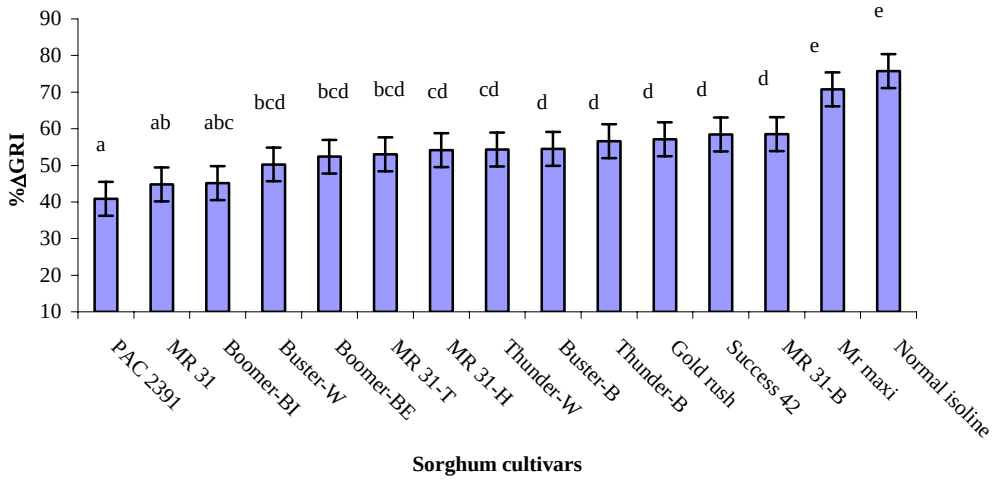
In the roller-milled grains, one barley sample (Tantangara-N) was removed prior to regression analysis due to high standard residuals (outlier). In sorghum, despite the high leverage of ΔGRI for cultivars Normal Isoline and Gold Rush, they were included in the regression analysis since they were still within the quartile range (Figure 6.4) of the data. The grain hardness index values of barley and sorghum but not wheat displayed positive linear relationships with their corresponding ΔGRI ($r^2=0.4$ and $r^2= 0.27$ respectively, $P<0.05$, Figure 6.4a and b).

In 2mm-milled grains, the grain hardness index of barley samples showed a significant relationship with the ΔGRI values ($r^2=0.26$, $P<0.05$, Figure 6.5a). In contrast the grain hardness index of sorghum and wheat samples did not show a significant relationship to their corresponding ΔGRI values (Figure 6.5b and c).

a) Barley




b) Sorghum




c) Wheat

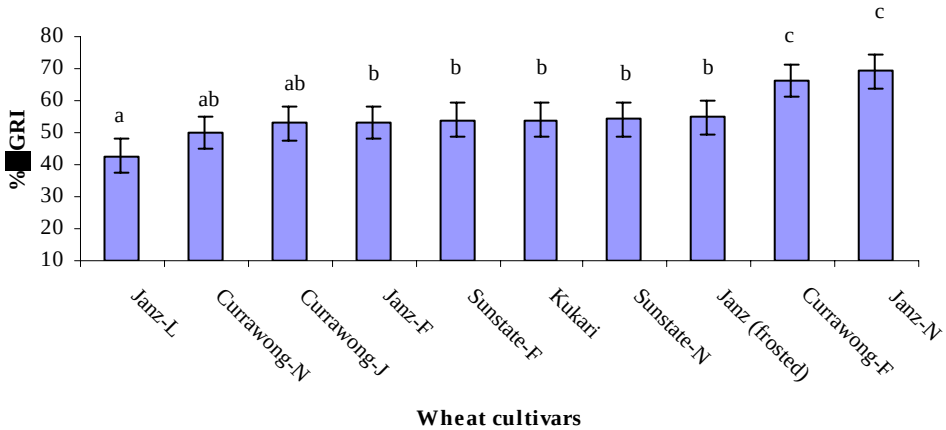
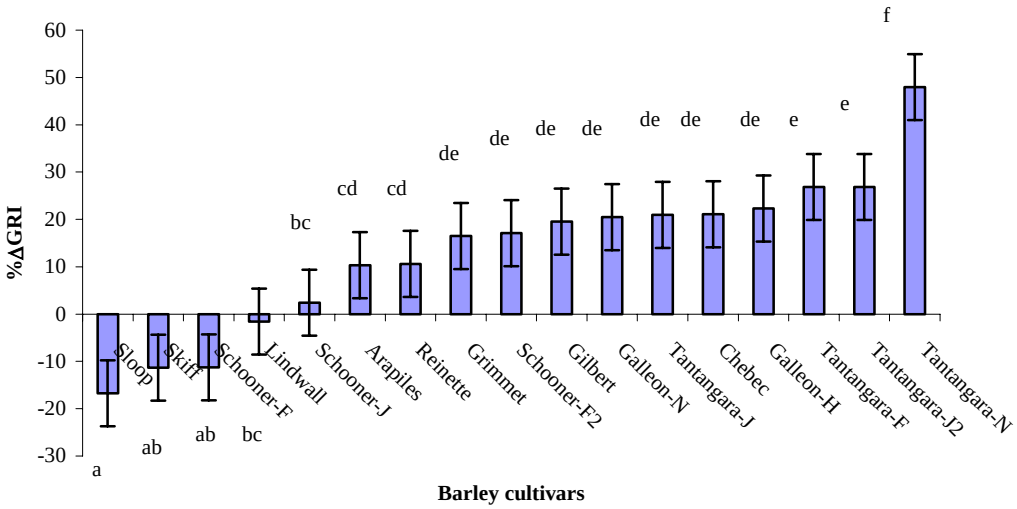
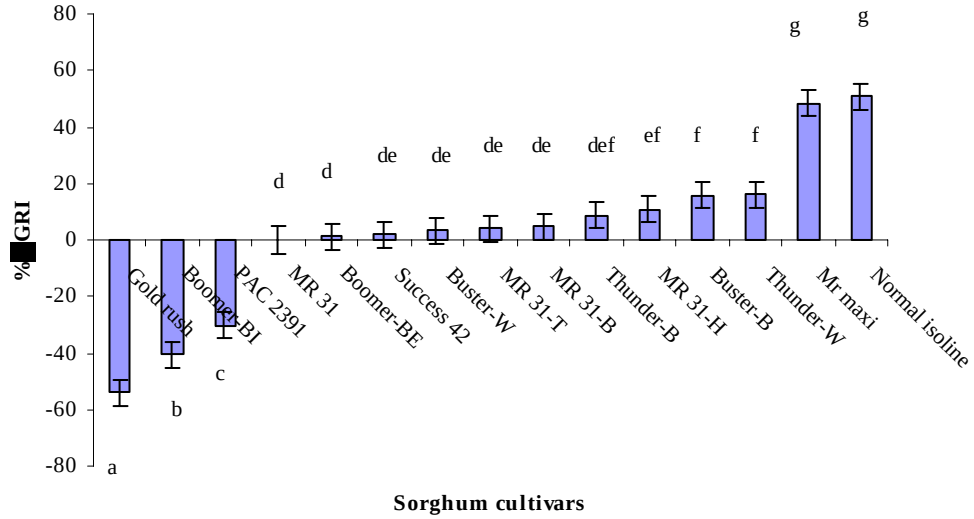



**Figure 6.1 The glucose release index (GRI) values for selected 2mm-milled barley, sorghum and wheat cultivars reported as the proportional difference (% Δ between 0.5mm-milled GRI values. Error bars indicate standard error. Bars with different alphabetical superscripts are significantly different from each other (P<0.001).**

a) Barley




b) Sorghum




c) Wheat

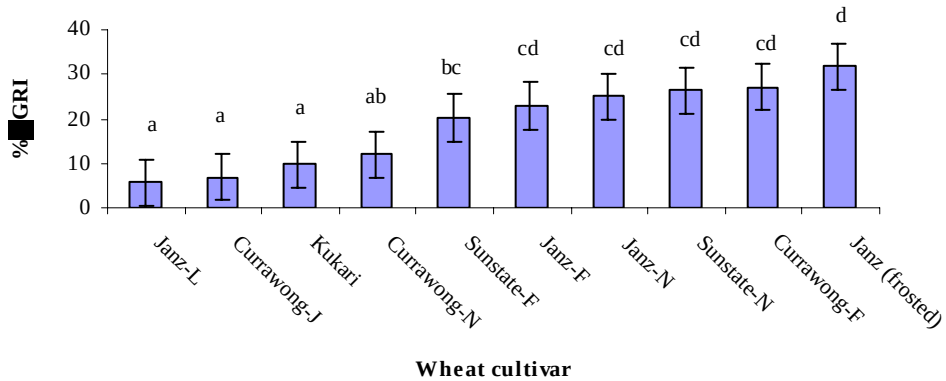



**Figure 6.2 The glucose release index values (GRI) values for selected roller-milled barley, sorghum and wheat cultivars, reported as the proportional difference (% Δ) between 0.5mm-milled GRI values. Error bars indicate standard error. Bars with a different alphabetical superscripts are significantly different from each other (P<0.001).**

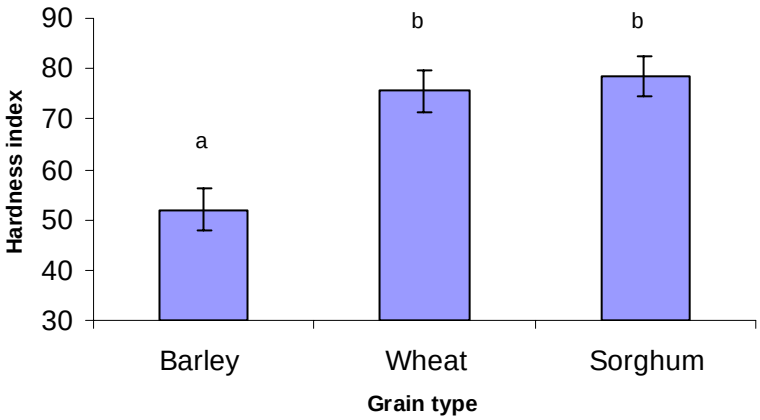
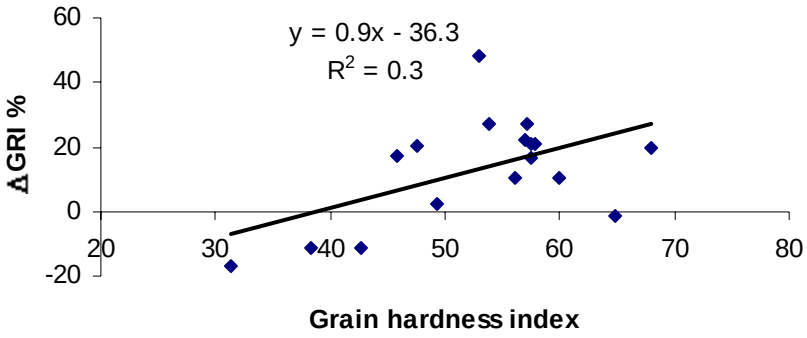
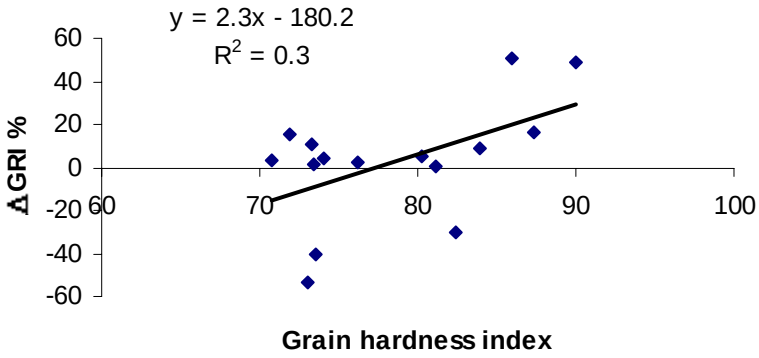



**Figure 6.3 The average comparison of grain hardness index values within the grain types (barley n=18, sorghum n=15 and wheat n=10). The bars with different superscripts are significantly different from each other (P<0.05).**

Barley




Sorghum




Wheat

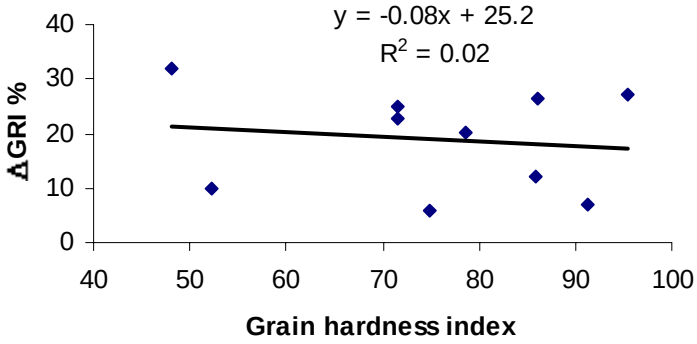
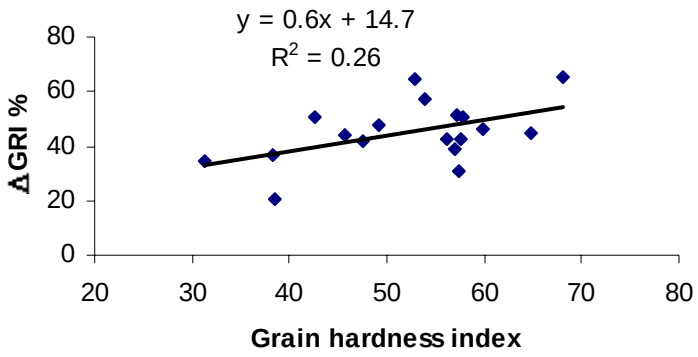
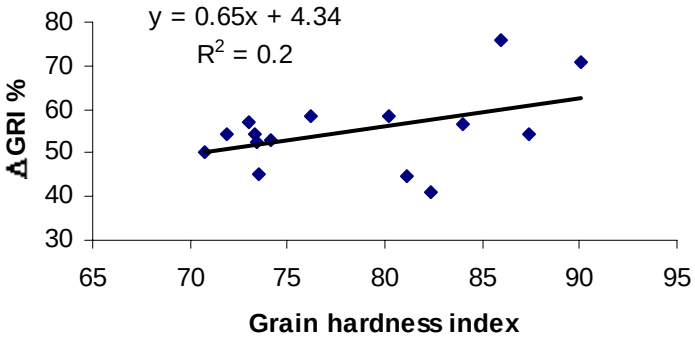



**Figure 6.4 The positive linear relationship between the proportion of GRI values for the roller milled samples in comparison to their corresponding GRI values for 0.5mm (ΔGRI) with their hardness index values (a=barley, n=17, P<0.05), (b= sorghum, n=16, P<0.05) and (c=wheat, P>0.05, n=10).**

Barley




Sorghum




Wheat

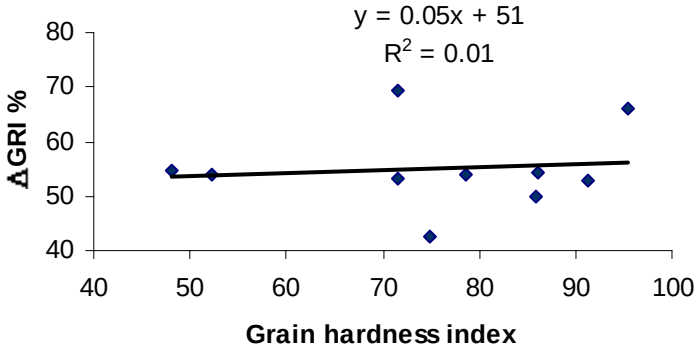



**Figure 6.5 The positive linear relationship between the proportional of GRI values for the 2mm milled samples in comparison to their corresponding GRI values for 0.5mm (ΔGRI) with their hardness index values (a=barley, n=18, P<0.05), (b= sorghum, n=16, P>0.05) and (c=wheat, P>0.05, n=10).**

## 6.4 Discussion

It has been demonstrated that decreasing the mean particle size of milled cereal grains increases their digestibility and utilisation by grower pigs (Ohh *et al.*, 1983; Goodband and Hines, 1988; Healy *et al.*, 1994), finisher pigs (Ivan *et al.*, 1974; Owsley *et al.*, 1981; Wondra *et al.*, 1995a; Flis *et al.*, 2000) and lactating sows (Wondra *et al.*, 1995e). More importantly, it has been demonstrated that the variation in particle size distribution of milled grain or its uniformity can influence feed intake, digestibility and growth performance in grower pigs (Wondra *et al.*, 1995c). Wondra *et al.* (1995c) indicated that milled corn with lower variation in particle size was digested more than milled corn with a higher variation in particle size, despite their similar mean particle size (850μm). Consequently in order to maximise the utilisation of diets by the animal and reduce the chance of gastro-intestinal ulcers for the pig industry, optimal particle sizes (Goodband and Hines, 1988; Albar *et al.*, 2000; Guillou and Landeau, 2000) as well as particle size uniformity of ground grains are required (Wondra *et al.*, 1995c).

