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Navy Bean Cotyledon Cells: Isolation, Characterisation and Their Application in Low Glycaemic Bread

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Abstract

There is an increasing consumer demand for low-glycaemic foods due to the rise in lifestyle diseases such as obesity and type 2 diabetes globally. Reducing the glycaemic response of starch-based foods is a challenge as it leads to a significant change in their texture. Pulses are one of the most popular foods and known to have a low glycaemic index (GI). Recently it has been suggested that their isolated cotyledon cells can play an important role in delaying the digestion rate of starch, due to their unique structure where starch granules are tightly embedded in a protein matrix surrounded by the intact cell walls. Studies have been reported on the lab-scale isolation of gelatinised as well as ungelatinised cotyledon cells from navy beans, but rarely investigated the mechanisms by which their cell structure (cell walls, intracellular proteins) controls the starch digestibility. In the present study, intact cotyledon cells from navy beans were extracted using different processing conditions including acid–alkali treatments, autoclaving and hydrothermal processing, and their properties were compared to their counterpart navy bean starch to understand changes in the starch structure and *in vitro* digestibility. Furthermore, the effect of partial substitution of wheat flour with isolated cells on physicochemical and textural properties, starch hydrolysis kinetics and digesta characteristics during oral, gastro-small intestinal digestion *in vitro* was studied. Light micrographs and SEM images of the isolated cells through acid–alkaline treatment showed the presence of intact cells containing ungelatinised starch granules embedded in the protein matrix. The *in vitro* starch digestion curves showed that cells with different treatments display different cell permeability and different digestion kinetics, thus affecting the starch digestibility. Regarding bread with cotyledon cells, adding 25% and 50% cotyledon cells to bread decreased the specific volume and whiteness of bread and increased breadcrumb and crust hardness. SEM observation evidenced that the integrity of the cell wall in bread was preserved in baked bread as well as digesta, and this led to a ~34% reduction *in vitro* digestibility compared to white bread when 50% of the wheat flour was replaced with intact cells. Selected samples of the bread were evaluated for their glycaemic indices through *in vivo* (human) trials. A similar *in vitro* digestion-like trend was observed during *in vivo* (human) digestion studies. The results of the study suggested that bread with 50% of wheat flour replaced by cotyledon cells may be considered as low GI bread. This study is expected to propose an optimized process suitable for commercial production of cotyledon cells from pulses and their application to develop low GI baked products such as bread.

Keywords: Cotyledon cell, Starch digestibility, Glycaemic response, Bread

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Abbreviations

%	Percent
A	Autoclaving
AA	Acid–alkaline treatment
AACC	American Association of Cereal Chemistry
ANOVA	Analysis of Variance
AOAC	Association of Official Analytical Chemists
B	Boiling
BRB	Boiling + 7-day retrogradation + fluid bed-drying
BRF	Boiling + 7-day retrogradation + freeze-drying
CB	Commercial bread
DSC	Differential scanning calorimeter
Dv10	The 10% of the cumulative volume distribution.
Dv50	The median for a volume distribution
Dv90	The 90% of the cumulative volume distribution.
eGI	Estimated GI
GI	Glycaemic index
N-P	Nitrogen-to-protein
NSPs	Non-starch polysaccharides
RDS	Rapidly digestible starch

RS	Resistant starch
S	Starch
SDS	Slowly digestible starch
SEM	Scanning electronic microscopy
SGF	Simulated gastric fluid
SIF	Simulated small intestinal fluid
SSF	Simulated saliva fluid
T_c	Conclusion temperature
T_o	Onset temperature
T_p	Peak temperature
WF	Wheat flour
α	Alpha
β	Beta
ΔH	Enthalpy of gelatinisation

CHAPTER ONE: Introduction

1.1 General introduction

Pulses are one of the largest food sources and are of increasing interest due to their richness in nutrients, such as carbohydrates, protein, dietary fibre, and minerals, as well as their low-fat content and low glycaemic response. It is suggested that regular consumption of pulses is associated with a reduced risk of diabetes and cardiovascular disease (Calles, 2016; Pallares Pallares et al., 2021; Rebello et al., 2014; Singh et al., 2021). Starch is the main carbohydrate in pulses and its composition and pasting properties will directly influence the digestive characteristics of the food product. Navy beans are one of the most popular pulses and are known as an excellent source of high-quality protein, minerals, dietary fibre and bioactive compounds. It is reported that navy beans contain twice the amount of protein as cereals (Liu et al. 2018). They contain a considerable amount of resistant starch, leading to a low glycaemic index. A combination of these compounds in navy beans may relate to anticancer action (Bazzano et al., 2011; Haydé et al., 2012; Yamada et al., 2005).

With the increasing population, changes in lifestyle, dietary habits, and food structure, and the ageing of society, the spectrum of human diseases has changed considerably. The proportion of insulin-related chronic diseases such as diabetes, cardiovascular diseases, obesity, hyperlipidaemia and hypertension is escalating and has become a major global public health problem. Amongst these diseases, the increase in the incidence of diabetes is particularly striking. It is estimated that the prevalence of diabetes will increase by 54% and annual deaths will climb by 38% by 2030 (Rowley et al., 2017). Thus, one of the greatest challenges and urgent needs for the food industry is to develop nutritious, tasty, and healthy products.

Previous research has identified that pulses are a natural source of slowly digestible starch and resistant starch that inhibits the rate of postprandial blood glucose rise

(Rizkalla et al., 2002). Therefore, pulses are effective in stabilising postprandial blood sugar fluctuations and minimizing the risk of chronic diseases (diabetes and obesity, etc.).

Moreover, recent studies have shown that starch digestibility plays a crucial role in building health and nutrition, and the relationship between the starch digestive kinetics and the microstructure of the starch molecule is gradually being revealed, i.e. impeding the approach of amylase to starch (Hoover & Ratnayake, 2002; Huang et al., 2007; Würsch et al., 1986; Zhou et al., 2004). Furthermore, the slow glycaemic response is not only related to microstructure and composition, but also due to the pulses cotyledon cell's unique structure. However, several studies have reported the isolation of cotyledon cells at laboratory scale and their properties, but the mechanism of how the cell wall affects the structure and digestibility of starch during different processing treatments is poorly understood, and rarely have studies focused on the possibility of developing low glycaemic food products using cotyledon cells.

This thesis attempts to show the role of the cell walls, using navy bean cotyledon cells as a model, and to assess the changes in structure and digestibility of starch granules after various processing. Another aim of this thesis is to study the addition of navy bean cotyledon cells in bread formulation and its influence on starch hydrolysis kinetics and digesta characteristics during oral, gastro-small intestinal digestion *in vitro*. This study will generate fresh insight into the potential of pulse cells as a novel ingredient in low GI food development.

1.2 Overview of thesis

The thesis has been organized in the following way.

Chapter 1 (Introduction) describes the association between pulses and health and their importance in our diet, drawing out their unique cell structure as a function to control starch digestion.

Chapter 2 (Literature review) gives a brief overview of the importance of legumes in sustainability, nutritious properties and health benefit, and the microstructure of cell walls. Then it illustrates the starch digestibility and the effect of different processing treatments on it. Also, the link between starch digestibility and glycaemic index is described.

Following the review section, intact cotyledon cells isolated using different extraction methods are evaluated (Chapters 3 & 4) for physicochemical and pasting properties and *in vitro* digestibility.

Chapter 5 explains the effect of replacing wheat flour with cotyledon cells on the bread quality, particularly their influence on starch digestibility of bread *in vitro* and glycaemic index of the same bread measured through *in vivo* human studies.

Finally, chapter 6 concludes with general conclusions, discussion, and recommendations for future work.

CHAPTER TWO: Literature review

2.1 Pulses

Pulses are dried edible seeds of the legume family, and common beans (*Phaseolus vulgaris* L.) are one of the most important pulses in the human diet. The Food and Agriculture Organization (FAO) recognises main types of pulses worldwide (Calles, 2016), as shown in Table 2.1. Generally, pulses play a crucial role in the human diet and regulate the human physiological metabolism because of their nutrient-richness and unique structural properties.

Table 2.1. Classification of pulses according to the FAO. As common vetches are only commonly grown as animal feed, they are not included in this table (Calles, 2016).

Category	Vernacular name	Species
Dry beans	Common bean (Kidney, Pinto, Navy, Cranberry)	*Phaseolus vulgaris* L
	Lima bean	*Phaseolus lunatus* L
	Scarlet runner bean	*Phaseolus coccineus* L
	Tepary bean	*Phaseolus acutifolius* A Gray
	Adzuki bean	*Vigna angularis* (Willd) Ohwi & H. Ohashi
	Mung bean	*Vigna radiata* (L) R Wilczek
	Mungo bean	*Vigna mungo* (L) Hepper
	Rice bean	*Vigna umbellata* (Thunb) Ohwi & H Ohashi
	Moth bean	*Vigna aconitifolia* (Jacq) Maréchal
Bambara beans	Bambara bean	*Vigna subterranea* (L) Verdc
Broad beans	Broad bean, Horse bean	*Vicia faba* L
Peas	Pea	*Pisum sativum* L
Chickpeas	Chickpea (Kabuli)	*Cicer arietinum* L

Cowpeas	Cowpea (Black-eyed pea)	*Vigna unguiculata* (L) Walp
Pigeon peas	Pigeon pea, Red gram bean	*Cajanus cajan* (L) Huth
Lentils	Lentil	*Lens culinaris* Medik
Lupins	Yellow lupin	Several *Lupinus* L species
Minor pulses	Hyacinth bean, Lablab bean	*Lablab purpureus* (L) Sweet
	Jack bean	*Canavalia ensiformis* (L) DC
	Winged bean	*Psophocarpus tetragonolobus* (L) DC
	Guar bean	*Cyamopsis tetragonoloba* (L) Taub
	Velvet bean	*Mucuna pruriens* (L) DC
	African yam bean	*Sphenostylis stenocarpa* (Hochst ex A Rich) Harms

2.1.1 Sustainability

Pulses are sustainable crops. Agricultural production and livestock breeding processes have historically consumed natural resources and caused environmental damage, including the consumption of land and water, and the use of pesticides and fertilisers, a model that is not conducive to sustainable development. In contrast to animal-based and other cereal foods, pulses consume less water and non-renewable energy per unit of production (Van Huis et al., 2013). Pulses are also able to fix nitrogen naturally and, in symbiosis with *Rhizobium* bacteria, their root nodules absorb nitrogen sources such as N_2O from the soil and synthesise them into nitrogen compounds available to plants, thus improving soil fertility, diminishing reliance on synthetic fertilisers and reducing greenhouse gas emissions (Das & Ghosh, 2012; Nulik et al., 2013; Werner & Newton, 2005). It has been found that pulses emit much less greenhouse gas per gram of protein than animal meat (Nijdam et al., 2012), as illustrated in Table 2.2. Furthermore, pulses facilitate crop rotation and improve soil quality through carbon sequestration and serve as effective cover crops to reduce soil erosion (Blanchart et al., 2006). Hence, the cultivation and integrated use of pulses are valuable for sustainable development, environmental preservation and climate change.