The present results were in support of the first hypothesis that the different types and extent of milling processes significantly influence the GRI of grains. This could be explained by differences in enzyme accessibility caused by the variation of the milling particle size through different milling conditions. Interestingly, the results also demonstrated that the effects of the milling process on GRI of grains varied significantly between the grain types and cultivars. Furthermore, the GRI of barley and sorghum cultivars displayed a significant positive relationship with their hardness indices in support of the second hypothesis of the study. Such a result could be due to softer grains having a significantly greater portion of large particles following milling (due to some particles being "flattened" rather than broken), in contrast to hard grains which generate more homogeneous particle sizes. Consequently the larger particles in milled soft grain samples may reduce enzymes accessibility and thus GRI.

It has been indicated that hard grain tends to fracture with relatively uniform larger particles compared with soft wheats (Osborne *et al.*, 2001). In the food industry, the hardness of a grain is the single most important characteristic in determining the milling quality (Anjum and Walker, 1991). Grain hardness is used to classify grain according to the way in which it fractures during milling and thus it is used for the prediction of particle sizes of milled grains (Williams *et al.*, 1998). Thus, it can be concluded that grains with different hardness produce different particle size distributions under similar milling

processes and this could affect the GRI. The positive correlation between grain hardness and the ΔGRI values in barley and sorghum samples support the above suggestion. The lack of a relationship between the ΔGRI and the hardness index values in wheat suggests that other characteristics of wheat kernels such as protein matrix or NSP composition may be the factors that are limiting the GRI.

In barley and sorghum, the grain hardness index may also be utilised to select an appropriate type of milling process for grains. For example, it could be suggested that on average, hammer-milling of barley grain with sieve sizes of 2mm may be sufficient to improve its starch digestibility because barley is relatively soft compared to wheat and sorghum. The lower ΔGRI values in hammer milled barley compared to sorghum and wheat lend support to the above conclusion. Barley will produce finer particles than wheat and sorghum under similar milling conditions.

It has been suggested that the selection of an appropriate milling process can improve the uniformity of grain particles following milling (Wondra *et al.*, 1995c) since different milling processes can produce different degrees of particle uniformity (Lawrence, 1970; Wu and Fuller, 1974). The current results revealed that the starch digestibility of different grain types and even different cultivars of the same grain type varied with the different type of milling processes (e.g., roller-mill vs hammer-mill) possibly due to differences in particle size distribution after milling. For example, the roller milling process was more effective for sorghum since on average the ΔGRI value of sorghum was around 5 and 7 times lower than barley and sorghum respectively. Therefore the combination of crushing and shearing force of roller milling may disrupt the tightly packed cells and starch granules in sorghum more effectively than the crushing force of hammer milling. The differences in cell size/shape, protein matrix, starch granule size and elasticity in sorghum kernel could be partially resulted to such differences from barley and wheat.

In order to minimise the variation in starch digestibility between and within grain types, an appropriate type of grinding process is needed for each grain type or cultivar. For instance, choosing the roller-milling process for barley and sorghum samples with negative ΔGRI values instead of hammer milling can improve the starch digestibility of milled grain, and at the same time, decrease the electric power consumption, since it has been demonstrated that the roller-milling process consumes 7 times less electricity than hammer-milling processing (Olsen *et al.*, 1980). Furthermore it is known that the particle size distribution and also particle shape of milled grain can influence its ability to flow,

mix and compress (Axe, 1999), which are important issues to feed manufacturing firms (Guillou and Landeau, 2000).

In summary, in order to provide a uniform and optimal particle size of milled grains, an appropriate milling process that is specific to individual grains may help to decrease variation in GRI between the grains. This is not currently practiced in milling animal feeds, but may become so based on experience in the human food industry. The grain hardness index in barley and sorghum can be used for prediction of endosperm texture and consequently selection of an appropriate milling process. Thus determining the ΔGRI and particle size distribution of milled grains could aid in the selection of an appropriate type of milling process.

In monogastric animals, the extent of the influence of protein matrix as one of the non-starch related factors influencing the effect of processing grains and starch digestibility of grains is still unclear. It has been hypothesized that variations in physical and chemical properties of the protein matrix in grains may affect the accessibility of digestive enzymes to starch granules and consequently its starch digestibility (Black, 2000). The protein matrix in the endosperm can influence the hardness (Stenvert and Kingswood, 1977) and consequently the milling quality of grains (Ellis *et al.*, 1992; Carter, 1996; Beecher *et al.*, 2001) and, as suggested in the present chapter, may influence starch digestibility of milled grains. In the following chapter, the influences of protein matrix on their starch digestibility between grain types and cultivars were investigated.

# Chapter 7 Influence of the protein matrix on glucose release index in barley, sorghum and wheat grains.

## 7.1 Introduction

In cereal grains, the majority of protein exists within the endosperm cell, and is located between and around the starch granules (Shewry, 1996). The quantity of protein can vary between different grains and also between cultivars of the same grain type (Shewry, 1996). For example, a range of 7 to 17% crude protein content within one wheat cultivar has been reported (Morris and Rose, 1996).

The quality or type of proteins in grains can also vary significantly within each grain type. As an example, the protein in some barley cultivars has approximately a 35% higher lysine content compared to other conventional cultivars (Jorgensen *et al.*, 1999). It is thought that such a variation in the protein quality may influence the extent of protein digestibility in grains by monogastric as well as ruminant animals (Hughes and Choct, 1999; van Barneveld, 1999a; van Barneveld, 1999c). For instance, sorghum has been documented to have low protein digestibility relative to other cereal grains (Weaver *et al.*, 1998). In sorghum, prolamine is the most abundant amino acid of the protein matrix, which has been shown to be resistant to enzymatic hydrolysis particularly in the corneous endosperm region (outside layer) (Rooney, 1996). Variations in protein digestibility have also been reported between different cultivars of barley, sorghum and wheat in monogastric animals (Büchmann, 1979; Bell and Keith, 1989; Rooney, 1996). Since most of the proteins in grains surround the starch granules, different protein digestibility may indirectly influence starch digestion.

In grains, the degree of digestibility of protein in the matrix that surrounds starch granules can influence the accessibility of bacterial enzymes to starch granules (Owens *et al.*, 1986; McAllister *et al.*, 1993; van Barneveld, 1999c). Therefore, starch digestibility of grains by ruminal microflora can vary depending on the chemical and physical characteristics of the protein matrix (McAllister *et al.*, 1992). It has been speculated that the protein matrix of cereal grains can also influence the starch digestibility in monogastric animals (Black, 2000). Several animal- and diet-related factors have been identified that may decrease the protease activity and consequently starch digestibility in pigs and poultry (O'Brien, 1999). The degree of encapsulation of starch granules by the protein matrix could significantly influence the accessibility of amylolytic enzymes to starch granules.

For instance, in barley and wheat grains several protease inhibitors (e.g., trypsin, chymotrypsin) have been reported (Boisen *et al.*, 1981; Boisen, 1983; Shewry, 1996), which can negatively influence the digestibility of the protein matrix and consequently decrease starch granule accessibility to digestive enzymes. Secondly, the degree of encapsulation of starch granules by the protein matrix could significantly influence the accessibility of amylolytic enzymes to starch granules.

The present experiment was conducted to investigate the extent of interaction between the protein matrix and starch digestion in grains under *in vitro* conditions. The hypothesis tested during this work was that pre-incubation of grain with protease enzymes would improve the GRI value in barley, sorghum and wheat.

To test this hypothesis, the objectives of this experiment were:

1: to determine the differences in the GRI values of grains treated with and without protease enzymes

2: to visually investigate the degree of encapsulation of starch granules by the protein matrix before and after protease treatment using scanning electron microscopy.

## 7.2 Materials and methods

### *7.2.1 Sample selection*

Three cultivars were selected from barley, sorghum and wheat samples. These cultivars displayed wide differences in their ileal digestible energy values for pigs, and therefore potentially would display the largest differences in starch digestibility (Table 7.1).

### *7.2.2 Determining the influence of protein matrix on the GRI from starch in cereal grains*

To determine the influence of the protein matrix on the GRI of starch, duplicate grain samples of 0.1g (0.5 mm milled, as described in section 3.2.1) were weighed into McCartney bottles (AdeLab, Australia) and the protein of grains was digested with 3ml of 0.67% (w/v) pepsin (porcine P-7000, Sigma Chemical Co. USA) at pH 2 for 60 minutes at 39°C in a shaking waterbath (as the pepsin protease is an enzyme with broad range enzyme activity, in order to digest and solubilise protein matrix in grain endosperm region). Following pepsin treatment, grain samples were analysed for GRI values as shown in

Figure 7.1. A duplicate set of non-pepsin-treated samples was also included in the GRI analysis. These samples were assayed in the same manner as described above, except that pepsin was omitted from the incubation buffer. Background GRI values were corrected by including duplicate sample blanks for the pepsin and non-pepsin treatments. Duplicate positive controls containing pure glucose (G-7528, Sigma Chemical Co. USA) and pure starch (102713R, BDH, Merck Pty. Ltd., Australia) were also used in each run of the GRI assay.

The GRI values were calculated as described in Chapter 3 Section 3.2.3 and the differences in value for samples treated with pepsin (GRI + pepsin) and without pepsin (GRI - pepsin) were plotted against the corresponding crude protein value to investigate the possible correlations.

### *7.2.3 Crude protein determination*

This assay was conducted by the Western Australian Chemistry Centre Laboratory for Premium Grains for Livestock Program. Crude protein was calculated by multiplying the nitrogen content for each grain sample by 6.25 (McDonald *et al.*, 1992a). The amount of nitrogen was measured by thermal conductivity (Dumas Nitrogen, as outlined in AOAC (1995) method 4.2.04.

### *7.2.4 Scanning electron microscopy of grains*

Two cultivars of each grain type (barley, sorghum and wheat) were also selected for scanning electron microscopy (as shown in Table 7.1). Nine kernels of each cultivar were randomly selected and cut longitudinally into halves. Three sectioned grains were placed on aluminium scanning electron microscopy stubs (two stubs used per cultivar) with the cut surface of the grain facing away from the surface of the stub. To avoid electrical charge formation on the surface of the grains, a carbon paint paste (Procitech Co, Australia) was applied. The stubs were prepared by one of the two following procedures:

1) Samples were not treated with pepsin and powder coated with gold-palladium-carbon (Procitech Co, Australia).
2) Samples were incubated with 10µl of 0.67% (w/v) pepsin (contained in 8.3% (v/v) HCl (UNIVAR Australia) + 0.74% (w/v) KCl (Ajax Chemicals Australia),

pH= 2) for two 30-minute periods with a wash in distilled water in between incubations. The samples were dried at 50°C in an oven for 48 hours and then powder coated with gold-palladium-carbon.

Starch granule shape, size and their arrangement inside endosperm cells was visually estimated for all samples using a Phillips KL30 field emission scanning electron microscope operated at an acceleration voltage of 10 kV. These experiments were conducted at the Centre for Electron Microscopy South Australia.

### *7.2.5 Statistical analysis*

The variations in the GRI values within and between samples treated with and without pepsin were tested by complete random design with 3x3x2 treatment structure using ANOVA and LSD (Genstat 4.2).

## 7.3 Results

### *7.3.1 Determining the GRI values in barley, sorghum and wheat with or without pepsin pre-treatment*

Pepsin treatment influenced the GRI values of cultivars of each grain type ($P<0.05$, Figure 7.2). The GRI values in barley decreased following pepsin treatment, however, this decrease was only significant for the Grimmet cultivar ($P<0.01$, Figure 7.2). In contrast to barley, sorghum and wheat displayed an increase in their GRI values following pepsin treatment ($P<0.01$, Figure 7.2), and this was significant in two of three cultivars of sorghum (Mr 31-B and Success 42) and wheat (Janz-frosted and Currawong-F) ($P<0.01$, Figure 7.2). Analysis of the average GRI values following pepsin pre-treatment revealed significant variation between barley, sorghum and wheat ($P<0.05$, Table 7.2).

The average GRI of both pepsin-treated wheat and sorghum increased approximately 1.1-fold compared to their non-pepsin treated GRI values. In barley, pepsin treatment decreased GRI values. Pepsin-treated sorghum displayed the lowest average GRI value compared to pepsin-treated barley and wheat, which had GRI values that were 1.3- to 1.4-fold higher than sorghum (Table 7.2).

**Table 7.1 Grain samples selected for investigating the influence of protein matrix on the glucose release index.**

| Grain type | Cultivar | Ileal DE/GE(%)[1] |
|---|---|---|
| Barley | *Grimmet*[2] | *50.1* |
| | *Mundah*[2] | *60.4* |
| | Galleon-N | 64.7 |
| Sorghum | *Boomer-BE*[2] | *69.2* |
| | *Success 42*[2] | *77.6* |
| | MR 31-B | 74.1 |
| Wheat | *Janz (frosted)*[2] | *57.8* |
| | *Janz-L*[2] | *65.6* |
| | Currawong-F | 78.3 |

[1] Ileal digestible energy / gross energy (DE/GE) was determined at the South Australian Research and Development Institute, Roseworthy for Premium Grains for Livestock Program (unpublished data).
[2] The grain cultivars shown in italics were also selected for the scanning electron microscopy study as outlined in Section 7.2.4.

**Table 7.2 A comparison of the average glucose release index values (GRI) of starch in barley wheat and sorghum with (+) or without (-) pepsin pre-treatment (significant interaction between grain type and pepsin treatment).**

| GRI (%) | Barley | Sorghum | Wheat | SED[1] |
|---|---|---|---|---|
| GRI + pepsin | 27.9 [b] | 21.1 [a] | 31.2 [c] | 1.02 |
| GRI - pepsin | 29.1 [b] | 18.9 [a] | 28.5 [b] | 1.02 |

[1] Standard error of difference between means
[a b c] GRI values within each row with a different superscript differ significantly at P<0.05.

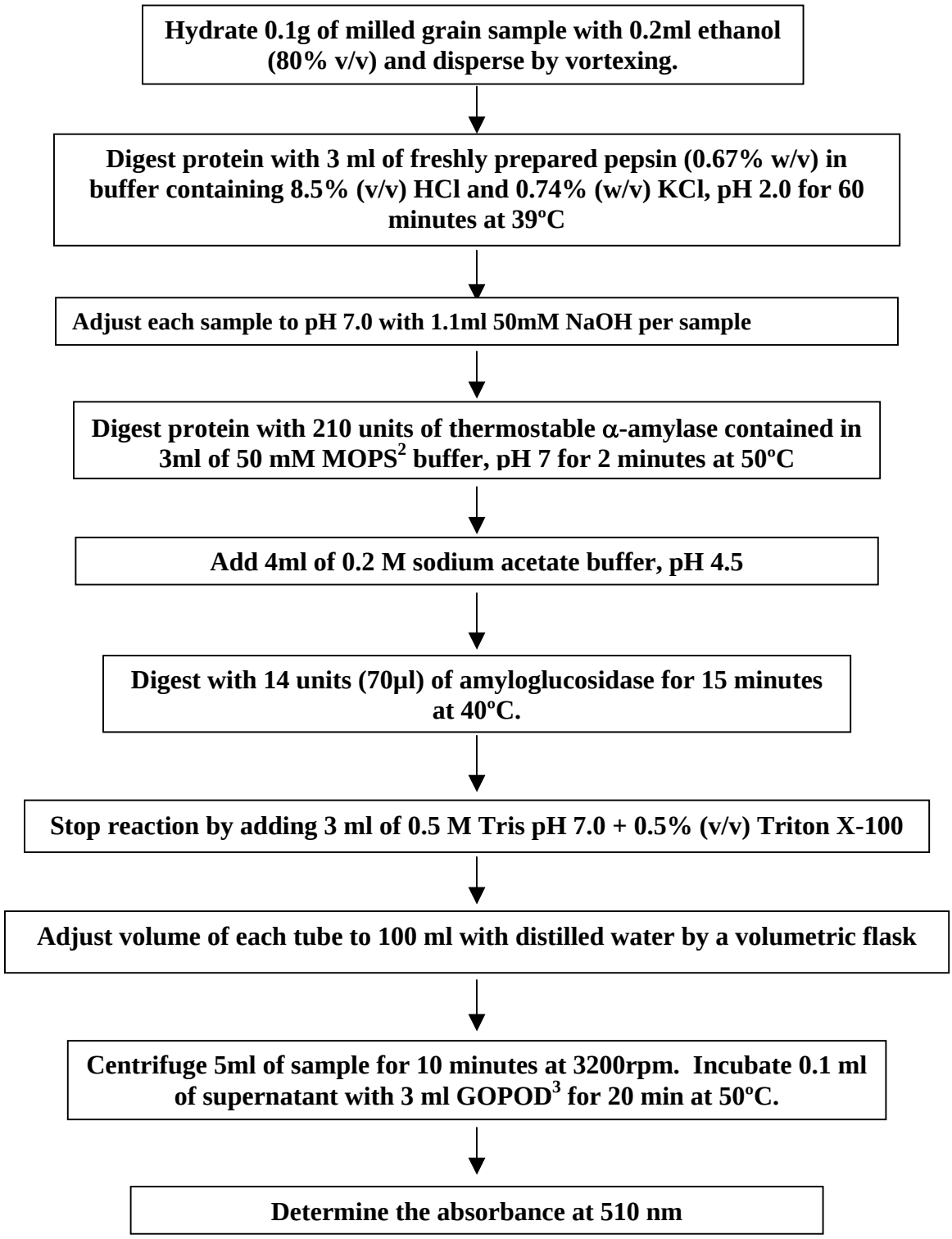



**Figure 7.1 A flow diagram of the glucose release index assay in cereal grains pre-treated with pepsin[1].**

- [1] The glucose release index for the non-pepsin treated samples was similarly determined except that pepsin was omitted from the incubation buffer.
- [2] MOPS buffer=also contains calcium chloride (5 mM) and sodium azide (0.02%).
- [3] GOPOD= glucose determination reagent = Glucose Oxidase + Peroxidase + 4-Aminoantipyrine.

In barley, sorghum and wheat, the difference in GRI values for the pepsin and non-pepsin treated grains was not related to crude protein contents of the grains (Appendix 7.1) (Figure 7.3).

### *7.3.2 Scanning electron microscopy*

Differences in starch granule shape, size and their arrangement within the endosperm cells were observed between barley, sorghum and wheat (Figure 7.4). Prior to pepsin treatment, the small starch granules (B-type) in barley and wheat were mainly embedded in the protein matrix, compared to the large starch granules (A-type), which remained relatively free of protein matrix (Figures 7.5 and 7.6). Following pepsin digestion, the protein matrix surrounding barley and wheat starch granules was no longer visibly detected (Figures 7.5 and 7.6). In sorghum, prior to pepsin treatment, starch granules in the corneous (Figure 7.7) and floury endosperm regions (Figure 7.7) displayed a tight arrangement with the protein matrix in comparison to barley and wheat (Figures 7.5 and 7.6). The corneous region (particularly close to the peripheral layer of endosperm) contained mainly B-type starch granules that were surrounded by a large amount of protein matrix (Figure 7.7).

Pepsin treatment did not completely remove the protein matrix surrounding starch granules in the corneous region of sorghum (Figure 7.7). The floury region in sorghum displayed a greater content of large starch granules (A-type) that appeared to be bound by a protein membrane (Figure 7.8). The protein matrix was removed in the floury region by pepsin treatment (Figure 7.8). In all samples, starch granules in the floury endosperm displayed pores in their surface following pepsin treatment (Figure 7.9).

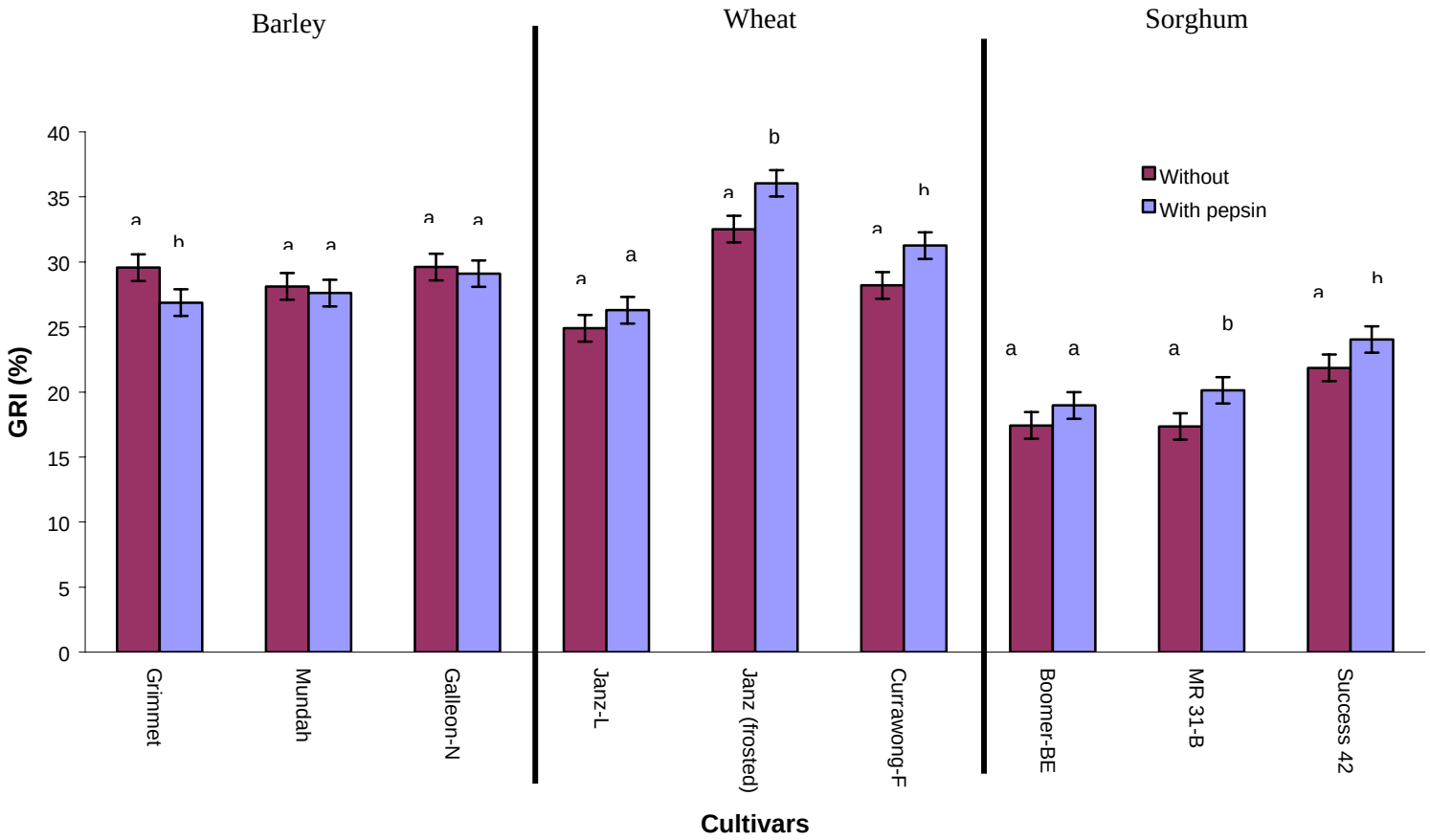



**Figure 7.2 A comparison of the glucose release index (GRI) with or without pepsin treatment of barley, wheat and sorghum. Bars with different superscripts differ significantly (P<0.01), error bars indicate standard errors.**


**Figure 7.3 A comparison of the difference in the glucose release index (GRI) values in barley, sorghum and wheat treated with and without pepsin to the percentage of crude protein content.**

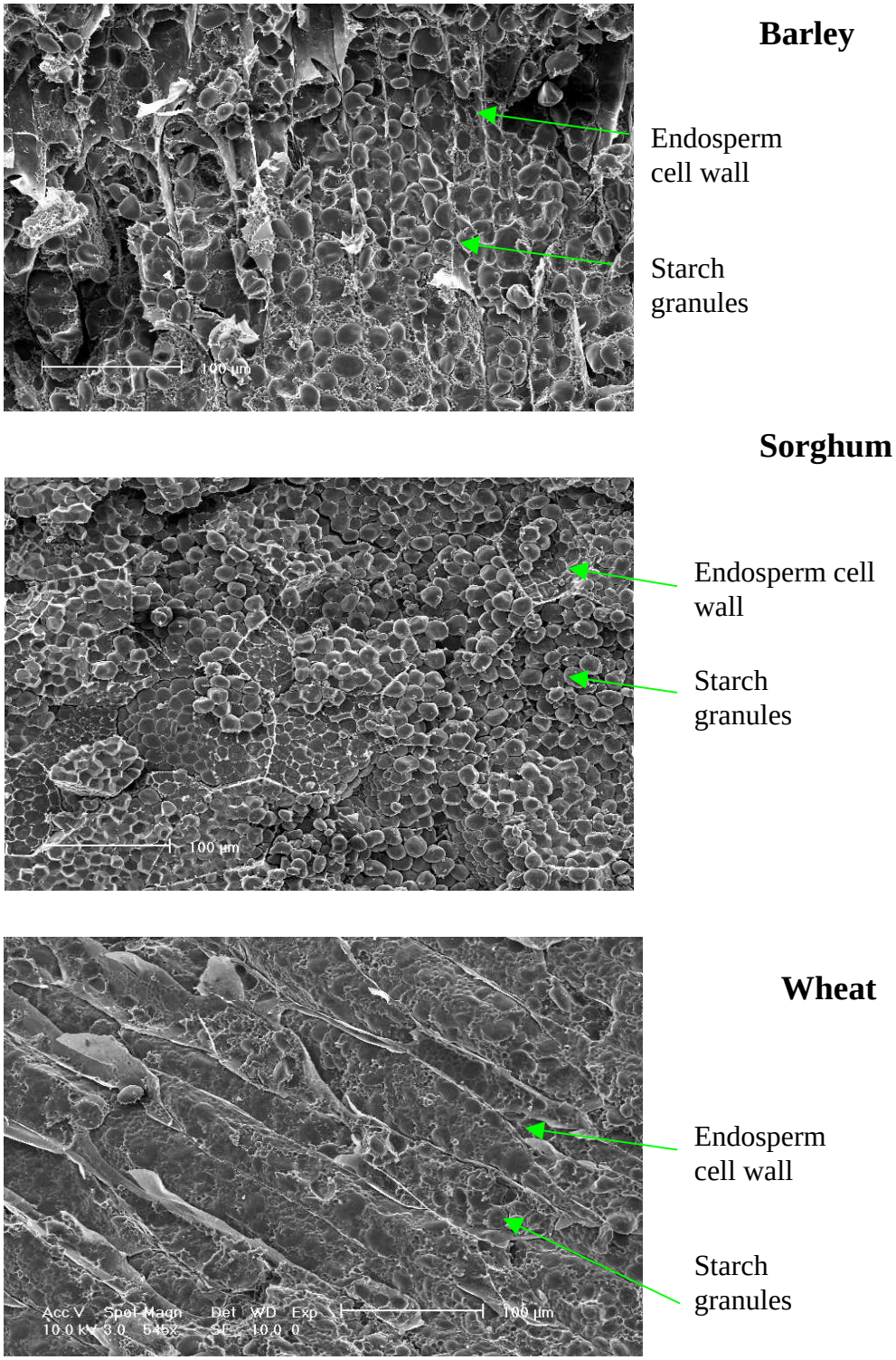
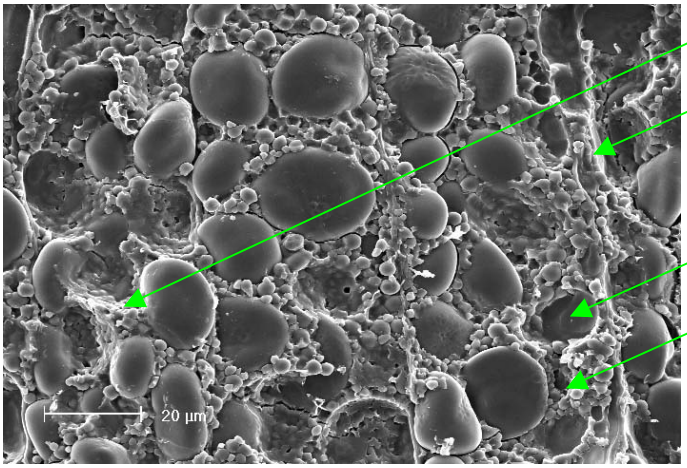



**Figure 7.4 Scanning electron microscopy of barley, sorghum and wheat showing endosperm cells filled with starch granules.**

Differences in the size and shape of endosperm cells and starch granules are visible between the different grain types. Starch granules are surrounded by protein matrix appearing as a white amorphous substance.