Table 2.2. Summary of the water and carbon footprints of some selected products. (Mekonnen & Hoekstra, 2012; Nijdam et al., 2012).

Food	Water footprint (L/g protein)	Carbon footprint (GHG kg CO_2-eq/kg protein)
Beef	112	46 to 640
Pork	57	20 to 55
Poultry	34	10 to 30
Eggs	29	15 to 42
Milk	31	28 to 43
Pulses	19	4 to 10

2.1.2 Nutritional properties

Pulses provide comparatively high levels of nutrients to the human diet compared with other field crops. Pulses are high in carbohydrates (55–70%), with starch accounting for 40–60% of total carbohydrates (Peña-Valdivia & Ortega-Delgado, 1986). The starch in pulses is a natural source of slowly digestible starch (SDS) and resistant starch (RS), which has lower digestibility than grain starch and has a positive effect on lowing glycaemic response (Liu et al., 2006; Los et al., 2018; Rizkalla et al., 2002). The presence of dietary fibre also helps to control blood sugar and cholesterol levels, promotes gastrointestinal motility and improves intestinal health (Brummer et al., 2015). In addition, the high protein (15–40%) and low fat (<1%) content, and the vitamin and mineral richness of pulses make them a high-quality plant-based food source with good health benefits.

Structurally, bean seeds, for example, consist of the seed coat, the cotyledons and the embryo, including the epicotyl, hypocotyl and radicle, as displayed in Fig. 2.1 (Pallares Pallares et al., 2021). The seed coat, which is rich in polyphenols, wraps around the embryo and cotyledons to protect them and to provide resistance against oxidation. When polyphenols combine with proteins and minerals, this impairs nutrient absorption, reducing the nutritional value of pulses (Karataş et al., 2017). The cotyledons are the most important component of the seeds and are the main nutrient storage centre, including carbohydrates and proteins, and also contain trace elements such as iron, calcium, and zinc (Berrios et al., 1998). The complex natural

microstructure of pulses plays a very important role in processing and human digestion.

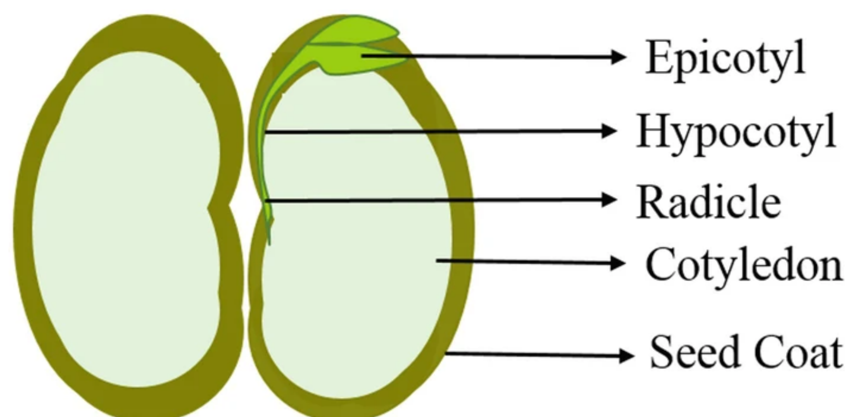

Fig. 2.1. Structure of a pulse seed (Mohan Naik et al., 2020).

2.1.2.1 Carbohydrates

Carbohydrates predominate in pulses, including starch, non-starch polysaccharides (NSPs), and some oligosaccharides, which provide the main sources of energy when consumed. Most pulse starch includes a high level of amylose (12–50%), although it is low in faba bean (Ambigaipalan et al., 2011). Moreover, the content of SDS and RS is high, which is beneficial for slowing starch hydrolysis and further lowering the glycaemic index (GI) of foods. However, these are also determined by changes such as processing and storage time (Chung et al., 2009). For instance, the GI value of canned chickpea is higher than that of raw chickpea (Atkinson et al., 2008).

Dietary fibre is an important component of pulses (found mainly in the hull and cell walls) and it is largely affected by the cultivar. Specifically, the total dietary fibre content of dry beans ranges from 23 to 32%, whereas in lentils, it varies between 18 and 20% (Tosh & Yada, 2010). Most is insoluble fibre consisting of cellulose, hemicellulose, and lignin, which are indigestible carbohydrates. Pectins, oligosaccharides and low molecular weight polysaccharides are soluble dietary fibres, which are found mainly in the cotyledon (Brummer et al., 2015). Dietary fibre contributes to lowering starch hydrolysis and has other health-related benefits, as shown in Table 2.3.

2.1.2.2 Protein

Pulses are higher in protein than cereals and tree nuts used as plant-based dairy substitutes. According to a study by Jeske et al. (2017), the protein content of these commercial milk substitutes is less than 1%, whereas the protein content of pulses

ranges from 17 to 30%, depending on the species and variety. There are two main proteins present in pulses: salt-soluble globulin and water-soluble albumin. Generally, in dry beans, the amount of protein accounted for by globulin and albumin ranges from 45 to 70% and from 10 to 30%, respectively (Sathe, 2002). Specifically, legumin (11S) and vicilin (7S) are forms of globulin, and albumin is composed of enzymatic proteins and lectins (Boye et al., 2010). They contain high amounts of amino acids such as lysine, and leucine, while low in methionine, cysteine, and tryptophan (Boye et al., 2010). Pulses contain approximately six to eight times more lysine than wheat, whereas wheat contains more methionine, cysteine, and tryptophan, which are low in pulses; hence intake of a combination of pulses and cereals is beneficial for balancing the amino acid profile to meet humans' essential amino acid requirements (Leinonen et al., 2019).

2.1.2.3 Vitamins and minerals

Pulses are also a good source of B vitamins, such as riboflavin (B2), thiamine (B1), niacin (B3), folic acid, and pyridoxine (B6), which play an essential role in energy metabolism as well as in fatty acid metabolic pathways (Depeint et al., 2006). Furthermore, several minerals (calcium, iron, potassium, magnesium, and zinc) are present in pulses. However, their bioavailability and bioaccessibility may be poor, as a result of processing and binding with antinutrients such as phytate and polyphenols (Sandberg, 2002). For instance, phytic acid is able to form stable complexes by binding with iron, zinc, and magnesium ions through an electrostatic interaction (Kumar et al., 2010). Additionally, processes such as soaking contribute to the loss of minerals, since water-soluble vitamins and some minerals (iron and zinc) tend to leach into the soaking medium (Afify et al., 2011).

2.1.2.4 Antinutrients

Pulses contain antinutrients, such as phytates, tannins, and some trypsin inhibitors, which can hinder nutrient absorption and negatively impact human health when consumed in large quantities (Champ, 2002). Numerous studies have illustrated that the presence of these antinutritional factors causes a decrease in the absorption of protein or minerals, and even causes health issues such as enlargement of the pancreas (Salgado et al., 2002). However, several studies also have discovered the bioactive effects of these antinutrients, such as antioxidation, blood sugar control, and anti-inflammatory effects as exhibited in Table 2.3 (Cassidy et al., 2006; Roy et al., 2010; Sievenpiper et al., 2009). Therefore, the balanced consumption of antinutritional factors has far-reaching significance for alleviating the prevalence of chronic diseases such as obesity, diabetes, and cancer.

Table 2.3. Associations between the components of pulses and health benefits.

Component	Health issue	Main results	References
SDS & RS	Diabetes	Lower postprandial glucose and insulin level;	Yamada et al. (2005)
	Colon cancer	Substrate for bacterial carbohydrate fermentation in the human colon; Increase faecal weight	Hylla et al. (1998)
Dietary fibre (soluble)	Diabetes	Activation of the glycogen synthesis process, leading to an increase in liver glycogen level and a reduction in blood glucose; Increase insulin sensitivity	Boby and Leelamma (2003)
	Cardiovascular disease	Reduce blood cholesterol and triglycerides	Bazzano et al. (2011)
Dietary fibre (insoluble)	Colon cancer	Exhibition of anti-proliferative activity and apoptosis in colon cancer cells	Haydé et al. (2012)
Polyphenolic compounds	Diabetes	α-amylase and α-glucosidase inhibitor;	Moreno-Valdespino et al. (2020)
	Cardiovascular disease Cancer	Antioxidant properties (scavenge free radicals) and inhibiting lipid peroxidation	Cheung et al. (2003)
Saponins	Diabetes	Inhibit α-amylase, α-glucosidase, and lipase activities	Ercan and El (2016)
	Cardiovascular disease	Hypocholesterolaemic activity	Afrose et al. (2009)
Protease inhibitors	Human immunodeficiency virus (HIV) Hypertension	Anti-inflammatory properties	Roy et al. (2010)

	Neurodegenerative disease		
Lectins	Immune system	Increase the phagocytosis activity of macrophages;	Hou et al. (2010)
		Inhibit HIV-1reverse transcriptase	Ye et al. (2001)
Phytosterols	Heart disease	Lower serum cholesterol and increase the saturation levels of cholesterol in the bile	Gylling and Miettinen (2005)
Phytic acid	Cancer Heart disease	Anticarcinogen (inositol hexaphosphate)	Shamsuddin (2002)
Isoflavones	Osteoporosis cardiovascular disease Menopause symptoms	Antioxidant properties	Cassidy et al. (2006)

2.1.3 Health-related benefits

There is a positive correlation between pulse intake and human health. A diet high in sugar, fat, and animal protein, as well as an unhealthy lifestyle, can cause blood glucose, body fat, and cholesterol levels to rise, leading to obesity and a high incidence of chronic diseases such as type 2 diabetes, cardiovascular disease and certain cancers (Esposito et al., 2010; Rebello et al., 2014; Winham et al., 2008). Table 2.4 summarises the health-related benefits of pulses.

Table 2.4. Summary of health-related benefits of pulses.

Chronic diseases	Main results	References
Cardiovascular disease	Reduced risk of coronary heart disease;	Bazzano et al. (2001)
	Reduced blood pressure;	Jayalath et al. (2014)
	Reduced total serum cholesterol and triglycerides;	Zhang et al. (2010)
	Increased high-density lipoprotein cholesterol, and decreased low-density lipoprotein and total cholesterol	Bazzano et al. (2011); Trinidad et al. (2010)
Diabetes	Improved glucose tolerance and insulin sensitivity; reduced risk of type 2 diabetes;	Livesey et al. (2008)
	Lower fasting blood glucose, insulin levels and glycosylated blood proteins	Sievenpiper et al. (2009)
Weight management	Increased duration of satiety;	Lunde et al. (2011)
	Reductions in waist circumference and obesity	Abete et al. (2019); Lin et al. (2011)
Cancer	Decreased risk of colorectal, breast, oesophagal and stomach cancer	Velie et al. (2005); Zhu et al. (2015)

Some specific components of pulses may be responsible for their ability to combat health issues. The high content of SDS and RS in pulses has been negatively associated with glycaemic response. Generally, SDS and RS have been shown to exhibit slow-digestion properties, thereby delaying the sharp rise in postprandial blood glucose (Yamada et al., 2005). Similarly, a negative correlation was found between dietary fibre and glycaemic response as well as colorectal cancers (Peters et al., 2003). Apart from SDS, RS and dietary fibre, other antinutritional components

such as saponins, protease inhibitors, phytic acid, and saponins also have a positive effect on human health, as shown in Table 2.3.