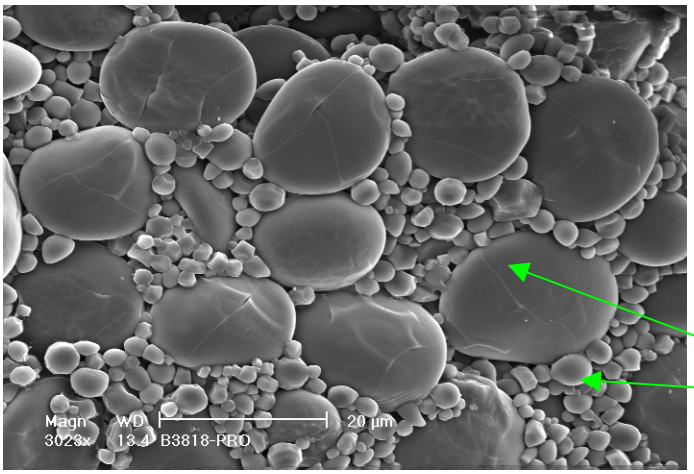
Without pepsin treatment




Protein matrix, white amorphous substance

Starch granules: A-type (large), B-type (small)

With pepsin treatment



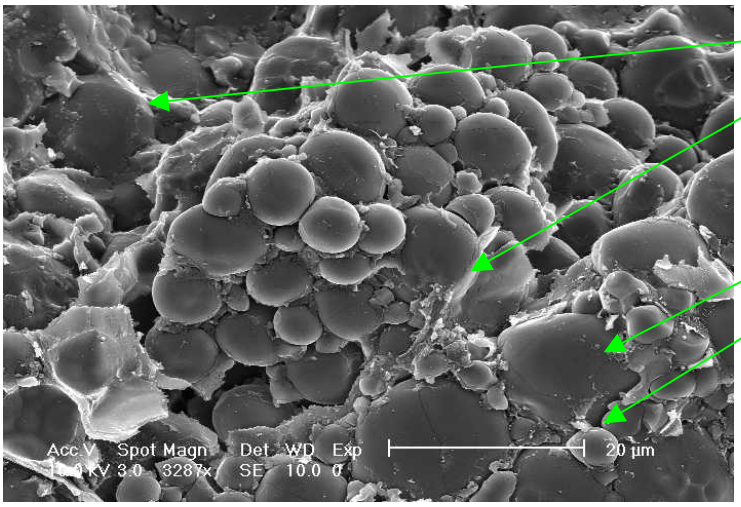

Visibly reduced appearance of protein matrix surrounding starch granules

Starch granules: A-type (large), B-type (small)

**Figure 7.5 Scanning electron microscopy of the endosperm region in barley, prior to and following pepsin digestion.**

The quantity of protein matrix surrounding starch granules in the endosperm region is visibly reduced following pepsin digestion.

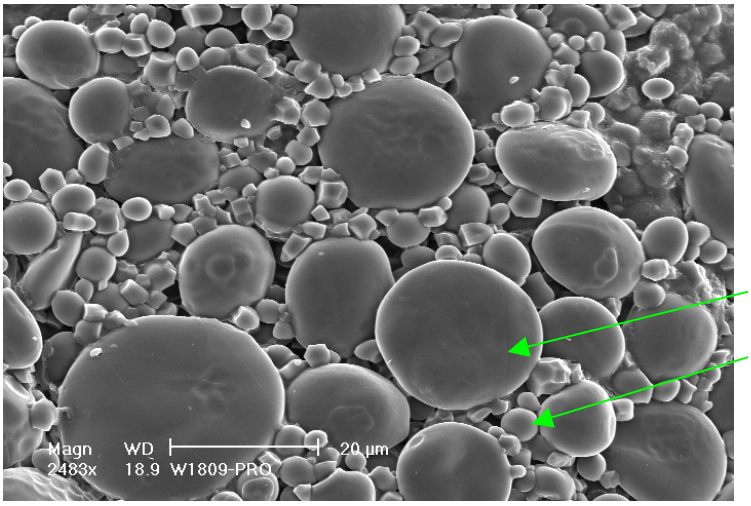
Without pepsin treatment




Protein matrix, white amorphous substance

Starch granules: A-type (large), B-type (small)

With pepsin treatment



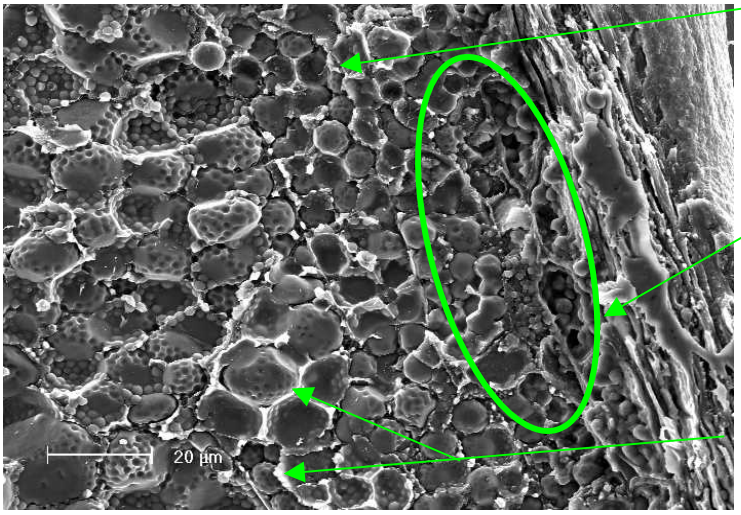

Visibly reduced appearance of protein matrix surrounding starch granules

Starch granules: A-type (large), B-type (small)

**Figure 7.6 Scanning electron microscopy of the endosperm region in wheat, prior to and following pepsin digestion.**

The quantity of protein matrix surrounding starch granules in the endosperm region is visibly reduced following pepsin digestion.

Without pepsin treatment

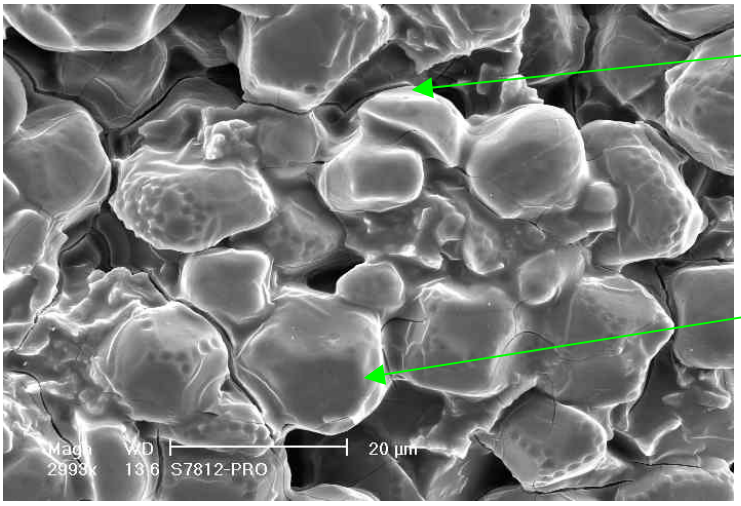



Starch granules

Corneous endosperm region and the peripheral layer

White amorphous protein matrix

With pepsin treatment



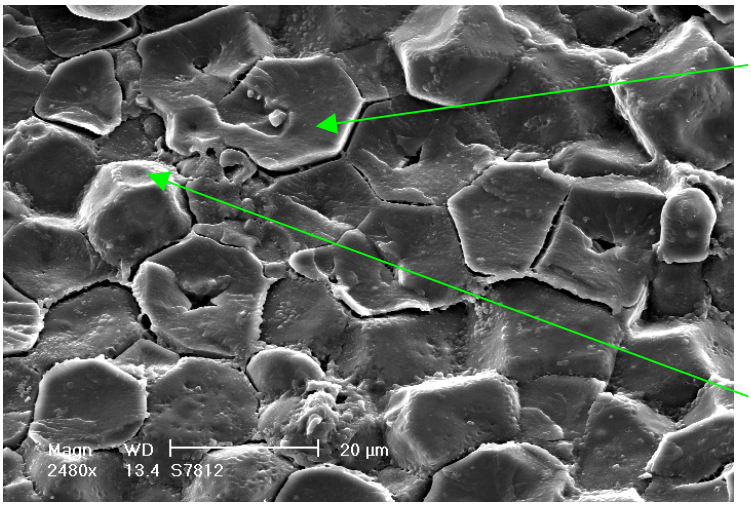

White amorphous protein matrix is still visible following pepsin digestion

Starch granule

**Figure 7.7 Scanning electron microscopy of the corneous endosperm region in sorghum, prior to and following pepsin digestion.**

The quantity of protein matrix surrounding starch granules in the corneous endosperm region is visibly reduced but not completely digested following pepsin treatment.

Without pepsin treatment

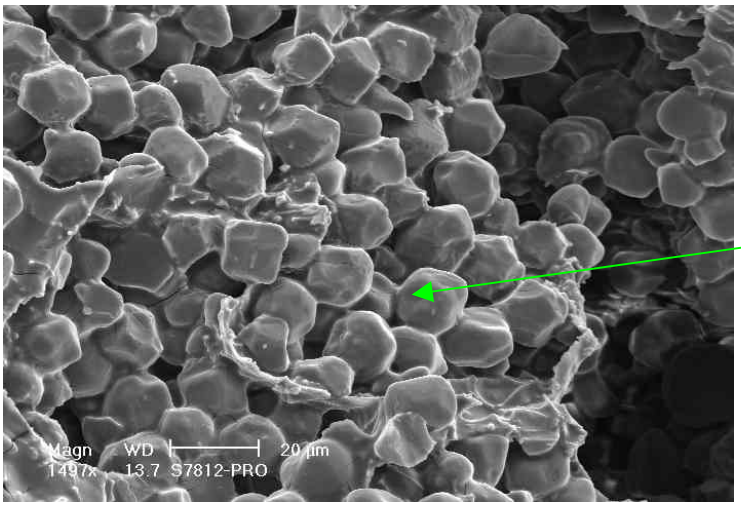



Starch granule

Floury endosperm region

White amorphous protein matrix

With pepsin treatment




Visibly reduced protein matrix following pepsin digestion

Starch granule

**Figure 7.8 Scanning electron microscopy of the floury endosperm region in sorghum, prior to and following pepsin digestion.**

Starch granules are polygonal in shape and are tightly cemented together by the surrounding protein matrix. The quantity of the surrounding protein matrix in the floury endosperm region is visibly reduced following pepsin treatment.

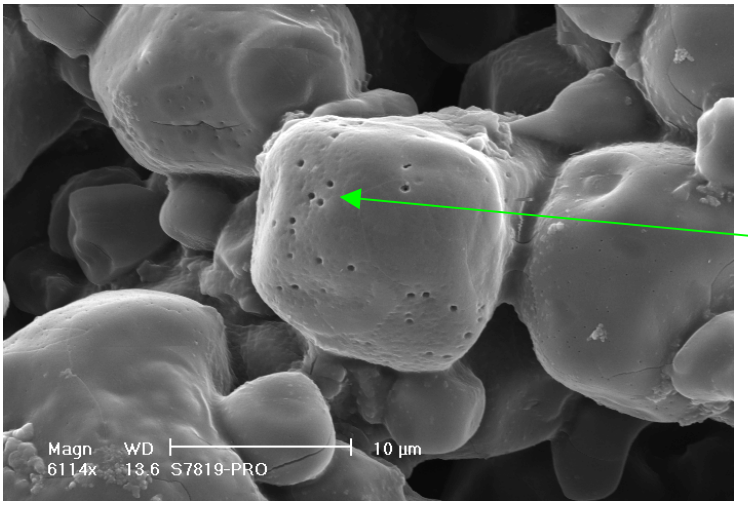



**Figure 7.9 Pores on the surface of starch granules in the endosperm region of sorghum following digestion by pepsin.**

## 7.4 Discussion

Black (2000) suggested that starch digestibility of grains by monogastric animals can be influenced by the extent of starch granule embedding in the protein matrix. Thus, in the current study, GRI assays were used to investigate the influence of protein matrix on the digestibility of starch in grains.

Pepsin treatment of grains influenced the GRI, indicating that protein matrix could affect starch digestibility. Differences between grain types and between cultivars in GRI were observed when treated with pepsin and these differences were particularly evident in sorghum and wheat. The differences in GRI following protease treatment suggest that there is variation in the susceptibility of protein to digestion between and within grains, in line with evidence reported by Rooney and Pflugfelder (1986) and Darlington *et al.* (2000) who showed that variation in protein digestibility could influence the starch digestibility of grains. Variation in protein digestibility by proteases may arise from differences in protein quantity and/or protein quality in grains, as previously reported by Ellis *et al.* (1992), Weaver *et al.* (1998) and Gaines *et al.* (2000). The increased GRI following pepsin pre-treatment in sorghum and wheat but not in barley partially supports the hypothesis of this study, and for sorghum, supports the findings of Lichtenwalner *et al.* (1978) who indicated that the digestion of protein in sorghum improves starch digestibility in an *in vitro* system.

The protein matrix surrounding starch granules in sorghum and wheat grains could influence starch digestion (as measured by the GRI) by one or a combination of the following mechanisms: 1) as observed in the scanning microscopy images, the removal of protein matrix by pepsin pre-treatment provides some spaces around starch granules (particularly around the small, B-type, starch granules that would assist enzyme accessibility to its substrate; 2) following pepsin pre-treatment, the appearance of pores in the surface of starch granules may assist the enzyme to penetrate inside the starch granules.

In barley, despite the ability of pepsin to remove the protein matrix (as displayed by scanning microscopy), the GRI values decreased. These results may partly be due to the degradation of the α-amylase and amyloglucosidase by pepsin. These negative effects of pepsin on α-amylase and amyloglucosidase enzymes in wheat and sorghum samples are also possible, however, the positive effect of pepsin in increasing the accessibility of α-amylase and amyloglucosidase enzymes to starch granules by digesting the surrounding protein matrix, may have been much greater and thus outweighed possible negative effects. Thus it could be suggested that the protein matrix in barley presents less of a physical

barrier to starch digestion than in wheat and sorghum. This conclusion is further supported by the result that non pepsin-treated barley grains exhibited the highest average GRI value compared to sorghum and wheat.