Pulses are increasingly being used in a wide range of applications that are not limited to traditional consumption (whole or split seeds) because of their nutrient-rich and environmentally friendly nature. Recently, several studies have been conducted that intact pulses' cotyledon cells are a potential novel ingredient for the production of low glycaemic response products, mainly due to the structural integrity of the cell walls (Abete et al., 2019; Bajka et al., 2021; Berg et al., 2012; Bhattarai et al., 2017; Boukid et al., 2019; Do et al., 2019; Edwards et al., 2020; Li et al., 2019, 2020; Rovalino-Córdova et al., 2019; Rovalino-Cordova et al., 2018; J. Singh et al., 2014; M. Singh et al., 2021; Xiong et al., 2019).

2.2 Pulse cell wall structure and starch characteristics

2.2.1 Pulse cell walls

The plant cell wall is considered to be a dynamic structure acting as a cytoskeleton. Its structure is largely subject to the osmotic swelling pressure of the cell and thus determines the textural properties of the plant tissue. It also plays a role in mechanical strength, cell shape maintenance and intercellular transport, etc (Waldron et al., 2003). The cell wall of pulses consists of two layers, the middle lamella and the primary wall, as demonstrated in Fig. 2.2. Pectin is the major component of this middle lamella, which has the primary function of adhering two adjacent cells together (Van Buggenhout et al., 2009). The primary cell wall is located between the middle lamella and the plasma membrane, where the cellulose microfibrils are embedded in the hemicellulose and pectin matrix, and completely cover the plasma membrane, which contributes to maintaining plant cell rigidity (Xiong et al., 2022).

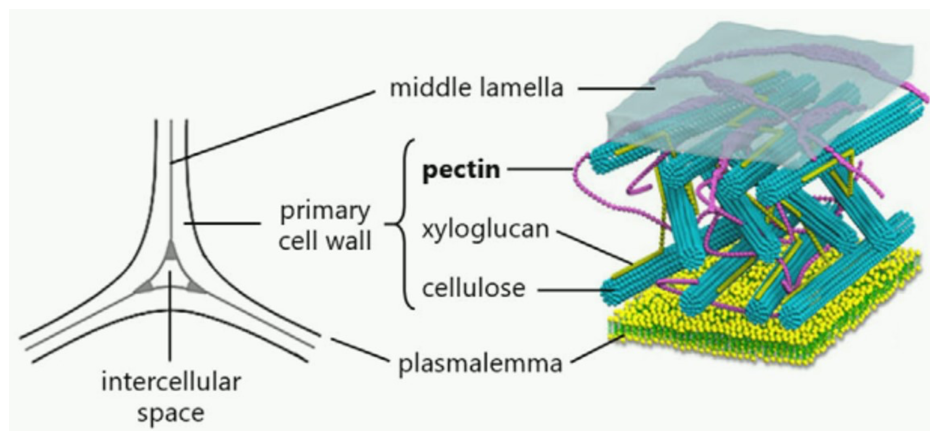

Fig. 2.2. Schematic diagram of the cell wall of a pulse. Adapted with permission from Pallares Pallares et al. (2021).

The strength and flexibility of the cell wall are mainly derived from the primary cell wall, which consists of cellulose microfibrils and xyloglucan. Each microfibril is made up of a bundle of glucose residues linked together by β (1→4) glycosidic bonds to form linear glucan chains. This arrangement connects a glucose residue to an adjacent glucose residue by rotating it 180° around its (1→4) axis. The overall framework of cellulose and xyloglucan is the most dominant component of the cell wall, accounting for approximately 50%, providing considerable strength (Holland et al., 2020).

In addition to cellulose and xyloglucan, pectin is also a major component of the cell wall, comprising approximately 30% of the wall mass, contributing to the flexibility of the cell wall. Specifically, the pectin polysaccharide network is mainly composed of galacturonic acid-rich polysaccharides including homogalacturonans (HGA), rhamnogalacturonan-I (RGI), and rhamnogalacturonan-II (RGII), which are linked by covalent bonds (Peña et al., 2001; Willats et al., 2001).

Cellulose, hemicellulose, pectin, and glucan are collectively known as non-starch polysaccharides (NSPs), which constitute the major components of the cell wall and are the primary source of dietary fibre (Selvendran & Robertson, 1990). In general, variation in the content of NSPs is mainly attributed to the cultivars and production factors such as dehulling (Bravo et al., 1998). Typically, they are divided into soluble and insoluble fractions (Choct, 1997). Table 2.5 demonstrates the content of NSPs of some pulses.

Table 2.5. Non-starch polysaccharides (NSPs) content (g/100g dry matter) of pulses.

Beans	Insoluble NSP	Soluble NSP	Total NSP	References
Navy bean, cooked	14.0	7.8	21.8	Anderson and Bridges (1988)
Navy bean, raw	11.7	5.7	17.4	Chang et al. (1989)
Pinto bean, raw	11.4	8.2	19.6	Anderson and Bridges (1988)
Chickpea, raw	7.6	2.0	9.6	Periago et al. (1997)
Red lentil, raw	4.0	1.5	5.5	Stephen et al. (1995)

2.2.2 Pulse starch

Most pulse starch granules have an ellipsoidal shape and have a relatively smooth, uncracked surface, as shown in Fig. 2.3 (Hoover & Sosulski, 1991). Their granule size generally ranges from about 4 to 85 µm, with some exceeding 100 µm or more, depending on species (Wang & Copeland, 2013).

Typically, most starch granules are composed of two structurally different polymers: linear amylose, and highly branched amylopectin with many clusters (Hoover et al., 2010). Pulse starch is rich in amylose, and the content of which varies considerably between cultivars. Previous studies have reported that amylose content in pulses generally ranges from 11.6 to 88.0%, with navy beans containing approximately 28–41% (Hoover et al., 2010; Hoover & Ratnayake, 2002). On the other hand, the branches of amylopectin are in clusters and are in the form of double helices forming many small crystalline regions (Pérez & Bertoft, 2010). It was found that different starch granules have different types of crystallinity, exhibiting three X-ray diffraction patterns (A, B and C). The C-type structure is a mixture of A-type (cereals) and B-type (tubers) type crystals, which is present primarily in pulses (Imberty et al., 1991; Wang et al., 2008). It was discovered that C-type starch is more resistant to amylase than A-type starch (Bogracheva et al., 1998). The difference in the crystallinity of pulse starches depends essentially on the arrangement of the crystals, the polycrystalline content, the size, and the moisture content (Zobel, 1988).

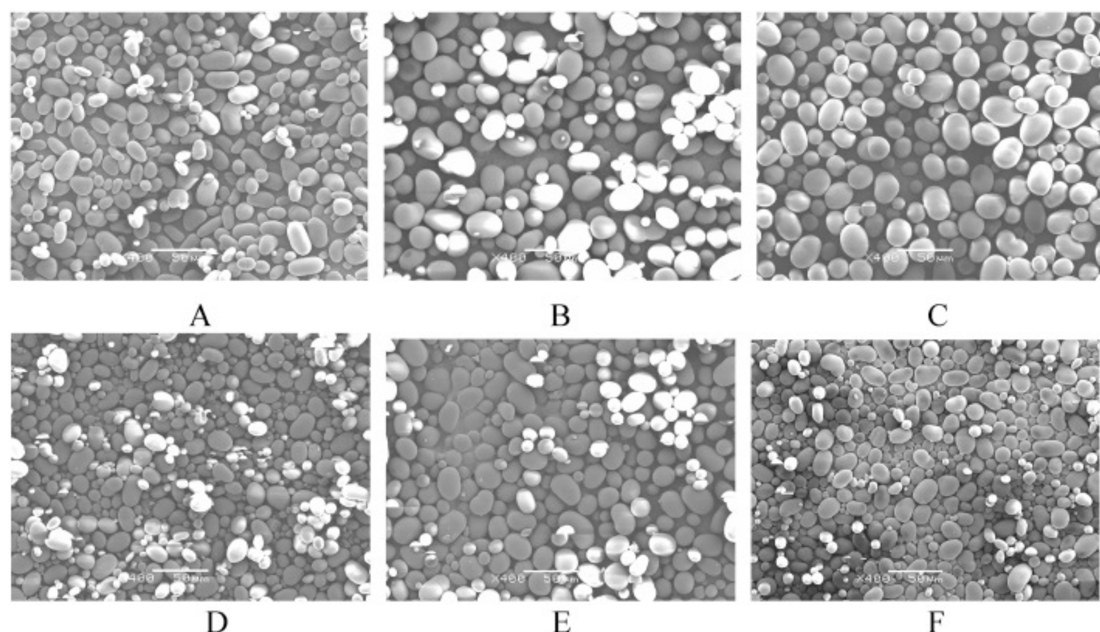

Fig. 2.3. Scanning electron microscopy (SEM) images of legume starch. Black eye bean starch (A); Baby lima bean starch (B); Lentil starch (C); Chick pea starch (D); Small red bean starch (E); Mung bean starch (F). Adapted with permission from Ma et al. (2017).

2.3 Intact cotyledon cells of pulses and their isolation

Traditionally, the nutritional properties of pulses are improved by grinding them into fine palatable pulse flour. During the milling process, mechanical forces break down the whole raw seed and even the cell walls, thus releasing nutrients such as starch and protein from the matrix for easier digestion and absorption by the body. In particular, the starch granules are more likely to be exposed and hydrolysed into glucose by amylase, leading to an increase in blood sugar levels after consumption (Berg et al., 2012; Do et al., 2019). However, because of increased awareness of health issues, the idea of delaying the release of blood sugar is becoming more relevant. More studies have paid attention to isolated intact cells from pulses with equivalent nutritional functionality, focusing on the mechanism of cotyledon cells and their cell walls, leading to a lower glycaemic response (Bajka et al., 2021; Berg et al., 2012).

Common extraction methods and processing conditions applied for isolating cells are summarised in Table 2.6. The basic principle of cell isolation is the removal or dissolution of the middle lamella, containing rich pectin that connects the cells. However, strong mechanical forces can break down the cell walls and liberate the free starch granules under dry conditions. Thus, a gentle thermomechanical process (including atmospheric and high-pressure heating) is a feasible and alternative method to remove the strong intercellular adhesions without physically damaging the cells and cell walls, preserving their integrity. After soaking and thermal treatment, a mild mechanical force is applied to extract the cells. Soaking, heating, and mechanical force all serve to soften the cotyledon matrix and intercellular adhesion. Specifically, heating allows for the dissolution of the pectin in the middle lamella, thereby considerably reducing intercellular adhesion and enabling the release of the intact cells more easily under minor mechanical force. The paste thus obtained is subsequently washed and sieved (the desired size depends on the pulses' cell size) to recover intact cotyledon cells, as shown in Fig. 2.4 (Berg et al., 2012; Dhital et al., 2016; Li et al., 2020; Pallares et al., 2018; Rovalino-Cordova et al., 2018; Xiong et al., 2019). However, different heating times and temperatures may affect the permeability of cell walls (Li et al., 2020).

In addition to physical hydrothermal processing, a mild acid–alkaline treatment (with HCl and NaOH) can be introduced to extract the cells as well. Typically, acid–alkaline treatment is carried out at room temperature, eliminating the effects of high temperature on the cell walls and native starch granules. The mild acid and alkali promote cell separation by softening the cell walls and through acid hydrolysis of pectin (Li et al., 2019; Sila et al., 2009). It is worth mentioning, however, that only limited studies have been conducted involving the acid–alkaline method and further

research is required to determine whether mild acid and alkali impact the porosity of the cell wall and starch structure.

Table 2.6. Various treatments to isolate intact cotyledon cells from pulses.

Pulses	Isolation treatment	References
Chickpea, green-lentil, red-lentil, butterbeans, red kidney beans	Soaking Hydrothermal treatment (100 °C for 90, 50, 30, 25 or 15 min) Grinding Wet-sieving (< 150 µm) Air drying	Edwards et al. (2020)
Garbanzo and pinto beans	Soaking in chilled water Dehulling Heating at 60, 70, 80, 90, and 100 °C for 30 min Mashing Wet-sieving (100–250 µm) Freeze-drying	Li et al. (2020)
Chickpea	**Acid–alkaline method** Dehulling and cutting into cubes Soaking in 0.05 M HCl for 6 h, then immersing in 0.05 M NaOH overnight Wet-sieving (90–150 µm) Pure ethanol for drying **Hydrothermal treatment** Soaking in 0.5% $NaCO_3$ and 1.5% $NaHCO_3$ Heating at 70, 80, 100 °C for 1 h Mashing	Li et al. (2019)

	Wet-sieving (90–150 μm) Pure ethanol for drying **Pressure-heating** Soaking in water Autoclaving (150 kPa) at 100 °C for 30 min Mashing Wet-sieving (90–150 μm) Pure ethanol for drying	
Adzuki bean, chickpea, lentil and lima bean	Soaking in 0.1 M HCl for 24 h Dehulling Soaking in 0.06 M NaOH for 24 h Mashing Wet-sieving (53–150 μm) Dehydration by graded ethanol then freeze-drying	Do et al. (2019)
Red kidney bean	Soaking overnight in chilled water Dehulling Boiling in water for 1 h Mashing Wet-sieving Storage in sodium azide (0.02%)	Rovalino-Cordova et al. (2018)
Pinto bean, garbanzo bean, green-split pea and black-eyed pea	Soaking in 4 °C water overnight Dehulling Heating at 95 °C for 1 h Mashing Wet-sieving (50–150 μm)	Xiong et al. (2018)

	Freeze-drying	
Pinto bean	Soaking in 0.5% $NaCO_3$ and 1.5% $NaHCO_3$ overnight Dehulling Heating at 60 °C for 1 h Mashing Wet-sieving (50–150 µm) Freeze-drying	Xiong et al. (2019)
Chickpea, mung bean and red kidney bean	Soaking in chilled water Dehulling Heating at 60, 95 °C for 1 h Mashing Wet-sieving (53–150 µm) Storage in 0.02% sodium azide solution	Dhital et al. (2016)
Common bean	Soaking 16 h Heating at 95 °C for 30, 60, 90, and 180 min Dehulling Mashing Wet-sieving (40–125 µm)	Pallares et al. (2018)
Red bean, smooth pea and mung bean	Soaking in 0.01 M HCl at 4 °C for 36 h Dehulling Soaking in 0.05 M NaOH at 40 °C for 36 h Wet-sieving (75–150 µm) Absolute ethanol for drying	Guo et al. (2020)

Apart from the popular extraction methods mentioned in Table 2.6, another alternative method that can facilitate separation is immersion in a salt solution such as sodium chloride and sodium carbonate or bicarbonate (Dhital et al., 2016). In comparison with water, the sodium ions in salt solutions will replace the calcium and magnesium ions in the cell wall, allowing more water molecules to penetrate into the cell walls, which results in faster softening of the cell walls. Consequently, the softer texture will assist in subsequent separation (Iyer et al., 1980).

Although the middle lamella can be cleaved effectively to achieve proper separation of the cell wall components, the permeability of the cell wall to the various processes, and whether they affect the physico-chemical properties and digestibility of the intracellular starch and protein matrix, are not fully understood. Therefore, attention will be given to this topic in the next section.

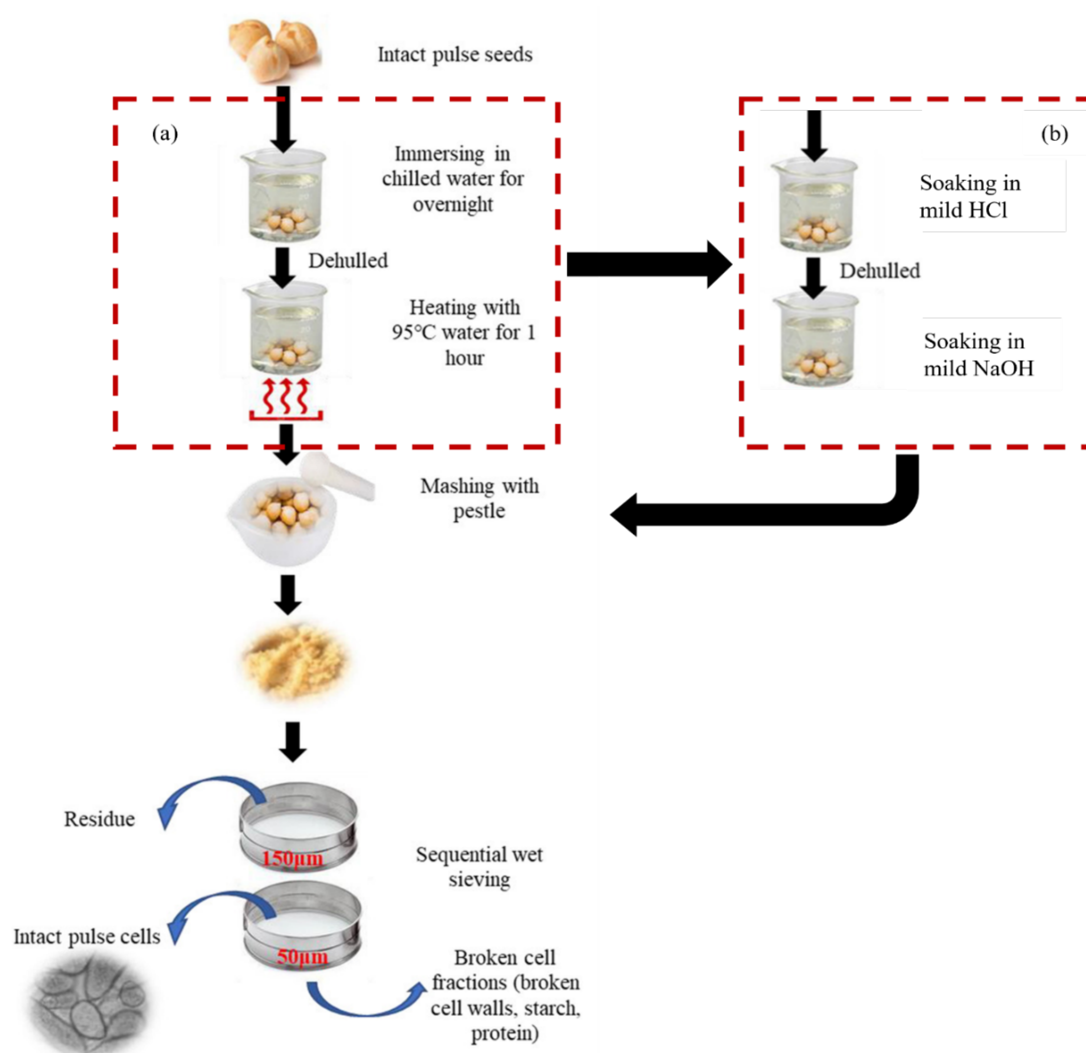

Fig. 2.4. Schematic of the isolation of intact cotyledon cells by hydrothermal processing (a) and acid–alkaline treatment (b). Adapted with permission from Xiong et al. (2022).

2.4 Starch digestion

The determinations of starch hydrolysis during digestion can be studied by *in vitro* and *in vivo* methods.

2.4.1 *In vitro* digestion

The *in vitro* digestion model is designed to simulate the biochemical environment, temperature, and pH of the human gastrointestinal tract. Typically, the model contains the oral, gastric and small intestinal stages as shown in Fig. 2.5. *In vitro* digestion has been used extensively for starch hydrolysis as well as for predicting GI value because of its efficiency and simplicity (Hasjim et al., 2010). Several methods of calculating starch digestibility *in vitro* are discussed below.