In sorghum, despite a significant increase in the average GRI value after protease treatment, this value was still significantly lower than in barley and wheat samples treated with pepsin. This is probably related to the lower surface area to volume ratio of starch granules in sorghum, which could restrict digestive enzyme accessibility to starch. Another important difference between sorghum and other grain types such as barley and wheat is its prolamine-rich protein matrix. There are three types of prolamines, namely α, β and γ kafirins (Rooney, 1996). The γ kafirin is mainly located in the peripheral area of protein bodies and is resistant to proteases (Weaver *et al.*, 1998). Also β and γ kafirins are known to form extensive intermolecular disulfide-bond complexes during seed development, and cooking or heating processes, that would consequently decrease their digestibility by proteases (Weaver *et al.*, 1998). Therefore, the protein matrix in sorghum, particularly in the peripheral area of the corneous endosperm region may not have been completely digested by the pepsin treatment, and thus enzyme accessibility to starch granules may not have been maximised. In support of this, the electron microscopy images indicated that the peripheral area of the corneous endosperm region in sorghum was not completely digested. Rooney and Pflugfelder (1986) have shown that the corneous region of endosperm, in comparison to the floury region, is more resistant to enzymic breakdown. However, because the current study indicated that the protein matrix in the remaining region of sorghum's endosperm (i.e., the floury region), which comprises the greater proportion of endosperm, was completely digested by pepsin treatment, the lower GRI in sorghum may not be solely related to the physicochemical properties of its protein matrix.

The current study showed that there was no relationship between the crude protein content and the GRI values for all grains. This suggests that the physical and chemical properties of the protein matrix such as its composition and structure, but not the absolute protein content, may play a role in starch digestibility. To understand this further, identifying those chemical and physical characteristics influencing the extent of protein digestibility of grains in conjunction with the GRI assay may provide a better insight into the effects of protein on starch digestibility in monogastric animals.

## Chapter Eight – Thesis Discussion

The studies reported in this thesis have shown wide differences in chemical and physical characteristics between barley, sorghum and wheat and between cultivars within each grain type, which are likely to influence energy availability. The glucose release index (GRI) was developed as a way to detect the potential influence of some chemical and physical characteristics of grains on starch digestibility. From the eight physical and chemical characteristics investigated, five in barley, four in sorghum, and three in wheat were associated with the GRI (Table 8.1). It can be concluded that the physical and chemical properties related to starch digestion were grain-type specific. Consequently the characteristics, which can be exploited in the selection of grains with high starch digestibility, are particular to the grain type. However, the GRI assay cannot be used in order to establish the order of importance of these factors in starch digestibility under *in vivo* conditions. The physical and chemical characteristics of the grains and their relationship to the GRI are discussed under two major categories: 1) starch-related factors and 2) non-starch-related factors.

### 8.1 Starch-related factors

The starch content of grains was not related to the GRI. However, the starch content was associated with the total glucose content between and within the grain types. For example, sorghum's starch content was higher than in barley and consequently more glucose was produced following its *in vitro* digestion compared to barley, despite sorghum's low GRI (Appendix 3.2, Chapter 3). This result reinforces the recommendation of Englyst *et al.*, (1992), that the starch digestibility index values of grains (such as the GRI used in this study), should be combined with information regarding their total starch content or their total glucose content in order to predict the glycaemic response and thus potentially the nutritional quality of grains for pigs or poultry.

The variation in starch granule size did not account for differences in the GRI between cultivars within each grain type. However, between grain types, the higher number of A-type (large) starch granules in sorghum compared to barley and wheat could partly explain the lower starch digestibility in sorghum. A lower ratio of starch granule surface area to volume (resulting from an increase in the number of A-type starch granules)

would reduce the contact between digestive enzymes and starch molecules, thus decreasing the GRI values of sorghum compared to barley and wheat.

In general, the variation in the physical and chemical characteristics of starch in wheat cultivars did not account for differences in GRI (Chapter 4). In contrast, the variation in the amylose to amylopectin ratio in barley and sorghum cultivars showed a significant relationship with their GRI values (Table 8.1). In addition, the gelatinisation properties of barley as assessed in Chapter 4 displayed a significant association with their GRI values (Chapter 4). Therefore, further characterisation of these physical and chemical properties of starch in barley and sorghum, but not in wheat, can potentially provide information on the nutritional quality of these grains.

## 8.2 Non-starch related factors

### *8.2.1 Non-starch polysaccharides*

The significance of the relationship between the physical and chemical properties of NSP with their anti-nutritional effects in pigs and poultry has been demonstrated in different grain types (Choct and Annison, 1990) and cultivars within a grain type (Taverner and Farrell, 1981; Choct *et al.*, 1993; Baidoo and Liu, 1998). In order to evaluate the physical properties of NSP in cereal grains, simple acid and water extract viscosity assays were conducted as indirect predictors of NSP anti-nutritional properties (Chapter 5). Results revealed that variation in the extract viscosity properties of NSP in cultivars of barley and wheat but not sorghum grains could be quantified using the current rapid assay. The low NSP content in sorghum grains could explain why significant differences in viscosity between cultivars of sorghum were not detected (Chapter 5).

It has been demonstrated in this thesis (Chapter 5) and by others (Rotter *et al.*, 1989) that the solubility of NSP and their viscous properties can vary under different pH conditions in grain extracts. It is known that the pH varies along the gastro intestinal tract of animals and therefore, it could be expected that the viscous properties of NSP could also change accordingly. Consequently, it was suggested that determining the viscous properties of NSP in barley and wheat under different pH extraction conditions (in addition to the conditions used in the present study) may provide a better insight into the anti-nutritional properties of NSP in pigs and poultry.

**Table 8.1 The relationship between physical and chemical characteristics of grains with their glucose release index, which is an indicator of starch digestibility of grains.**

| | | | Between grain types | Within grains types (different cultivars) | | |
|---|---|---|---|---|---|---|
| **Origin** | **Characteristics of grains** | | | **Barley** | **Sorghum** | **Wheat** |
| Starch related factors | Chemical | Starch content | √ [1] | x [2] | x | x |
| | Physical | Starch granules size and distribution | √ | x | x | x |
| | Chemical | Amylose / amylopectin ratio | x | √ | √ | x |
| | Both | Gelatinisation properties | √ | √ | x | x |
| Non-starch related factors | Both | Viscosity | √ | √ | x | √ |
| | Chemical | NSP content and compositional profile | √ | √ | √ | √ |
| | Physical | Hardness and milling properties | √ | √ | √ | x |
| | Chemical | Protein matrix | √ | x | √ | √ |

[1] Associated with starch digestibility
[2] No significant relationship with starch digestion

### *8.2.2 Milling quality of grains*

Results in Chapter 6 indicated that in cultivars of each grain type, different milling processes influenced the GRI. This result highlights the importance of kernel hardness on the ultimate starch digestion of grains. Variation in grain hardness may result in variations in particle size distribution following milling which, in turn, can influence starch digestibility by influencing the available surface area for accessibility of digestive enzymes. However, The significance of variation in grain particle size distribution an pig production have been demonstrated (Wondra *et al.*, 1995c). However the relationship between grain hardness and particle size distribution following milling requires further investigation.

The variation in grain hardness between cultivars of barley and sorghum, but not in wheat, was associated with variation in GRI. Therefore in barley and sorghum, a grain hardness index can potentially be used for predicting the performance of grains after milling. Such information may be used to select the most appropriate milling process to maximise the starch digestion of grains, or to select cultivars that have more desirable milling qualities.

### *8.2.3 Protein matrix*

It has been demonstrated that variations in the properties of the protein matrix surrounding grain starch granules can influence starch digestibility in animals (Hibberd *et al.*, 1985; Owens *et al.*, 1986; Garnsworthy and Wiseman, 2000). Results in Chapter 7 revealed that between cultivars of sorghum and wheat, the digestion of protein matrix was positively associated with the GRI values. Thus investigations to assess variations in the physical and chemical properties of protein matrix in sorghum and wheat grains may be used to predict more accurately the available energy values of grains for pigs and poultry. For barley, digestion of the protein matrix reduced the GRI, suggesting that the protein matrix surrounding starch granules was not limiting starch digestion for this grain type. The added pepsin may in fact, have reduced the activity of enzymes such as α-amylose.

## 8.3 Future research direction

The results presented in the current work have opened up avenues for further investigations into two major areas as follows:

1) To assess the consequences of variations in starch digestibility between grain types and cultivars on pig and poultry performance. For example, it has been indicated that the availability of glucose can influence feed efficiency in animals by affecting the synchronisation in the supply of glucose and amino acids to animal tissues (Rerat *et al.*, 1979; George *et al.*, 1988). Furthermore, variation in the blood glucose levels as a result of the variation in starch digestibility of a diet can influence the blood insulin concentration (Behall *et al.*, 1988), and plasma lipid content (Topping *et al.*, 1988; Behall and Howe, 1995; Lerer-Metzger *et al.*, 1996). Therefore, a better understanding of the effects of the variation of grain starch digestibility on the metabolic status of pigs and poultry can be utilised to modify diets (e.g. formulating diets with specific starch digestibility properties, similar to human food industries) in order to increase feed utilisation and profitability. In order to achieve this, the first step is to conduct appropriate *in vivo* tests to determine the portal flux of glucose and volatile fatty acids in pigs and poultry such as those reported by Rerat *et al.* (1984b), van Leeuwen *et al.* (1995) and Bach Knudsen *et al.* (1997).

2) Minimising the variation of starch digestibility from grains fed to pigs and poultry by modifying the physical and chemical characteristics of grains that can influence starch digestibility. A reduction in the digestion of feed by animal digestive enzymes can result in microbial overgrowth and more importantly the ratios of microbial species that coexist in the gut would be changed along the gastrointestinal tract (Choct *et al.*, 1996; Smits and Annison, 1996; de Lange, 2000) that could affect the available energy of diets and the gut health of animals (Pluske *et al.*, 1996). Therefore, minimising variation in starch digestibility of a diet would be even more critical when antibiotics are removed from pig and poultry diets (Bedford, 2000b), since gut health through nutrition will become increasingly important.

Some potential examples for reducing the variation of starch digestibility between grain types and cultivars are listed below:

1) The results reported for the gelatinisation properties of starch in barley and potentially sorghum may provide a guide for the administration of the appropriate heat treatment during diet manufacturing since over-heating of grains can cause the formation of resistant starch and consequently lower the digestibility (Atwell *et al.*, 1988).

2) The viscosity of grain extracts is mainly attributed to the soluble NSP content (Lzydorczk *et al.*, 1991; Saulnier *et al.*, 1995). A highly viscous diet could depress starch and protein digestion by animals and increase the microfloral population in the gastro intestinal tract (Bedford, 2000b). Currently, in order to reduce the viscous properties of NSP in grains, an exogenous carbohydrase enzyme is commonly added to pig (Partridge, 2001) and poultry diets (Choct, 2001). It has been indicated that grain extract viscosity can be used to predict the response of viscosity-reducing enzymes (Choct, 2001). The current studies demonstrate that the simple acid and water extract methods can be used as rapid and reproducible *in vitro* techniques for determining the possible response of exogenous NSP-degrading enzymes in barley and wheat grains for pigs and poultry.

3) The ΔGRI values, grain hardness index and, potentially, particle size distribution, can all be used to predict the variation in the milling quality of grains. This information can then be used to select the most appropriate grinding process for each grain or to select grains with desirable milling quality in order to maximise energy utilisation of grains and minimise their variation in digestibility.

## 8.4 Conclusion

In conclusion, the results showed that the GRI of grains varied significantly between grain types and even within each grain type. In support of the main hypothesis of this work, the variation in physical and chemical characteristics of grains can influence their starch digestion. However, the physical and chemical properties were grain type specific. Based on these findings, it is suggested that grain-specific assays are required to predict the available energy values of grains. The classification of grains based on their physical and chemical properties which can influence their starch digestibility may be used as an indirect predictor of their available energy values. In addition, achieving a better understanding of physical and chemical properties of grains may also be helpful in reducing their variation in available energy through the use of appropriate treatments and feed processing.

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# Appendices

**Appendix 1.1 The place of conduct, statistical analyses used and original purpose of the assays presented in the chapters of the current thesis.**

| Location | Assay | Conducted by | Statistical analyses | Purpose |
|---|---|---|---|---|
| Chapter 3 | Total starch content | UNE[1] | Author[7] | PGLP[6] |
| | Total digestible starch content | UNE[1] | Author | PGLP |
| | Glucose release index | Author | Author | Current thesis |
| Chapter 4 | Determination of starch granule surface area | Author | Author | Current thesis |
| | Starch viscoelasticity | BRI[2] | Author | PGLP |
| | Amylose content of starch | BRI[2] | Author | PGLP |
| Chapter 5 | NSP[3] chemical composition | UNE[1] | Author | PGLP |
| | Acid extract viscosity | Author | Author | Current thesis |
| | Water extract viscosity | Author | Author | Current thesis |
| Chapter 6 | ΔGRI[4] | Author | Author | Current thesis |
| | Grain hardness index | BRI[2] | Author | PGLP |
| Chapter 7 | GRI[5]+ protease treatment | Author | Author | Current thesis |
| | Crude protein | Western Australian chemistry Center laboratory | Author | PGLP |
| | Electron microscopy of grains | Author | Author | Current thesis |

[1] School of rural science and agriculture at the University of New England
[2] Bread Research Institute
[3] non-starch polysaccharides
[4] proportional differences of glucose release index (GRI 0.5mm milled grains – GRI roller milled or 2mm milled grains)
[5] glucose release index
[6] Premium Grains for Livestock Program
[7] Author = M R Zarrinkalam