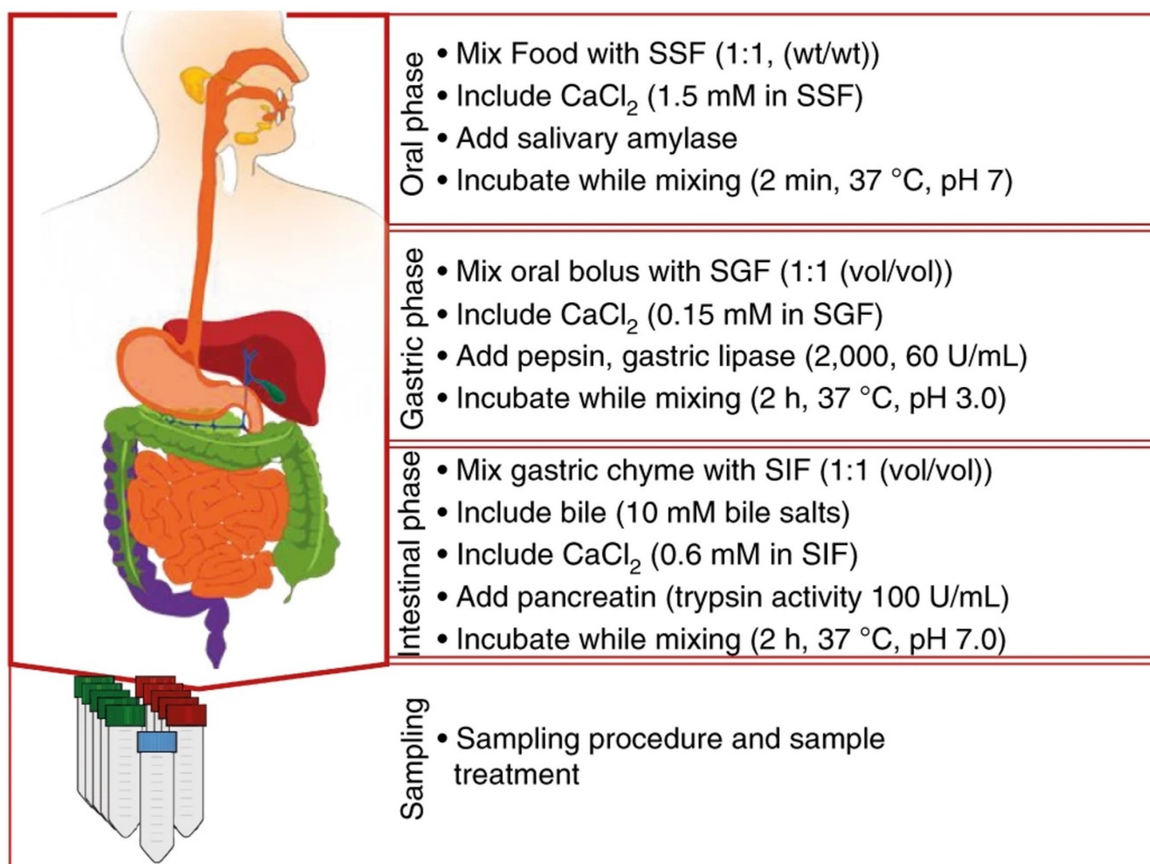

Fig. 2.5. Schematic of the *in vitro* digestion procedure. Simulated saliva fluid (SSF), simulated gastric fluid (SGF), and simulated intestinal fluid (SIF). Adapted with permission from Brodkorb et al. (2019).

2.4.1.1 The Englyst method

Englyst et al. (1992) were the first to model the digestion of CHO *in vitro* by simulating the hydrolysis of starch in the gastric and small intestine, measuring the

glucose released during starch hydrolysis at different time points (20 min and 120 min) and calculating the corresponding starch hydrolysis rates. Equations for determining rapidly digestible starch (RDS), SDS, and RS have been proposed:

$$RDS = 0.9 \times G20 \times 100\% \quad (2.1)$$

$$SDS = 0.9 \times (G120 - G20) \times 100\% \quad (2.2)$$

$$RS = 0.9 \times (TG - G120) \times 100\% \quad (2.3)$$

where *RDS* is starch that is digested and absorbed in the small intestine within 20 min; *SDS* is starch that is completely digested and absorbed in the small intestine from 20 min to 120 min; *RS* is starch that is not digested and absorbed in the small intestine within 120 min, but reaches the colon and is fermented by colonic microorganisms and then has an effective physiological effect; *TG* is total glucose.

This method for determining starch digestibility is only able to calculate the amount of glucose released during the reaction to obtain the amount of RDS, SDS, and RS, but it does not reveal the relationship between the rate of glucose release and digestion time.

2.4.1.2 The first-order kinetic method

To overcome the limitations of the Englyst method, the first-order kinetic method was developed to fit the starch digestion curve (Goñi et al., 1997):

$$C_t = C_\infty(1 - e^{-kt}) \quad (2.4)$$

where C_t is the percentage of digested starch at digestion time *t*, C_∞ is the equilibrium concentration, *k* is the first-order rate constant (min^{-1}) and *t* is the selected time.

If the enzymatic reaction occurs at a defined concentration of amylase, then $\ln(C_\infty - C_t/C_\infty)$ shows a linear function of *t* with a slope of -*k*.

2.4.1.3 Limitations

The *in vitro* digestion method is mainly used to determine starch digestibility, but the type of food, processing, and food composition can have a major impact on starch hydrolysis. Moreover, sugar alcohols should be considered, because they may contribute to the malabsorption of other nutrients in the diet, such as carbohydrates and fat, altering the starch digestibility and glucose release (Lal et al., 2021).

2.4.2 *In vivo* digestion

2.4.2.1 Human clinical trials

Human clinical trials are the standard international method for determining GI. These require around 10 healthy volunteers who have passed an oral glucose tolerance test to consume food containing 50 g of glucose or an equivalent amount of CHO, then measuring their blood glucose values after fasting and at 15, 30, 45, 60, 90 and 120 minutes after the meal. These data are collected to plot the area under the curve (AUC) and calculate the GI values (FAO/WHO, 1998; Wolever et al., 1990).

The measurement of GI in human clinical trials is influenced by a variety of factors, including the health conditions of the volunteers, the number of subjects, pre-test meal requirements, the available CHO content, the timing and method of blood collection, the method of AUC calculation and data processing. Moreover, the structure, particle size, processing, and texture of the test food will affect the GI value as well (Brand et al., 1985; Granfeldt et al., 1994; Thorne et al., 1983).

2.4.2.2 Animal models

Animal models such as mice and pigs are now commonly used to study the digestibility of starch *in vivo* (Belobrajdic et al., 2017; Guilloteau et al., 2010). Pigs provide a good digestive model because their gastrointestinal tract structure, metabolic mechanisms as well as nutrient absorption and utilisation are more similar to those of humans (Guilloteau et al., 2010; Roura et al., 2016). The pigs are trained to undergo an oral glucose tolerance test for a period of time as a way to come closer to human standards (Manell et al., 2016).

However, it must be noted that there are still differences between animal models and humans. Taking the pig model as an example, it has been observed that pigs have no discernible sensor for diets containing high-intensity sweeteners (Roura et al., 2016). Hence, it is assumed that the same food with high-intensity sweeteners is not as enjoyed by humans as it is by pigs, thereby indirectly influencing the amount of food consumed and the postprandial glycaemic response.

2.4.2.3 Limitations

Generally, it is still challenging to develop adequate human clinical trials and animal models for accurately predicting GI values and glycaemic responses. There are certain inevitable flaws, including the accuracy of the methods, the way blood is obtained, the method of food preparation and the number of subjects. For example, the concentration of glucose in the blood will be different if blood is collected from the fingertip capillary or vein (Belobrajdic et al., 2017; Nielsen et al., 2014). In particular,

in vivo digestion is costly compared with *in vitro* digestion, and may involve ethical issues and animal rights.

Overall, determination of the GI value is critical for the development of low GI foods but it can also be challenging. *In vivo* digestion is an accurate portrayal of human digestion, but it has restrictions caused by individual variation, high costs and complex sampling, whereas *in vitro* digestion has the relative benefit of being more widely available, cheaper, more efficient, and less morally objectionable.

2.5 Factors influencing starch digestion

2.5.1 Starch characteristics

Different types of starch in the diet may have different rates of starch hydrolysis. Starch is generally classified into three categories according to its digestibility and bioavailability: RDS is digested and absorbed in the small intestine within 20 min, SDS is completely digested and absorbed in the small intestine within 20 min to 120 min and RS is not digested and absorbed in the small intestine within 120 min, but reaches the colon and is fermented by colonic microorganisms, which have a physiological effect (Englyst et al., 1992). This means that SDS and RS are hydrolysed by digestion at a much slower rate than RDS. Therefore increasing the SDS and RS content in food can be used as a way of reducing glucose absorption and lowering the postprandial glycaemic response (Osorio-Dıaz et al., 2002; Zaman & Sarbini, 2016). Practically, the addition of RS as an ingredient in the diet has great potential for the prevention and control of diabetes and obesity.

Furthermore, the morphology of starch granules also affects enzymatic hydrolysis. Specifically, large granule size and starch digestibility show a negative correlation. For example, Kaur et al. (2007) discovered that small-sized potato starch granules hydrolyse more quickly than larger ones because of their greater specific surface area, increasing the degree of binding to the enzyme and thus their higher susceptibility. Furthermore, the surface characteristics of different sources of starch are different. Many studies have shown that the surface of wheat, maize and rice starch granules is not as smooth as that of tuber and legume starch granules. “Pinholes” and pores were observed on their surface, which may directly lead to the penetration of amylase into the interior through these pores, facilitating hydrolysis (Dreher et al., 1984; Li et al., 2001).

In addition to the starch morphology, the amylose and amylopectin content can also affect the starch digestibility. Cereals, such as wheat and maize, contain 20–30% amylose and 70–80% amylopectin, whereas pulses contain approximately 30–40%

amylose and 60–70% amylopectin. Interestingly, the digestibility of pulses is much lower than that of cereals (Hoover & Zhou, 2003). Therefore, high amylose content is believed to postpone starch hydrolysis. It can be assumed that the high amylose content delays the starch hydrolysis, as amylose has longer chains and stronger hydrogen bonds between glucose molecules, forming a more stable structure and thus making it more resistant to enzymatic attack. Moreover, the surface area of a single molecule of amylopectin is larger than that of amylose, making it more accessible to amylase (Kaur et al., 2016; Singh et al., 2010).

2.5.2 Proteins, lipids and dietary fibre

The presence of protein may affect the rate of starch digestion. It has been found that protein denaturation and hydrolysis can inhibit starch hydration and enzyme cleavage. Firstly, there are interactions between protein and α-amylase, and Bhattarai et al. (2016) observed that protein can partially bind to α-amylase, thereby reducing enzyme availability. Similarly, López-Barón et al. (2017) revealed that protein limits the metabolic enzymes of starch hydrolysis during digestion and enhances the starch–protein interaction. Moreover, the proteins form a network structure that wraps around the starch granules tightly, resulting in a need for protease to first hydrolyse the outer layer of proteins before amylase can hydrolyse the starch, thus delaying starch hydrolysis. Protein is glued into the matrix surrounding the starch granules and acts as a barrier to starch digestion, blocking the catalytic binding of amylase on the starch granules' surface (Wang, Luo, et al., 2014). Furthermore, endogenous protein is also responsible for reducing starch hydrolysis. The starch granules are exposed through the addition of pronase enzyme to hydrolyse the protein matrix, and therefore the digestibility increases significantly (Rooney & Pflugfelder, 1986). Similarly, studies of starch–protein interactions via confocal microscopy have suggested that protein attaches to and surrounds the starch granules, which limits the starch swelling and blocks the access and binding of digestive enzymes to starch (Edwards et al., 2020). However, the effect on endogenous protein and additional protein is different. Jenkins et al. (1987) concluded that the additional gluten added to gluten-free flour to make white bread did not reduce the starch hydrolysis as much as the addition of regular wheat flour. Thus, endogenous protein and its structure play a key role in starch digestibility.

Apart from the effect of protein, lipids are also equally important for starch digestibility. Generally, free fatty acids are likely to form complexes with amylose, and the hydrocarbon portion of the lipid is located in the helical cavity of amylose (Fig. 2.6) (Crowe et al., 2000). The complexes may reduce the swelling power of starch, increase the gelatinisation temperature and enhance the resistance to digestive

enzymes. For example, Annor et al. (2013) investigated the *in vitro* digestibility of defatted millet flour, millet flour, and millet starch, and discovered that defatted millet flour had much higher starch hydrolysis. The effect of lipid–amylose complexes on slowing the rate of starch digestion, delaying the rate of starch absorption in the small intestine, and lowering the postprandial glycaemic response and the insulin response has been demonstrated in the literature (Gelders et al., 2005; Holm et al., 1983; Murray et al., 1999; Panyoo & Emmambux, 2017). Gelders et al. (2005) revealed through *in vitro* digestion that the number of complexes was inversely related to the degree of amylase digestion, and that enzyme resistance improved with an increase in the lipid chain length and the degree of polymerisation of amylose. Moreover, lipids reduce starch gelatinisation, and have an inhibitory effect on amylase, preventing the binding of starch to amylase, thus affecting glycaemic effect (Gulliford et al., 1989; Moghaddam et al., 2006; Sun et al., 2014).

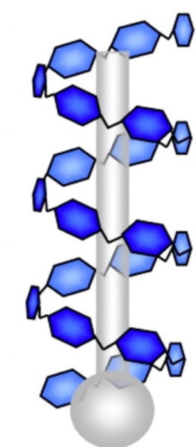

Fig. 2.6. Schematic diagram of an amylose–lipid complex. Adapted with permission from Putseys et al. (2010).

In addition, dietary fibre is mainly derived from the cell walls and is resistant to the action of hydrolytic enzymes present in the digestive tract. It affects the absorption of fat and the metabolism of glucose. In general, the higher the amount of dietary fibre in food, the lower the starch hydrolysis rate (Cummings et al., 2004; Grundy et al., 2016; Peters et al., 2003). Specifically, dietary fibre increases the viscosity of the digestive system, which in turn decreases the rate of starch digestibility and slows the release of glucose (Regand et al., 2011). Furthermore, the addition of dietary fibre can alter the starch ratio in food. It may, for example, lead to a rise in the amount of resistant starch (Borczak et al., 2012; Reshmi et al., 2017). This trend is similar to that reported by Oh et al. (2014), who suggest that adding insoluble dietary fibre increases the SDS content and drops the RDS content. Also, soluble dietary fibre contains a large proportion of hydrophilic groups which compete with starch for water absorption,

resulting in reduced starch gelatinisation and digestibility (Boulos et al., 2000). On the other hand, dietary fibre can also form a network that envelops the starch granules and hinders the attack of amylase (Minemoto et al., 2002).

2.5.3 Other internal factors

Other significant factors such as various types of glycogens, anti-nutrients, minerals, etc. will influence starch digestibility. In particular, pulses are often rich in anti-nutrients such as tannins, lectins, phytic acid, and enzyme inhibitors, the presence of which reduces the rate of starch digestion and glucose release (Carmona et al., 1996). Phytic acid, for instance, binds Ca^{2+} and impacts digestibility by binding to some amino acid residues as well as influencing amylase biological activity (Yoon et al., 1983). Kumar et al. (2020) found that the higher the phytic acid content, the lower the rate of starch hydrolysis in rice. Besides, the synthesis, secretion, and content of insulin are closely related to minerals, especially chromium and zinc. Vervuert et al. (2010) detected that chromium significantly increased insulin response levels and improved glucose metabolism.

2.5.4 Processing

The processing has a significant impact on starch digestion as well as the components. The degree of starch gelatinisation, protein denaturation, and fat hydrolysis vary according to the processing technique, thus it is critical to evaluate the impact of different processing on starch digestibility.

Hydrothermal treatment causes starch gelatinisation and the inactivation of amylase inhibitors, thus improving the starch *in vitro* digestibility. The crystalline structure is disrupted when starch granules are cooked in excess water, resulting in a rise in swelling and solubility, as shown in Fig. 2.7, and even the surface of starch granules is found to be rough and porous, thereby causing it to be more easily available for amylase hydrolysis (Debet & Gidley, 2007). A study by Rehman and Salariya (2005) found that the starch digestibility of black grams, chickpeas, and lentils increased from 40.44–42.00% to 75.00–77.29% after traditional cooking compared with the uncooked forms. This may be related to starch gelatinisation, inactivation of amylase inhibitors, and denaturation of the protein matrix that surrounds the starch granules. During cooling, the molecules rearrange their structural conformation and are attracted by hydrogen bonds to form insoluble precipitates or gels, a process known as starch retrogradation (Ding et al., 2021). Although hydrothermal treatment increases the RDS content, in combination with starch retrogradation, it is effective in increasing the RS content, which further lowers the hydrolysis of starch. Berg et al. (2012) discovered that cooking navy beans followed by refrigeration for 7 days was

effective in reducing the RDS content and *in vitro* starch digestibility. Similarly, the application of low-temperature storage (starch retrogradation) to potato starch increased the RS content and dramatically reduced enzymatic accessibility (Chen et al., 2020).

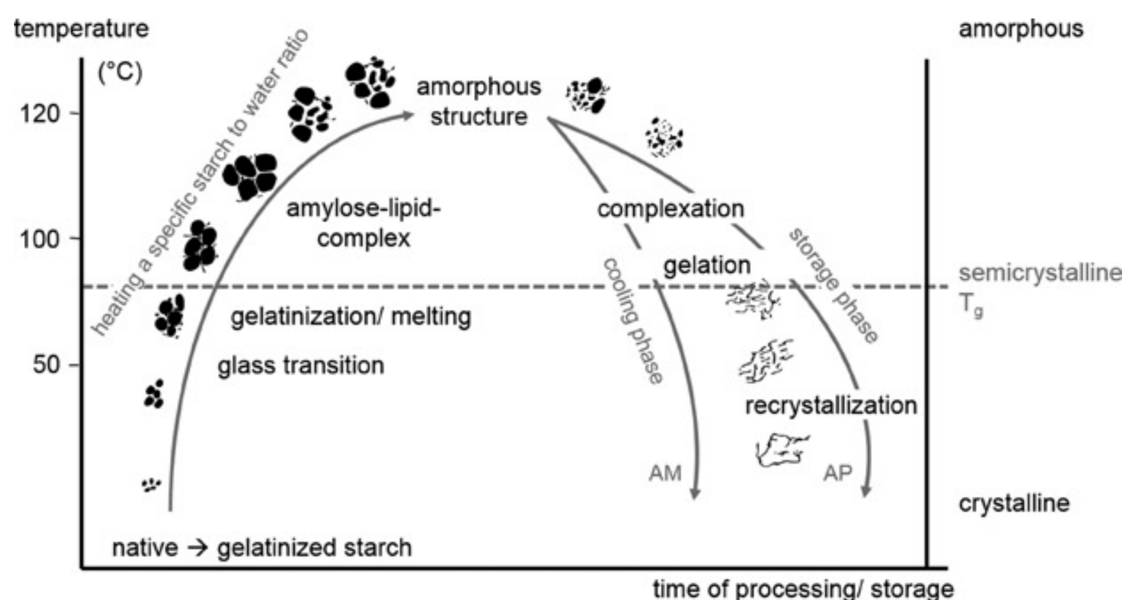

Fig. 2.7. Schematic representation of starch swelling and gelatinisation. Adapted with permission from Schirmer et al. (2015).

Autoclaving is commonly used in food processing, because it promotes the gelatinisation of starch granules, and alters their structure, thus increasing the digestibility of starch. For instance, Berg et al. (2012) discovered that autoclaved navy beans with partially ruptured cell walls and a denatured protein matrix, as well as swollen starch granules, had increased their digestibility by approximately 20%, compared with hydrothermal treatment. These effects of autoclaving on starch digestibility are similar to those found by other researchers (Ezeogu et al., 2005, 2008; Miao et al., 2010). Generally, autoclaving followed by cooling can also increase RS content through starch retrogradation (Colussi et al., 2018).

Pulse starch granules are affected by acid–alkaline treatment primarily through their cell walls. The acid–alkaline-treated cotyledon cells are less permeable than those subjected to thermal processing, thus leading to significant differences in the thermal properties (gelatinisation peaks and gelatinisation transition temperatures) and digestibility of starch. Li et al. (2019) observed that cells treated with acid and alkali had higher enthalpy and starch crystallinity than heat-processed cells. Moreover, the cells isolated by acid–alkaline treatment have a wider gelatinisation range and lower swelling power and capacity than pure starch from the same botanical sources (Do et al., 2019). In terms of starch digestibility, the cells isolated by acid–alkaline treatment

have limited digestibility, because the lower permeability of the cell walls reduced the enzyme accessibility. Specifically, cells extracted by acid–alkaline treatment exhibited lower digestibility and kinetic parameters compared with hydrothermal processing (Do et al., 2019; Li et al., 2019). However, limited studies have been published regarding acid–alkaline treatment. There is some understanding of how the acid–alkaline treatment affects isolated cells and the permeability of cell walls, but the effects on the intracellular protein matrix related to digestibility are still not completely understood. Further studies are required.

Extrusion cooking enhances starch digestibility. The combined effects of high temperature, high pressure, and high shear during extrusion lead to the breakage of hydrogen bonds between starch molecules and significant changes in food structure and texture, increasing the sensitivity of starch to digestive enzymes (Dushkova et al., 2012; Singh et al., 2010). Bouasla et al. (2017) observed that extrusion led to a reduction in particle size, which accelerated the starch hydrolysis rate and increased the glucose release. However, the starch hydrolysis rate of some grains can sometimes be reduced after extrusion, possibly due to the formation of amylose-lipid complexes during processing (Bhattacharya, 1997; Singh et al., 2010).

Baking is a dry heat processing method using heat radiation, where protein denaturation and starch gelatinisation occur in food, it is an essential step in bakery products such as bread and cake. Most foods have a markedly higher glycaemic response after baking than before baking. This is probably attributable to the fact that starch is gelatinised and cleaved into dextrin and maltose, which are more easily absorbed and digested (Allen et al., 2012). However, somehow baking can also decrease RDS content and decline the starch hydrolysis rate (Roopa & Premavalli, 2008).

Frying also impacts the starch digestive properties. Bahado-Singh et al. (2011) compared the effects of different conventional cooking ways on the starch digestion of sweet potatoes and found that fried sweet potatoes had a lower starch hydrolysis rate compared to baked ones. Likewise, Odenigbo et al. (2012) concluded the starch digestibility of sweet potatoes was decreased after frying. Thus, it can be assumed that frying does not significantly increase the starch digestion of the food, which might be attributed to the added fat content, resulting in delayed starch gelatinisation. On the other hand, frying provokes a rise in RS content and limits the starch hydrolysis, compared to boiling (Fernandes et al., 2005).

2.5.5 Food product characteristics

Food characteristics such as consistency, particle size, and texture will affect starch digestion as well. A large particle and firm texture are generally inversely correlated to starch hydrolysis rate (Choy et al., 2021; Gao et al., 2019; Gao et al., 2021). Loose and porous bread, for example, cannot prevent the penetration of digestive enzymes and is more susceptible to hydrolysis into glucose, which has a higher starch hydrolysis rate than bread with a compact texture (Fardet et al., 2006).