**Appendix 3.1. The source, location and cultivars of the selected barley, sorghum and wheat samples sourced from the Premium Grains for Livestock Program.**

| Grain type | Unique ID | Sample | Source | Location | Cultivar |
|---|---|---|---|---|---|
| BARLEY | FG96B.3801 | 3801 | LADLOW | HORSHAM | ARAPILES |
| BARLEY | FG96B.3804 | 3804 | GOLDER | BRIM | CHEBEC |
| BARLEY | FG96B.3807 | 3807 | LADLOW | HORSHAM | GALLEON |
| BARLEY | FG96B.3808 | 3808 | PBI | NARRABRI | GRIMMET |
| BARLEY | FG96B.3811 | 3811 | HART | JUNEE | SCHOONER |
| BARLEY | FG96B.3814 | 3814 | HART | JUNEE | SKIFF |
| BARLEY | FG96B.3815 | 3815.1 | HART | JUNEE | TANTANGARA |
| BARLEY | FG97B.3817 | 3817 | ESDAILE | MOREE | LINDWALL |
| BARLEY | FG97B.3818 | 3818 | NOKES | FORBES | MUNDAH |
| BARLEY | FG97B.3819 | 3819 | NOKES | FORBES | SCHOONER |
| BARLEY | FG97B.3820 | 3820 | NOKES | FORBES | SLOOP |
| BARLEY | INC98NB.3702 | 3702 | UNKNOWN | NARRABRI | REINETTE |
| BARLEY | LSF97B.3904 | 3904 | 1997 | FORBES | SCHOONER |
| BARLEY | LSF97B.3906 | 3906 | 1997 | FORBES | TANTANGARA |
| BARLEY | LSJ97B.3909 | 3909 | 1997 | JUNEE | TANTANGARA |
| BARLEY | LSN97B.3902 | 3902 | 1997 | NARRABRI | GALLEON |
| BARLEY | LSN98B.3912 | 3912 | 1998 | NARRABRI | TANTANGARA |
| BARLEY | NT98B+N.3951 | 3951 | 200 KG/HA | NARRABRI | GILBERT |
| SORGHUM | FG97S.7801 | 7801 | ALAN McTAGGART | BILOELA | BUSTER |
| SORGHUM | FG97S.7802 | 7802 | HERMITAGE | WARWICK | BUSTER |
| SORGHUM | FG97S.7804 | 7804 | HERMITAGE | WARWICK | MR 31 |
| SORGHUM | FG97S.7805 | 7805 | HERMITAGE | WARWICK | THUNDER |
| SORGHUM | FG97S.7811 | 7811 | ALAN McTAGGART | BILOELA | BOOMER |
| SORGHUM | FG97S.7812 | 7812 | RATHIE | BELLATA | BOOMER |
| SORGHUM | FG98S.7814 | 7814 | ESDAILE | MOREE | GOLD RUSH |
| SORGHUM | FG98S.7815 | 7815 | RATHIE | BELLATA | MR 31 |
| SORGHUM | FG98S.7816 | 7816 | PHILIP BRODIE GRAINS | TOOWOOMBA | MR 31 |
| SORGHUM | FG98S.7817 | 7817 | TONY McCOSKER | HERMITAGE | MR 31 |
| SORGHUM | FG98S.7818 | 7818 | RATHIE | BELLATA | PAC 2391 |
| SORGHUM | FG98S.7819 | 7819 | RATHIE | BELLATA | SUCCESS 42 |
| SORGHUM | FG98S.7820 | 7820 | ALAN McTAGGART | BILOELA | THUNDER |
| SORGHUM | FG98S.7827 | 7827 | B.HENSELL | BILOELA | NORMAL ISOLINE |
| SORGHUM | FG99S.7830 | 7830 | P.BARDSLEY | MOREE | MR MAXI |
| WHEAT | FG97W.1810 | 1810 | HOFFMAN | LOCKHART | JANZ |
| WHEAT | FG98W.1809 | 1809 | GOLLASCH | WALLACETOWN | JANZ (FROSTED) |
| WHEAT | FG99W.1818 | 1818 | SARDI | ROSEDALE | KUKARI |
| WHEAT | LSF97W.1906 | 1906 | 1997 | FORBES | CURRAWONG |
| WHEAT | LSF98W.1913 | 1913 | 1998 | FORBES | SUNSTATE |
| WHEAT | LSF98W.1914 | 1914 | 1998 | FORBES | JANZ |
| WHEAT | LSJ97W.1909 | 1909 | 1997 | JUNEE | CURRAWONG |
| WHEAT | LSN97W.1901 | 1901 | 1997 | NARRABRI | SUNSTATE |
| WHEAT | LSN97W.1902 | 1902 | 1997 | NARRABRI | JANZ |
| WHEAT | LSN97W.1903 | 1903 | 1997 | NARRABRI | CURRAWONG |

**Appendix 3.2 The glucose release index of the selected barley, sorghum and wheat samples sourced from the Premium Grains for Livestock Program.**

| Id | Cultivars | % Released Glucose[1] | %Total released Glucose[2] | % GRI[3] |
|---|---|---|---|---|
| FG96B.3801 | Arapiles | 21.0 | 56.2 | 37.3 |
| FG96B.3804 | Chebec | 20.2 | 54.9 | 36.9 |
| FG96B.3807 | Galleon-H | 21.2 | 55.7 | 38.0 |
| FG96B.3808 | Grimmet | 16.5 | 48.8 | 33.7 |
| FG96B.3811 | Schooner-J | 17.8 | 52.8 | 33.8 |
| FG96B.3814 | Skiff | 18.9 | 51.9 | 36.3 |
| FG96B.3815 | Tantangara-J2 | 19.2 | 53.1 | 37.1 |
| FG97B.3817 | Lindwall | 15.9 | 52.9 | 29.6 |
| FG97B.3818 | Mundah | 15.8 | 50.0 | 31.5 |
| FG97B.3819 | Schooner-F | 13.7 | 49.7 | 27.5 |
| FG97B.3820 | Sloop | 13.7 | 51.8 | 27.2 |
| INC98NB.3702 | Reinette | 14.8 | 54.3 | 27.3 |
| LSF97B.3904 | Schooner-F2 | 17.0 | 52.6 | 32.2 |
| LSF97B.3906 | Tantangara-F | 18.6 | 52.8 | 35.2 |
| LSJ97B.3909 | Tantangara-J | 17.7 | 52.8 | 33.5 |
| LSN97B.3902 | Galleon-N | 15.8 | 53.5 | 29.4 |
| LSN98B.3912 | Tantangara-N | 23.7 | 53.0 | 44.8 |
| NT98B+N.3951 | Gilbert | 22.8 | 51.2 | 44.6 |
| FG97S.7801 | Buster-B | 21.9 | 69.7 | 31.5 |
| FG97S.7802 | Buster-W | 20.5 | 72.0 | 28.5 |
| FG97S.7804 | MR 31 | 19.0 | 69.1 | 27.5 |
| FG97S.7805 | Thunder | 21.7 | 68.8 | 31.5 |
| FG97S.7811 | Boomer-BI | 19.6 | 68.8 | 28.4 |
| FG97S.7812 | Boomer-BE | 12.9 | 66.6 | 24.4 |
| FG98S.7814 | Gold rush | 17.1 | 65.7 | 26.0 |
| FG98S.7815 | MR 31-B | 20.3 | 70.3 | 28.8 |
| FG98S.7816 | MR 31-T | 18.5 | 65.7 | 28.2 |
| FG98S.7817 | MR 31-H | 19.9 | 65.1 | 30.5 |
| FG98S.7818 | PAC 2391 | 16.9 | 66.0 | 25.6 |
| FG98S.7819 | Success 42 | 21.2 | 65.8 | 32.2 |
| FG98S.7820 | Thunder | 17.5 | 64.3 | 27.2 |
| FG98S.7827 | Normal isoline | 35.4 | 65.5 | 54.0 |
| FG99S.7830 | Mr maxi | 36.9 | 67.7 | 54.5 |
| FG97W.1810 | Janz-L | 17.9 | 56.1 | 31.9 |
| FG98W.1809 | Janz (frosted) | 23.9 | 47.1 | 50.7 |
| FG99W.1818 | Kukari | 22.4 | 55.1 | 40.6 |
| LSF97W.1906 | Currawong-F | 24.5 | 58.3 | 42.1 |
| LSF97W.1909 | Currawong-J | 20.5 | 59.7 | 34.3 |
| LSF98W.1913 | Sunstate-F | 29.2 | 62.4 | 46.9 |
| LSF98W.1914 | Janz-F | 25.4 | 60.7 | 41.9 |
| LSN97W.1901 | Sunstate-N | 24.2 | 60.8 | 39.8 |
| LSN97W.1902 | Janz-N | 30.9 | 58.3 | 52.9 |
| LSN97W.1903 | Currawong-N | 21.7 | 58.1 | 37.4 |

[1]% released glucose from starch, defined using the enzymic conditions described in section 3.2.3

[2] % total glucose released from the complete hydrolysis of starch using the standard enzymic conditions described in section 3.2.2

[3] GRI = Glucose Release Index: is the proportion of the released glucose[1] to the total glucose[2].

**Appendix 3.3 The proportion of dry mater, total starch, resistant starch and total digestible starch content. Data sourced from the Premium Grains for Livestock Program.**

| Type | Id | Cultivars | % DM[1] | %Total Starch[2] | %Resistant Starch[3] | %Digestible Starch[4] |
|---|---|---|---|---|---|---|
| Barley | FG96B.3801 | Arapiles | 89.0 | 63.2 | 2.9 | 60.3 |
| Barley | FG96B.3804 | Chebec | 89.2 | 61.6 | 0.2 | 61.4 |
| Barley | FG96B.3807 | Galleon-H | 88.4 | 63.0 | 2.4 | 60.7 |
| Barley | FG96B.3808 | Grimmet | 88.7 | 55.0 | 2.3 | 52.7 |
| Barley | FG96B.3811 | Schooner-J | 88.9 | 59.3 | 2.3 | 57.0 |
| Barley | FG96B.3814 | Skiff | 88.9 | 58.3 | 1.3 | 57.0 |
| Barley | FG96B.3815 | Tantangara-J2 | 88.9 | 59.7 | 1.3 | 58.4 |
| Barley | FG97B.3817 | Lindwall | 89.0 | 59.5 | 2.7 | 56.8 |
| Barley | FG97B.3818 | Mundah | 90.0 | 55.6 | 1.9 | 53.6 |
| Barley | FG97B.3819 | Schooner-F | 90.9 | 54.7 | 0.4 | 54.3 |
| Barley | FG97B.3820 | Sloop | 89.9 | 57.7 | 3.3 | 54.4 |
| Barley | INC98NB.3702 | Reinette | 89.7 | 60.5 | 2.8 | 57.7 |
| Barley | LSF97B.3904 | Schooner-F2 | 90.2 | 58.3 | 0.0 | 58.3 |
| Barley | LSF97B.3906 | Tantangara-F | 90.1 | 58.6 | 0.4 | 58.1 |
| Barley | LSJ97B.3909 | Tantangara-J | 90.0 | 58.6 | 0.0 | 58.6 |
| Barley | LSN97B.3902 | Galleon-N | 90.3 | 59.3 | 2.6 | 56.7 |
| Barley | LSN98B.3912 | Tantangara-N | 90.1 | 58.8 | 1.9 | 56.9 |
| Barley | NT98B+N.3951 | Gilbert | 89.4 | 57.3 | 2.6 | 54.7 |
| Sorghum | FG97S.7801 | Buster-B | 87.4 | 79.7 | 3.7 | 76.0 |
| Sorghum | FG97S.7802 | Buster-W | 88.2 | 81.6 | 2.8 | 78.9 |
| Sorghum | FG97S.7804 | MR 31 | 88.0 | 78.5 | 2.2 | 76.3 |
| Sorghum | FG97S.7805 | Thunder | 88.2 | 78.0 | 0.6 | 77.4 |
| Sorghum | FG97S.7811 | Boomer-BI | 88.1 | 78.1 | 4.7 | 73.4 |
| Sorghum | FG97S.7812 | Boomer-BE | 89.3 | 74.5 | 3.7 | 70.8 |
| Sorghum | FG98S.7814 | Gold rush | 89.0 | 73.9 | 1.8 | 72.1 |
| Sorghum | FG98S.7815 | MR 31-B | 89.5 | 78.6 | 3.5 | 75.1 |
| Sorghum | FG98S.7816 | MR 31-T | 88.6 | 74.2 | 0.5 | 73.7 |
| Sorghum | FG98S.7817 | MR 31-H | 88.3 | 73.7 | 3.1 | 70.7 |
| Sorghum | FG98S.7818 | PAC 2391 | 89.7 | 73.5 | 3.2 | 70.4 |
| Sorghum | FG98S.7819 | Success 42 | 89.7 | 73.3 | 3.2 | 70.1 |
| Sorghum | FG98S.7820 | Thunder | 88.0 | 73.1 | 0.1 | 73.0 |
| Sorghum | FG98S.7827 | Normal isoline | 89.0 | 73.6 | 1.3 | 72.4 |
| Sorghum | FG99S.7830 | Mr maxi | 89.5 | 75.7 | 0.0 | 75.7 |
| Wheat | FG97W.1810 | Janz-L | 89.9 | 62.5 | 4.9 | 57.6 |
| Wheat | FG98W.1809 | Janz (frosted) | 90.6 | 52.0 | 0.0 | 52.0 |
| Wheat | FG99W.1818 | Kukari | 89.0 | 61.9 | 5.4 | 56.5 |
| Wheat | LSF97W.1906 | Currawong-F | 89.9 | 64.9 | 8.6 | 56.3 |
| Wheat | LSF97W.1909 | Currawong-J | 90.1 | 66.3 | 2.6 | 63.7 |
| Wheat | LSF98W.1913 | Sunstate-F | 90.0 | 69.3 | 3.2 | 66.1 |
| Wheat | LSF98W.1914 | Janz-F | 90.1 | 67.3 | 3.7 | 63.6 |
| Wheat | LSN97W.1901 | Sunstate-N | 89.4 | 68.1 | 4.2 | 63.9 |
| Wheat | LSN97W.1902 | Janz-N | 89.5 | 65.1 | 3.3 | 61.8 |
| Wheat | LSN97W.1903 | Currawong-N | 90.0 | 64.6 | 7.3 | 57.3 |

[1] Dry Matter

[2 3 4] Data reported as a proportion of dry matter

**Appendix 4.1 The physical characteristics of A-type and B-type starch granules in selected barley samples.**

| Unique ID | Barley Cultivars | Total area of A-type SGs[1] ($\mu m^2$) | Mean area of A-type SGs[1] ($\mu m^2$) | Median of A-type SGs[1] ($\mu m^2$) | Total area of B-type SGs[1] ($\mu m^2$) | Mean area of B-type SGs1 ($\mu m^2$) | Median of B-type SGs[1] ($\mu m^2$) | Ratio of A-type to B-type SGs[1] |
|---|---|---|---|---|---|---|---|---|
| FG96B.3801 | Arapiles | 48879 | 239.4 | 213.95 | 20226 | 8.5 | 5.36 | 9.7 |
| FG96B.3804 | Chebec | 55540 | 271.5 | 231.10 | 17026 | 7.0 | 4.43 | 9.9 |
| FG96B.3807 | Galleon-H | 139048 | 268.6 | 245.2 | 17598 | 10.1 | 6.2 | 3.8 |
| FG96B.3808 | Grimmet | 75989 | 279.5 | 243.70 | 18901 | 8.2 | 5.13 | 6.8 |
| FG96B.3811 | Schooner-J | 36210 | 217.8 | 192.85 | 16616 | 6.7 | 3.50 | 12.1 |
| FG96B.3814 | Skiff | 72717 | 347.1 | 316.65 | 20884 | 8.7 | 5.83 | 11.9 |
| FG96B.3815 | Tantangara-J2 | 35791 | 306.2 | 262.90 | 19724 | 7.8 | 5.83 | 17.0 |
| FG97B.3817 | Lindwall | 85981 | 310.7 | 274.30 | 17400 | 7.5 | 4.43 | 6.5 |
| FG97B.3818 | Mundah | 29357 | 310.0 | 299.55 | 19653 | 7.6 | 5.60 | 21.6 |
| FG97B.3819 | Schooner-F | 73013 | 224.9 | 201.95 | 17561 | 7.9 | 4.20 | 6.4 |
| FG97B.3820 | Sloop | 159172 | 313.3 | 295.00 | 16294 | 8.1 | 4.90 | 4.0 |
| INC98NB.3702 | Reinette | 110286 | 353.5 | 340.00 | 15649 | 8.8 | 5.10 | 5.7 |
| LSF97B.3904 | Schooner-F2 | 64191 | 210.8 | 189.45 | 17214 | 7.7 | 3.96 | 8.4 |
| LSF97B.3906 | Tantangara-F | 46353 | 303.3 | 260.25 | 20589 | 8.4 | 5.60 | 13.5 |
| LSJ97B.3909 | Tantangara-J | 35095 | 287.2 | 261.15 | 19702 | 7.8 | 5.60 | 21.9 |
| LSN97B.3902 | Galleon-N | 34575 | 228.6 | 199.45 | 17381 | 6.9 | 4.90 | 13.6 |
| LSN98B.3912 | Tantangara-N | 60873 | 299.7 | 266.15 | 20250 | 8.5 | 5.83 | 9.2 |
| NT98B+N.3951 | Gilbert | 91979 | 288.2 | 260.45 | 22237 | 10.3 | 6.76 | 6.4 |