2.6 Cell walls and their influence on starch digestion

Generally, the low starch hydrolysis during digestion of pulse flour is related not only to its starch structure but also to the structure of its non-starch composition. Pulse starch is physically encapsulated by the cell wall and protein matrix, which acts as a barrier preventing amylase from entering the cell and catalysing starch hydrolysis. Furthermore, intact cell walls limit the complete absorption of water and the swelling of starch granules, which reduces the effect of digestive enzymes, as shown in Fig. 2.8. In contrast, the cell walls are disrupted to various degrees by traditional grinding and domestic cooking, thus liberating the starch granules and making them easier to bind to amylase and be hydrolysed (Dhital et al., 2016).

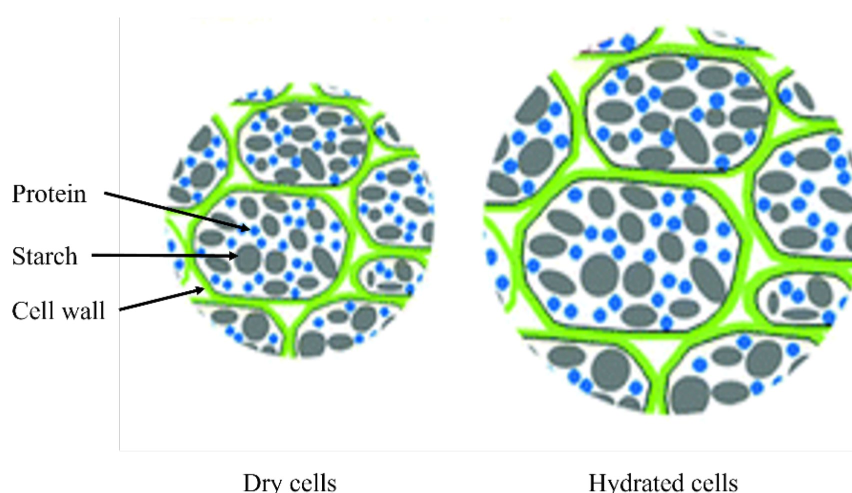

Fig. 2.8. Schematic diagram of pulse cells. Adapted with permission from Dhital et al. (2016).

Traditionally, the intact cotyledon cells of pulses are isolated during cooking. Würsch et al. (1986) identified the role of cell walls as a barrier during starch digestion. Interestingly, pectin is resistant to solubility during cooking, which, in turn, prevents the amylase from binding to the starch in the cells, thus hindering starch hydrolysis (Frost et al., 2016). Dhital et al. (2016) also revealed that cotyledon cells extracted from chickpea, pea, mung bean, and kidney bean retain their cell wall integrity after

cooking, and the intracellular starch is not fully gelatinised and keeps some crystalline structure. The intact cell walls directly block the enzyme from binding to the starch and resisting enzymatic hydrolysis, but once the cell walls have been destroyed, the starch hydrolysis rate will increase (Berg et al., 2012; Do et al., 2019; Edwards et al., 2020; Rovalino-Córdova et al., 2019). Likewise, Bhattarai et al. (2017) found that the integrity of cell walls was still maintained after gastrointestinal digestion in mice, which demonstrates the protective role of cell walls during digestion.

However, different species, mechanical forces, and heating can affect the permeability and porosity of cell walls, influencing enzyme accessibility (Li et al., 2019; Pallares et al., 2018). It has been found that in addition to the barrier role of cell walls, the protein matrix, and insoluble cell wall polysaccharides also assist in limiting starch hydrolysis. The protein matrix can be regarded as a second barrier, reducing the exposure of starch to enzymes, and dietary fibre can bind amylase, thereby inhibiting access to starch (Brummer et al., 2015; Qiu et al., 2017). Overall, intact cell walls, intracellular protein and dietary fibre all play a vital role in limiting starch hydrolysis and reducing digestibility.

2.7 Glycaemic index and low GI foods development

2.7.1 Definition

The glycaemic index (GI) was first introduced by Jenkins et al. (1981) to reflect the extent to which food affects blood glucose levels in humans. GI can be defined as the ratio of the AUC of food containing 50 g of available carbohydrate to the AUC of a reference food (white bread or glucose) containing an equivalent amount of available carbohydrate. Generally, foods can be classified into three categories according to the GI value: low-GI food ($GI \leq 55$), medium-GI food ($55 < GI \leq 70$), and high-GI food ($GI > 70$) (Brand et al., 1991).

The estimated GI (eGI) is measured by *in vitro* digestion via the following equation:

$$eGI = 39.71 + 0.549HI \quad (2.5)$$

where the hydrolysis index (*HI*) is determined as a percentage of the difference in the AUC between a food and a reference food (white bread) (Goñi et al., 1997).

Low-GI foods take longer to digest in the gastrointestinal tract, resulting in a slower rise in blood glucose, thus maintaining a sense of satiety and reducing postprandial insulin secretion, and improving insulin sensitivity. Therefore, low-GI foods are effective in lowering the risk of diseases such as diabetes and obesity (Rizkalla et al., 2002).

2.7.2 Low GI foods development

Low GI foods will hold a huge market potential in the future due to the key role of dietary interventions and recommendations in diabetes prevention and control. Current research on low GI foods is focused on adding low glycaemic index ingredients such as legume flour or oat bran to high GI foods (e.g. bread and cake) to reduce the GI value of food. Many studies have been conducted on staple foods such as bread, pasta, biscuits, and rice.

Bread is one of the most popularly consumed staple foods worldwide and is usually made from wheat flour and is considered to have a high GI value. Zafar et al. (2015) prepared bread by incorporating different levels of chickpea flour into wheat flour and compared them to white bread in terms of acceptability, satiety, and blood glucose levels, showing that bread with 35% chickpea flour remained palatable, had acceptable texture and reduced the glycaemic effect. Similarly, the addition of dietary fibre to bread formulations is also effective in reducing GI value. For example, Papakonstantinou et al. (2018) fortified bread with bran and carob seed flour, and the enhanced bread had higher dietary fibre content and lower GI value according to nutritional profile. Chhavi and Sarita (2012) substituted 60% of wheat flour with millet flour and the GI value dropped from 67.82 to 41.43 (low GI).

The low-GI concept is also popular in other baked products such as biscuits and cakes. A low GI biscuit was produced with pigeon pea-wheat flour, achieving a good taste with a GI value of 51.67 at 75% pigeon pea substitution (Gbenga-Fabusiwa et al., 2018). Meanwhile, Oku et al. (2009) investigated the cake made from whole soy powder and measured the GI value. The results indicated that the addition of whole soy flour delayed starch hydrolysis rate and resulted in a low-GI cake (GI=22±6). Generally, wheat flour can be substituted in a high proportion in biscuits and cakes, but this is unlikely for bread. This is probably because high substitution levels do not form gluten networks easily and tend to change the porous and fluffy texture.

In addition to baked products, pasta and rice are also favoured sources of carbohydrates in many European and Asian countries. It is reported that the incorporation of legume flour can improve their nutritional value and affect the glycaemic effect (Giuberti et al., 2015). One study showed that adding chickpea flour to spaghetti resulted in a significant boost in protein content and lowered the GI value from 73±5 to 58±6 (Goñi & Valentín-Gamazo, 2003). Also, polyphenol and flavonoid content are increased due to legume flour (Turco et al., 2019). In the case of rice, Zeng et al. (2014) incorporated sea tangle and olive oil into Thai Jasmin rice to reduce the GI value from 70.94 to 52.9. Overall, research in low GI foods is gaining attention

and popularity, not only in the area of staple food but also in other areas such as low GI snacks.

2.8 Research gap, questions and hypotheses

Although much attention has been received on the changes in the structure and digestibility of isolated pulse starch, the interactions between cellular components such as intracellular proteins, cell wall, and starch on the functional properties including starch digestibility have not been sufficiently investigated. The mechanism by which pulses cell walls affect the starch structure and digestibility during processing and treatment is particularly unknown. In addition, only limited studies have explored the properties of intact pulses cotyledon cells and their application to develop low glycaemic foods. Therefore, the present study investigates the effect of different isolation methods on the structure and *in vitro* starch digestibility of those cotyledon cells. The effects of the addition of isolated cotyledon cells in the bread formulations and its influence on starch hydrolysis kinetics and digesta characteristics during oral, gastro-small intestinal digestion *in vitro* and glycaemic index (*in vivo* human) were also studied. Also, this study is expected to contribute towards the development of an optimised process suitable for commercial production of cotyledon cells from legumes and pulses and their application in different starch-based foods to develop their low GI versions.

Having identified the research gap, the following research questions and hypotheses were developed, as highlighted in Fig. 2.9.

	Research questions	Hypotheses
Chapter 3 & 4	What is the role of cotyledon cell walls in limiting starch hydrolysis after undergoing different types of processing?	The cotyledon cell walls retain their integrity after processing and act as a physical barrier along with intracellular protein matrix to inhibit α-amylase access to starch and therefore lower starch hydrolysis.
Chapter 5	Can intact cotyledon cells remain intact after secondary processing and still capable of restricting amylase digestion of starch in a low GI bread?	The secondary processed cell walls of cotyledons are robust enough and can be used as a novel ingredient to design low GI foods.

Fig. 2.9. Research questions and hypotheses.

CHAPTER THREE: Isolation of cotyledon cells from navy beans

3.1 Introduction

Common beans (*Phaseolus vulgaris L.*), peas (*Pisum sativum L.*), lentils (*Lens culinaris L.*) and chickpeas (*Cicer arietinum L.*) are the most typical pulses, belonging to the legume family. They are grown and consumed in large quantities worldwide as indispensable crops, particularly for their nutritional features (they are rich in complex carbohydrates, protein, dietary fibre, vitamins and minerals), as an ideal source of protein in some materially deprived areas, and for their low-fat content and high dietary fibre content, which can help to lower cholesterol, increase satiation, aid blood sugar control and prevent cancer (de Almeida Costa et al., 2006; Rochfort & Panozzo, 2007). Owing to these qualities, they are a good nutrient source for some people suffering from cardiovascular disease and type II diabetes (Tharanathan & Mahadevamma, 2003). The low digestibility of starch in pulses is attributable to several factors, including the nature of the starch itself; the interactions of starch with lipids, proteins and dietary fibres; and the presence of various anti-nutritional compounds including amylase inhibitors, phytic acid and polyphenols (Reddy et al., 1984; Singh et al., 2010). Besides, a growing amount of evidence has recognised the importance of cotyledon cell structure for low starch digestibility (Berg et al., 2012; Do et al., 2019; Edwards et al., 2020; Hoover & Zhou, 2003; Singh et al., 2014).

Bean starches, regardless of their intrinsic enzymatic susceptibility, are not readily accessible to digestive enzymes. The cell wall prevents amylase from entering the cell and catalysing starch hydrolysis; hence, it can be considered to safeguard the starch encapsulated in the cell. Additionally, the starch granules are tightly embedded in a protein matrix surrounded by the cell. At the same time, this unique structure (the intact cell wall) also limits the water absorption, swelling and gelatinisation of the

starch granules and the spillage of starch granules during the swelling process, thus reducing the action of digestive enzymes (Berg et al., 2012). However, after different treatments such as grinding or cooking, the integrity of the cell wall is disrupted to varying degrees and the starch is readily hydrolysed by amylase (Berg et al., 2012; Do et al., 2019; Edwards et al., 2020).