[1] starch granules

**Appendix 4.2 The physical characteristics of A-type and B-type starch granules in selected sorghum samples.**

| Unique ID | Barley Cultivars | Total area of A-type SGs[1] ($\mu m^2$) | Mean area of A-type SGs[1] ($\mu m^2$) | Median of A-type SGs[1] ($\mu m^2$) | Total area of B-type SGs[1] ($\mu m^2$) | Mean area of B-type SGs1 ($\mu m^2$) | Median of B-type SGs[1] ($\mu m^2$) | Ratio of A-type to B-type SGs[1] |
|---|---|---|---|---|---|---|---|---|
| FG97S.7801 | Buster-B | 244071.3 | 277.3 | 253.0 | 38250.1 | 28.3 | 18.6 | 1.6 |
| FG97S.7802 | Buster-W | 174273.9 | 240.0 | 219.8 | 38876.9 | 29.3 | 20.1 | 1.9 |
| FG97S.7804 | MR 31 | 222411.8 | 225.1 | 210.8 | 36803.8 | 33.4 | 24.3 | 1.2 |
| FG97S.7805 | Thunder | 227675.2 | 239.4 | 225.6 | 31779.6 | 28.9 | 19.2 | 1.3 |
| FG97S.7811 | Boomer-BI | 311668.1 | 300.8 | 281.9 | 33208.2 | 32.0 | 22.3 | 1.0 |
| FG97S.7812 | Boomer-BE | 344049.9 | 256.2 | 241.3 | 29178.2 | 34.4 | 26.4 | 0.7 |
| FG98S.7814 | Gold rush | 250879.5 | 222.2 | 205.2 | 40505.1 | 33.6 | 26.5 | 1.1 |
| FG98S.7815 | MR 31-B | 251063.7 | 253.7 | 240.7 | 28365.1 | 27.5 | 16.4 | 1.1 |
| FG98S.7816 | MR 31-T | 195682.3 | 241.2 | 228.0 | 31392.6 | 25.3 | 14.9 | 1.6 |
| FG98S.7817 | MR 31-H | 169361.1 | 213.3 | 192.2 | 38530.3 | 30.4 | 21.0 | 2.3 |
| FG98S.7818 | PAC 2391 | 138950.8 | 218.3 | 196.6 | 39911.7 | 24.3 | 12.6 | 3.3 |
| FG98S.7819 | Success 42 | 182271.2 | 230.3 | 210.9 | 39855.9 | 26.3 | 15.7 | 1.7 |
| FG98S.7820 | Thunder | 235600.8 | 234.1 | 216.7 | 35489.4 | 32.0 | 23.1 | 1.0 |
| FG98S.7827 | Normal isoline | 245556.7 | 255.3 | 219.9 | 33484.8 | 29.8 | 17.5 | 1.2 |
| FG99S.7830 | Mr maxi | 253370.6 | 283.8 | 248.0 | 30236.1 | 25.9 | 13.8 | 1.3 |

[1] starch granules

**Appendix 4.3 The physical characteristics of A-type and B-type starch granules in selected wheat samples.**

| Unique ID | Barley Cultivars | Total area of A-type SGs[1] (μm$^2$) | Mean area of A-type SGs[1] (μm$^2$) | Median of A-type SGs[1] (μm$^2$) | Total area of B-type SGs[1] (μm$^2$) | Mean area of B-type SGs1 (μm$^2$) | Median of B-type SGs[1] (μm$^2$) | Ratio of A-type to B-type SGs[1] |
|---|---|---|---|---|---|---|---|---|
| FG97W.1810 | Janz-L | 41565.8 | 241.5 | 204.50 | 165543.1 | 14.3 | 8.63 | 11.8 |
| FG98W.1809 | Janz (frosted) | 61495.2 | 255.5 | 207.95 | 33832.2 | 15.9 | 10.26 | 9.0 |
| FG99W.1818 | Kukari | 24931.6 | 304.0 | 250.40 | 26660.1 | 13.6 | 17.49 | 26.6 |
| LSF97W.1906 | Currawong-F | 46966.1 | 302.2 | 240.35 | 30671.9 | 15.0 | 9.76 | 14.0 |
| LSF97W.1909 | Currawong-J | 42626.1 | 307.9 | 242.85 | 34297.1 | 15.8 | 10.50 | 15.7 |
| LSF98W.1913 | Sunstate-F | 49882.5 | 320.3 | 258.00 | 25544.0 | 13.4 | 8.86 | 12.4 |
| LSF98W.1914 | Janz-F | 33929.7 | 317.8 | 273.05 | 38655.1 | 18.1 | 14.23 | 18.1 |
| LSN97W.1901 | Sunstate-N | 54317.9 | 280.6 | 231.50 | 28947.8 | 15.0 | 18.89 | 11.2 |
| LSN97W.1902 | Janz-N | 51413.5 | 351.3 | 265.10 | 30125.4 | 14.8 | 10.03 | 16.2 |
| LSN97W.1903 | Currawong-N | 79287.2 | 384.5 | 301.75 | 28328.2 | 15.5 | 10.50 | 9.3 |

[1] starch granules

**Appendix 4.4 The amylose/amylopectin ratio, and viscoelasticity properties of starch in barley, sorghum and wheat samples. Data sourced from the Premium Grains for Livestock Program**

| Unique ID | Cultivar | Amlyose / amylopectin | Peak Viscosity (RVU[1]) | Peak time (minute) | Holding viscosity (RVU[1])r | Final viscosity (RVU[1]) |
|---|---|---|---|---|---|---|
| FG96B.3801 | Arapiles | 0.50 | 86 | 5.9 | 71 | 116 |
| FG96B.3804 | Chebec | 0.55 | 87 | 5.9 | 63 | 110 |
| FG96B.3807 | Galleon-H | 0.54 | 117 | 5.8 | 83 | 138 |
| FG96B.3808 | Grimmet | 0.54 | 89 | 6.1 | 66 | 96 |
| FG96B.3811 | Schooner-J | 0.54 | 102 | 5.9 | 73 | 145 |
| FG96B.3814 | Skiff | 0.52 | 139 | 6.1 | 102 | 163 |
| FG96B.3815 | Tantangara-J2 | 0.50 | 128 | 6.2 | 94 | 138 |
| FG97B.3817 | Lindwall | 0.55 | 94 | 5.7 | 57 | 111 |
| FG97B.3818 | Mundah | 0.56 | 95 | 6.0 | 70 | 124 |
| FG97B.3819 | Schooner-F | 0.62 | 51 | 6.5 | 45 | 70 |
| FG97B.3820 | Sloop | 0.62 | 64 | 6.4 | 52 | 70 |
| INC98NB.3702 | Reinette | 0.54 | 116 | 6.4 | 93 | 139 |
| LSF97B.3904 | Schooner-F2 | 0.60 | 66 | 5.9 | 52 | 92 |
| LSF97B.3906 | Tantangara-F | 0.61 | 59 | 6.0 | 48 | 72 |
| LSJ97B.3909 | Tantangara-J | 0.55 | 104 | 5.9 | 73 | 120 |
| LSN97B.3902 | Galleon-N | 0.55 | 125 | 6.2 | 87 | 165 |
| LSN98B.3912 | Tantangara-N | 0.51 | 130 | 6.2 | 99 | 165 |
| NT98B+N.3951 | Gilbert | 0.52 | 119 | 6.3 | 91 | 137 |
| FG97S.7801 | Buster-B | 0.49 | 142 | 5.4 | 112 | 152 |
| FG97S.7802 | Buster-W | 0.53 | 138 | 5.5 | 112 | 143 |
| FG97S.7804 | MR 31 | 0.52 | 138 | 5.4 | 109 | 147 |
| FG97S.7805 | Thunder | 0.47 | 137 | 5.3 | 104 | 141 |
| FG97S.7811 | Boomer-BI | 0.55 | 127 | 5.4 | 106 | 146 |
| FG97S.7812 | Boomer-BE | 0.56 | 143 | 5.3 | 105 | 146 |
| FG98S.7814 | Gold rush | 0.55 | 137 | 5.4 | 110 | 146 |
| FG98S.7815 | MR 31-B | 0.57 | 134 | 5.4 | 103 | 138 |
| FG98S.7816 | MR 31-T | 0.52 | 138 | 5.6 | 112 | 158 |
| FG98S.7817 | MR 31-H | 0.53 | 139 | 5.4 | 112 | 161 |
| FG98S.7818 | PAC 2391 | 0.56 | 140 | 5.2 | 106 | 158 |
| FG98S.7819 | Success 42 | 0.53 | 137 | 5.3 | 106 | 161 |
| FG98S.7820 | Thunder | 0.51 | 136 | 5.3 | 106 | 152 |
| FG98S.7827 | Normal isoline | 0.46 | 154 | 6.1 | 103 | 157 |
| FG99S.7830 | Mr maxi | 0.56 | 160 | 5.5 | 123 | 179 |
| FG97W.1810 | Janz-L | 0.52 | 97 | 5.8 | 68 | 145 |
| FG98W.1809 | Janz (frosted) | 0.50 | 149 | 5.7 | 95 | 195 |
| FG99W.1818 | Kukari | 0.52 | 114 | 5.9 | 84 | 177 |
| LSF97W.1906 | Currawong-F | 0.60 | 88 | 5.8 | 62 | 126 |
| LSF97W.1909 | Currawong-J | 0.59 | 98 | 6.1 | 75 | 130 |
| LSF98W.1913 | Sunstate-F | 0.54 | 160 | 6.3 | 119 | 201 |
| LSF98W.1914 | Janz-F | 0.53 | 84 | 5.8 | 62 | 121 |
| LSN97W.1901 | Sunstate-N | 0.50 | 134 | 6.1 | 95 | 179 |
| LSN97W.1902 | Janz-N | 0.56 | 82 | 6.1 | 68 | 115 |
| LSN97W.1903 | Currawong-N | 0.58 | 78 | 6.1 | 63 | 108 |

[1] Rapid Visco Analyser Units: 1RVU=10 mPa s

**Appendix 5.1 The percent of soluble and insoluble non starch polysaccharides (NSP) in barley samples based on dry matter (DM) of grains. Data sourced from the Premium Grains for Livestock Program**

| Unique ID | Cultivar | % Insoluble NSP (DM) | | | | % Soluble NSP (DM) | | | | % Total (DM) |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Arabinoxylan | Cellulose | Other[1] | In[2]-Total | Arabinoxylan | B-Glucan | Other[1] | So[3] Total | |
| FG96B.3801 | Arapiles | 6.9 | 3.8 | 0.61 | 11.3 | 0.5 | 0.0 | 0.2 | 0.8 | 12.0 |
| FG96B.3804 | Chebec | 7.4 | 4.2 | 0.6 | 12.2 | 0.6 | 3.0 | 0.2 | 3.8 | 16.0 |
| FG96B.3807 | Galleon-H | 8.1 | 4.9 | 0.6 | 13.6 | 0.5 | 4.5 | 0.2 | 5.2 | 18.7 |
| FG96B.3808 | Grimmet | 7.7 | 4.8 | 0.7 | 13.2 | 0.6 | 5.4 | 0.2 | 6.2 | 19.4 |
| FG96B.3811 | Schooner-J | 7.1 | 4.3 | 0.6 | 12.1 | 0.1 | 4.0 | 0.1 | 4.2 | 16.3 |
| FG96B.3814 | Skiff | 5.7 | 3.5 | 0.5 | 9.7 | 0.1 | 4.2 | 0.1 | 4.5 | 14.2 |
| FG96B.3815 | Tantangara-J2 | 6.5 | 4.4 | 0.5 | 11.4 | 0.4 | 4.1 | 0.2 | 4.8 | 16.1 |
| FG97B.3817 | Lindwall | 6.9 | 3.9 | 0.5 | 11.4 | 0.5 | 4.8 | 0.2 | 5.5 | 16.9 |
| FG97B.3818 | Mundah | 7.8 | 5.6 | 0.6 | 14.0 | 0.2 | 5.3 | 0.1 | 5.6 | 19.6 |
| FG97B.3819 | Schooner-F | 7.9 | 5.3 | 0.6 | 13.9 | 0.1 | 3.6 | 0.2 | 4.0 | 17.9 |
| FG97B.3820 | Sloop | 7.9 | 5.7 | 0.6 | 14.2 | 0.2 | 2.9 | 0.3 | 3.3 | 17.5 |
| INC98NB.3702 | Reinette | 6.8 | 4.2 | 0.8 | 11.8 | 0.6 | 4.4 | 0.2 | 5.2 | 17.0 |
| LSF97B.3904 | Schooner-F2 | 6.6 | 3.8 | 0.7 | 11.1 | 0.5 | 4.2 | 0.3 | 5.0 | 16.1 |
| LSF97B.3906 | Tantangara-F | 7.5 | 3.7 | 0.8 | 12.0 | 0.5 | 4.3 | 0.3 | 5.1 | 17.0 |
| LSJ97B.3909 | Tantangara-J | 7.4 | 4.0 | 0.8 | 12.2 | 0.5 | 4.0 | 0.3 | 4.8 | 17.0 |
| LSN97B.3902 | Galleon-N | 5.5 | 3.2 | 0.8 | 9.5 | 0.6 | 4.2 | 0.2 | 5.0 | 14.5 |
| LSN98B.3912 | Tantangara-N | 6.2 | 3.5 | 0.6 | 10.3 | 0.5 | 4.1 | 0.2 | 4.8 | 15.1 |
| NT98B+N.3951 | Gilbert | 6.8 | 4.0 | 0.7 | 11.5 | 0.6 | 5.7 | 0.2 | 6.6 | 18.1 |

[1]NSP components exist as minor heteropolyers like xyluglucans, arabinogalactans etc.
[2] insoluble
[3] soluble

**Appendix 5.2 The percent of soluble and insoluble non starch polysaccharide (NSP) content in sorghum samples based on dry matter (DM) of grains. Data sourced from the Premium Grains for Livestock Program**

| Unique ID | Cultivar | % Insoluble NSP (DM) | | | | % Soluble NSP (DM) | | | | % Total (DM) |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Arabinoxylan | Cellulose | Other[1] | In[2]-Total | Arabinoxylan | B-Glucan | Other[1] | So[3] Total | |
| FG98S.7815 | MR 31-B | 2.58 | 2.66 | 0.42 | 5.66 | 0.09 | 0.26 | 0.15 | 0.49 | 6.15 |
| FG98S.7818 | PAC 2391 | 1.48 | 1.71 | 0.76 | 3.96 | 0.08 | 0.28 | 0.11 | 0.47 | 4.42 |
| FG97S.7811 | Boomer-BI | 2.94 | 1.50 | 8.33 | 12.77 | 0.09 | 0.25 | 0.15 | 0.49 | 13.26 |
| FG97S.7812 | Boomer-BE | 2.57 | 2.58 | 0.30 | 5.45 | 0.08 | 0.20 | 0.15 | 0.42 | 5.88 |
| FG97S.7804 | MR 31 | 1.90 | 2.19 | 0.32 | 4.41 | 0.08 | 0.20 | 0.15 | 0.43 | 4.85 |
| FG97S.7802 | Buster-W | 2.34 | 2.36 | 0.39 | 5.09 | 0.08 | 0.24 | 0.15 | 0.47 | 5.56 |
| FG97S.7805 | Thunder | 2.58 | 2.14 | 0.29 | 5.01 | 0.07 | 0.22 | 0.12 | 0.41 | 5.41 |
| FG97S.7801 | Buster-B | 2.53 | 2.78 | 0.31 | 5.62 | 0.10 | 0.10 | 0.21 | 0.41 | 6.03 |
| FG98S.7819 | Success 42 | 2.66 | 2.34 | 0.38 | 5.38 | 0.03 | 0.23 | 0.06 | 0.32 | 5.70 |
| FG99S.7830 | Mr maxi | 2.30 | 2.19 | 0.45 | 4.94 | 0.07 | 0.09 | 0.14 | 0.30 | 5.24 |
| FG98S.7816 | MR 31-T | 2.31 | 2.87 | 0.35 | 5.53 | 0.07 | 0.20 | 0.15 | 0.42 | 5.95 |
| FG98S.7817 | MR 31-H | 2.70 | 2.75 | 0.34 | 5.78 | 0.09 | 0.31 | 0.20 | 0.59 | 6.38 |
| FG98S.7827 | Normal isoline | 2.11 | 1.89 | 0.43 | 4.43 | 0.06 | 0.11 | 0.13 | 0.30 | 4.74 |
| FG98S.7820 | Thunder | 3.08 | 2.48 | 0.31 | 5.86 | 0.04 | 0.28 | 0.11 | 0.43 | 6.30 |
| FG98S.7814 | Gold rush | 3.45 | 3.79 | 0.49 | 7.72 | 0.08 | 0.25 | 0.17 | 0.50 | 8.23 |

[1]NSP components exist as minor heteropolyers like xyluglucans, arabinogalactans etc.
[2] insoluble
[3] soluble

**Appendix 5.3 The percent of soluble and insoluble non starch polysaccharide (NSP) content in wheat samples based on dry matter (DM) of grains. Data sourced from the Premium Grains for Livestock Program**

| Unique ID | cultivar | % Insoluble NSP (DM) | | | | % Soluble NSP (DM) | | | | % Total (DM) |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Arabinoxylan | Cellulose | Other[1] | In[2] Total | Arabinoxylan | B-Glucan | Other[1] | So[3] Total | |
| FG97W.1810 | Janz-L | 7.23 | 2.76 | 0.39 | 10.38 | 0.46 | 0.69 | 0.30 | 1.45 | 11.84 |
| FG98W.1809 | Janz (frosted) | 8.94 | 3.45 | 0.74 | 13.13 | 0.67 | 0.91 | 0.30 | 1.88 | 15.00 |
| FG99W.1818 | Kukari | 5.94 | 2.50 | 0.40 | 8.84 | 0.65 | 0.67 | 0.27 | 1.59 | 10.43 |
| LSF97W.1906 | Currawong-F | 6.27 | 2.45 | 0.37 | 9.10 | 0.50 | 0.73 | 0.29 | 1.52 | 10.62 |
| LSF98W.1913 | Sunstate-F | 5.95 | 2.59 | 0.41 | 8.94 | 0.56 | 0.84 | 0.32 | 1.72 | 10.65 |
| LSF98W.1914 | Janz-F | 6.41 | 2.48 | 0.39 | 9.28 | 0.50 | 0.73 | 0.28 | 1.52 | 10.79 |
| LSJ97W.1909 | Currawong-J | 4.65 | 2.10 | 0.49 | 7.24 | 0.79 | 0.68 | 0.26 | 1.72 | 8.97 |
| LSN97W.1901 | Sunstate-N | 5.20 | 2.08 | 0.40 | 7.68 | 0.54 | 1.04 | 0.29 | 1.87 | 9.55 |
| LSN97W.1902 | Janz-N | 6.59 | 3.01 | 0.46 | 10.06 | 0.50 | 0.75 | 0.33 | 1.58 | 11.64 |
| LSN97W.1903 | Currawong-N | 5.82 | 2.57 | 0.43 | 8.82 | 0.45 | 0.81 | 0.31 | 1.58 | 10.40 |

[1]NSP components exist as minor heteropolyers like xyluglucans, arabinogalactans etc.
[2] insoluble
[3] soluble

**Appendix 6.1 The glucose release index (GRI) for 0.5mm-milled, 2mm-milled and roller-milled barley, sorghum and wheat.**

| Grain types | Id | Cultivar | GRI for 0.5 mm | GRI for Roller | GRI for 2mm |
|---|---|---|---|---|---|
| Barley | FG96B.3801 | Arapiles | 37.3 | 33.5 | 19.9 |
| Barley | FG96B.3804 | Chebec | 36.9 | 29.1 | 21.5 |
| Barley | FG96B.3807 | Galleon-H | 38.0 | 29.5 | 23.2 |
| Barley | FG96B.3808 | Grimmet | 33.7 | 28.2 | 23.3 |
| Barley | FG96B.3811 | Schooner-J | 33.8 | 33.0 | 17.7 |
| Barley | FG96B.3814 | Skiff | 36.3 | 40.5 | 18.0 |
| Barley | FG96B.3815 | Tantangara-J2 | 37.1 | 27.1 | 18.0 |
| Barley | FG97B.3817 | Lindwall | 29.6 | 30.1 | 16.4 |
| Barley | FG97B.3818 | Mundah | 31.5 | Not available | 24.9 |
| Barley | FG97B.3819 | Schooner-F | 27.5 | 30.6 | 17.5 |
| Barley | FG97B.3820 | Sloop | 27.2 | 31.7 | 17.8 |
| Barley | INC98NB.3702 | Reinette | 27.3 | 21.6 | 15.7 |
| Barley | LSF97B.3904 | Schooner-F2 | 32.2 | 21.6 | 18.1 |
| Barley | LSF97B.3906 | Tantangara-F | 35.2 | 25.8 | 15.2 |
| Barley | LSJ97B.3909 | Tantangara-J | 33.5 | 26.5 | 16.4 |
| Barley | LSN97B.3902 | Galleon-N | 29.4 | 23.4 | 17.1 |
| Barley | LSN98B.3912 | Tantangara-N | 44.8 | 23.3 | 15.7 |
| Barley | NT98B+N.3951 | Gilbert | 44.6 | 35.9 | 15.4 |
| Sorghum | FG97S.7801 | Buster-B | 31.5 | 22.3 | 14.3 |
| Sorghum | FG97S.7802 | Buster-W | 28.5 | 27.6 | 14.2 |
| Sorghum | FG97S.7804 | MR 31 | 27.5 | 27.5 | 15.2 |
| Sorghum | FG97S.7805 | Thunder | 31.5 | 26.4 | 14.4 |
| Sorghum | FG97S.7811 | Boomer-BI | 29.2 | 40.9 | 16.0 |
| Sorghum | FG97S.7812 | Boomer-BE | 29.4 | 29.0 | 14.0 |
| Sorghum | FG98S.7814 | Gold rush | 26.0 | 40.0 | 11.1 |
| Sorghum | FG98S.7815 | MR 31-B | 28.8 | 27.4 | 12.0 |
| Sorghum | FG98S.7816 | MR 31-T | 28.2 | 27.0 | 13.2 |
| Sorghum | FG98S.7817 | MR 31-H | 30.5 | 27.3 | 14.0 |
| Sorghum | FG98S.7818 | PAC 2391 | 25.6 | 35.6 | 15.1 |
| Sorghum | FG98S.7819 | Success 42 | 32.2 | 25.8 | 13.4 |
| Sorghum | FG98S.7820 | Thunder | 27.2 | 24.9 | 11.8 |
| Sorghum | FG98S.7827 | Normal isoline | 54.0 | 26.6 | 13.1 |
| Sorghum | FG99S.7830 | Mr maxi | 54.5 | 28.1 | 15.9 |
| Wheat | FG97W.1810 | Janz-L | 31.9 | 30.0 | 18.3 |
| Wheat | FG98W.1809 | Janz (frosted) | 50.7 | 34.6 | 22.9 |
| Wheat | FG99W.1818 | Kukari | 40.6 | 35.0 | 18.7 |
| Wheat | LSF97W.1906 | Currawong-F | 42.1 | 30.7 | 14.2 |
| Wheat | LSF97W.1909 | Currawong-J | 34.3 | 32.0 | 16.2 |
| Wheat | LSF98W.1913 | Sunstate-F | 46.9 | 37.4 | 21.6 |
| Wheat | LSF98W.1914 | Janz-F | 41.9 | 32.3 | 18.8 |
| Wheat | LSN97W.1901 | Sunstate-N | 39.8 | 26.6 | 18.2 |
| Wheat | LSN97W.1902 | Janz-N | 52.9 | 39.7 | 16.3 |
| Wheat | LSN97W.1903 | Currawong-N | 37.4 | 31.3 | 18.7 |

**Appendix 6.2 The grain harness index in barley, sorghum and wheat samples. Data sourced from the Premium Grains for Livestock Program.**

| Grain type | Unique ID | Cultivars | Grain hardness index | SD[1] |
|---|---|---|---|---|
| Barley | FG97B.3820 | Sloop | 31.27 | 12.74 |
| Barley | FG97B.3819 | Schooner-F | 38.26 | 15.28 |
| Barley | FG97B.3818 | Mundah | 38.53 | 13.24 |
| Barley | FG96B.3814 | Skiff | 42.63 | 14.18 |
| Barley | LSF97B.3904 | Schooner-F2 | 45.78 | 14.33 |
| Barley | LSN97B.3902 | Galleon-N | 47.49 | 15.75 |
| Barley | FG96B.3811 | Schooner-J | 49.27 | 16.33 |
| Barley | LSN98B.3912 | Tantangara-N | 52.95 | 14.29 |
| Barley | LSF97B.3906 | Tantangara-F | 53.86 | 15.55 |
| Barley | INC98NB.3702 | Reinette | 56.19 | 18.39 |
| Barley | FG96B.3807 | Galleon-H | 57.03 | 12.45 |
| Barley | FG96B.3815 | Tantangara-J2 | 57.18 | 15.1 |
| Barley | FG96B.3808 | Grimmet | 57.44 | 10.56 |
| Barley | FG96B.3804 | Chebec | 57.51 | 11.45 |
| Barley | LSJ97B.3909 | Tantangara-J | 57.85 | 14.88 |
| Barley | FG96B.3801 | Arapiles | 59.95 | 11.6 |
| Barley | FG97B.3817 | Lindwall | 64.77 | 17.15 |
| Barley | NT98B.N.3951 | Gilbert | 67.99 | 17.44 |
| Sorghum | FG97S.7802 | Buster-W | 70.74 | 17.07 |
| Sorghum | FG97S.7801 | Buster-B | 71.9 | 21.1 |
| Sorghum | FG98S.7814 | Gold rush | 73.06 | 21.52 |
| Sorghum | FG98S.7817 | MR 31-H | 73.31 | 19.8 |
| Sorghum | FG97S.7812 | Boomer-BE | 73.44 | 21.63 |
| Sorghum | FG97S.7811 | Boomer-BI | 73.5 | 20.11 |
| Sorghum | FG98S.7816 | MR 31-T | 74.1 | 14.55 |
| Sorghum | FG98S.7819 | Success 42 | 76.25 | 18.08 |
| Sorghum | FG98S.7815 | MR 31-B | 80.19 | 19.83 |
| Sorghum | FG97S.7804 | MR 31 | 81.12 | 18.77 |
| Sorghum | FG98S.7818 | PAC 2391 | 82.4 | 19.55 |
| Sorghum | FG98S.7820 | Thunder | 83.98 | 19.81 |
| Sorghum | FG98S.7827 | Normal isoline | 85.97 | 21.75 |
| Sorghum | FG97S.7805 | Thunder | 87.36 | 15.36 |
| Sorghum | FG99S.7830 | Mr maxi | 90.03 | 20.02 |
| Wheat | FG98W.1809 | Janz (frosted) | 48.16 | 21.05 |
| Wheat | FG99W.1818 | Kukari | 52.35 | 17.43 |
| Wheat | LSF98W.1914 | Janz-F | 71.46 | 16.93 |
| Wheat | LSN97W.1902 | Janz-N | 71.58 | 17.45 |
| Wheat | FG97W.1810 | Janz-L | 74.91 | 16.22 |
| Wheat | LSF98W.1913 | Sunstate-F | 78.66 | 17.23 |
| Wheat | LSN97W.1903 | Currawong-N | 85.79 | 16.25 |
| Wheat | LSN97W.1901 | Sunstate-N | 86.04 | 16.74 |
| Wheat | LSF97W.1909 | Currawong-J | 91.21 | 17.21 |
| Wheat | LSF97W.1906 | Currawong-F | 95.53 | 17.66 |

[1] Standard deviation

**Appendix 7.1 The crude protein content of barley, sorghum and wheat samples. Data sourced from the Premium Grains for Livestock Program.**

| Grain type | Unique ID | Cultivars | Crude protein content (% based on DM[1]) |
|---|---|---|---|
| Barley | FG97B.3818 | Mundah | 12.33 |
| | LSN97B.3902 | Galleon-N | 15.84 |
| | FG96B.3808 | Grimmet | 15.67 |
| Sorghum | FG97S.7812 | Boomer-BE | 10.41 |
| | FG98S.7819 | Success 42 | 10.48 |
| | FG98S.7815 | MR 31-B | 10.95 |
| Wheat | FG98W.1809 | Janz (frosted) | 18.10 |
| | FG97W.1810 | Janz-L | 18.02 |
| | LSF97W.1906 | Currawong-F | 14.68 |

[1] dry matter