Several methods can be used to obtain intact cotyledon cells. Dissolving the pectin-rich middle lamella between the cells is the key to separation of intact cotyledon cells (Sila et al., 2009). The first intact cotyledon cells were isolated from pulses by Würsch et al. (1986) in a simulated cooking process, but the digestion properties of starch granules inside the cells have been studied less often in the following decades. Only recently food researchers have been evaluating the relationship between the plant cell wall and digestibility and the absorption of nutrients contained therein, resulting in extensive literature describing the nutritional and functional properties of cotyledon cells, and using and developing the isolation method of Würsch et al. (1986), although, in general, the cotyledon cells are still obtained by melting the pectin through hydrothermal processing (Berg et al., 2012; Bhattarai et al., 2018; Edwards et al., 2020; Eyaru et al., 2009; Li et al., 2020; Rovalino-Cordova et al., 2018; Xiong et al., 2019). Alternatively, Kugimiya (1990) found that changing the pH of the soaking solution by successively using hydrochloric acid and sodium hydroxide changed the middle lamella and cell wall, such as by the hydrolysis of pectin. This avoided the irreversible effects of elevated temperatures on cells and starch granules, leading to the successful isolation of cotyledon cells with ungelatinised starch granules. The acid–alkaline method has also been successfully applied to isolate intact cotyledon cells from chickpea, lentil, adzuki bean, lima bean, kidney bean and peas, and so on. The properties of their cotyledon cells all share similar characteristics, such as retention of ungelatinised starch granules, lower swelling power and digestibility (Do et al., 2019; Guo et al., 2020). One issue noted by Do et al. (2019) was that acid

and alkali can alter the natural structure of the cell wall to some extent, and the yields are so low that they cannot be used in commercial production.

This study is aimed to propose an optimised isolation process that is suitable for commercial production of cotyledon cells from legumes and investigate their morphological properties, compared to their counterpart navy bean starch. Therefore, this project provides an important opportunity to advance the understanding of various isolation methods of cotyledon cells and their properties for application in commercial industry.

3.2 Materials and Methods

3.2.1 Materials

Whole dry navy beans (*Phaseolus vulgaris*) were purchased from Bin Inn, Waitara, New Zealand. Their physical appearance is shown in Fig. 3.1. Navy beans were stored in sealed thick plastic bags at 4 °C throughout the study.

Fig. 3.1. Navy beans.

3.2.2 Extraction of cotyledon cells

Five different methods were used.

3.2.2.1 Acid–alkaline treatment

Intact, raw navy bean cotyledon cells were obtained through successive treatment with acid and alkali solutions following a modified version of Do et al. (2019). Separation of navy bean cells under mild acid and alkaline conditions minimised damage to the cell wall. The cells were separated by successive soaking in mild acid and alkaline solutions to remove the middle lamella layer of pectin. Dry navy beans were soaked thoroughly in a 0.1 M hydrochloric acid (HCl) solution and stored at 4 °C in a chiller for 24 hours to allow the shell to swell and loosen sufficiently, followed by peeling the testa and hypocotyls manually, then the cotyledons, which had been split into two parts, were washed to remove the residual acid and transferred to the 0.06 M sodium hydroxide (NaOH) solution for soaking at room temperature (20 °C) for 24 hours with gentle stirring. A mincer (Kenwood, Ltd, Havant, UK) was used to gently mash the softened, alkali-treated cotyledons to a uniform paste. Following thorough washing with water, the resulting paste was passed through 150- and 53-µm sieves. The cell extracts were collected on the 53-µm sieve and then freeze-dried. The dry powders were sealed and kept at room temperature until further testing. The acid and alkali were both food grade.

3.2.2.2 Autoclaving

The intact navy beans cotyledon cells were extracted following a modification of the method reported by Berg et al. (2012). Dry beans were immersed in water for 1 hour, then hydrated beans were rinsed and placed in a can. After adding water, the can was sealed and placed in an autoclave (Steriflow, Roanne, France). The first phase was heating to 121 °C, holding for 15 min, and then cooling down to room temperature. The autoclaved beans were gently mashed with the mincer described above to make a consistent paste, followed by passing the mixture through 150- and 53-µm sieves, washing with extensive water until no free starch was released, and freeze-drying. The dry powders were sealed and kept at room temperature until further testing.

3.2.2.3 Hydrothermal processing

The beans were soaked in water overnight at 4 °C to inhibit enzyme activity. They were boiled for 30 min to soften the beans, imitating home cooking. This was followed by the procedure described in Section 3.2.2.2, mashing to a uniform paste and wet sieving (150 and 53 µm), and freeze-drying. The dry powders were sealed and kept at room temperature until further testing.

3.2.2.4 Retrogradation of cooked navy bean cells

Dry beans were soaked in water overnight at 4 °C, then boiled for 30 min. They were placed in cans and sealed after cooling, then stored in a 4 °C chiller for 7 days to achieve starch retrogradation, subsequent mashing and wet-sieving to harvest the cotyledon cells (53–150 µm), followed by freeze-drying (boiling–retrogradation–freeze-drying; BRF), or fluid-bed drying (boiling–retrogradation–bed drying; BRB) at 30 °C to gather a dry powder with intact cells. The 2 different types of dry powders were sealed and kept at room temperature until further testing.

3.2.3 Isolation of navy bean starch granules

Navy bean starch granules were extracted according to a slight modification of the processing method described by Berg et al. (2012). Dry beans were steeped in 0.5% sodium metabisulphite at 4 °C for 18 h. The hydrated beans were rinsed and wet-ground with water in a laboratory blender (PB7950, Sunbeam, New Zealand) after the soaking liquid had been removed. The milky suspension was filtered through sieves of 150 and 100 µm in succession. The residue remaining on the 100-µm sieve was washed thoroughly with water until no starch was visible. The collected filtrate slurry was then centrifuged at 1500 × *g* for 15 min to obtain two-layer solid sediment after discarding the supernatant. This was followed by scraping off the protein-rich top layer with a spatula, and the starch solution was centrifuged again with water. The same step (centrifugation and washing) was performed three times. The starch

sediment was then laid flat in a tray and air-dried for 18 h at 40 °C. The dry powders were sealed and kept at room temperature until further testing.

3.2.4 Determination of the morphological properties

3.2.4.1 Light microscopy

Light micrographs were captured with a microscope (Leica CME, Wetzlar, Germany) equipped with a digital camera (OMRX 18 MP USB 3.0, China) and the application suite of Leica software at 20× magnification. A scanning electron microscope (FEI Quanta 200 FEI Electron Optics, Eindhoven, The Netherlands) was used to collect representative micrographs of bean starch and various cotyledon cells. Samples of starch and cell powders were dispersed on double-sided sticky tape mounted on an aluminium stub and then sputtered-coated with gold.

3.2.4.2 Particle size distribution

The granule size distribution was measured by a laser diffraction particle size analyser (Malvern Mastersizer 2000, Malvern Instruments Ltd., UK). The refractive indices chosen for cell powders (or starch) and the dispersant (water) were 1.530 and 1.330, respectively. Particle absorption was 0.1. Each sample was mixed thoroughly with water and slowly added until the obscuration reached 15 ± 1%. Each determination was run in triplicate. Dv10, Dv50, Dv90, span, and specific surface area were collected.

3.2.5 Determination of yield

The yield was defined as the weight of the final amount of cell powder as a percentage of the weight of the original dry beans:

$$\%Y = \frac{W_f}{W_o} \times 100 \quad (3.1)$$

where $\%Y$ is the percentage of yield (%), W_f is the final weight of cell powder (g) and W_o is the original weight of the beans (g).

3.2.6 Statistical analysis

All data were analysed by Tukey's test and variance (ANOVA) in Minitab 19 software, and the confidence level of the difference between the means was 95% ($p < 0.05$). The results were expressed as means and standard deviations of triplicates.

3.3 Results and Discussion

3.3.1 Isolation methods and morphological properties

Extra care is required during extraction to preserve the integrity of the cell wall and minimise damage, due to the specificity and complexity of the cotyledon cell structure. The cotyledon cells are bound together by a thin pectin-rich middle that is packed with cellulose, hemicellulose and pectin (Carpita & Gibeaut, 1993). Theoretically, cotyledon cell extraction is a matter of loosening or dissolving the middle lamella while avoiding harm to the nutrient-coated cell walls.

Individual cells were isolated via the acid–alkaline method according to the methods of previous studies (Dhital et al., 2016; Do et al., 2019). Navy beans were successively immersed in acid and alkaline solutions, and the starch granules within the cells were not gelatinised. The soaking process was accelerated by adding low concentrations of acid to soften the cotyledon cell tissue and remove divalent cations from the cross-linking of the pectin, particularly calcium and magnesium ions (Ca^{2+}, Mg^{2+}). The subsequent alkaline solution led to middle lamella dissolution and thus cell separation by the β-elimination reaction (Kugimiya, 1990; Njoroge et al., 2016). This extraction method has the advantages of obtaining ungelatinised starch cells and avoiding the effects of high temperatures on the cell wall and cellular components, such as water absorption and cell wall swelling, as well as denaturation of the proteins, which would alter the morphology and physicochemical properties of the original cell. On the other hand, acid and alkaline solutions may also have an impact on the structure and permeability of the cell wall by altering or interacting with certain

components. As a result, the structure and functionality of cells extracted by this method deviate from those of natural cell walls to some extent (Do et al., 2019).

In addition to the acid–alkaline method, thermal processing (boiling and autoclaving) can also be used to separate cells effectively. Heating partially dissolves the thin pectin layer between the cells, decreasing the intercellular adhesion, which, together with physical grinding, facilitates the separation of intact cells. Furthermore, through an increase in temperature, the turgor pressure within and outside the cell is considerably reduced as the lipid bilayer of the cell membrane dissolves, allowing water molecules to easily travel between tissue structures. Additionally, the gelatinisation of starch granules within the cells can facilitate cell separation by imposing mechanical tension on the tissue (Carpita & Gibeaut, 1993; De Belie et al., 2000; Dhital et al., 2016). Despite these benefits, one of the disadvantages of this method is that high temperatures also alter the starch and cell walls' morphology, porosity and permeability; the permeability of the cell wall is positively correlated with cooking time, temperature and pressure (Li et al., 2019; Pallares et al., 2018; Pallares Pallares et al., 2021). Hence, there is a high possibility that the cotyledon cells extracted by these methods will differ from natural cell tissue in some way.

Figures 3.2 and 3.3 summarise the appearances of navy bean starch and cotyledon cells obtained by each extraction method and their particle size distribution. Navy bean starch granules were round (small size) and oval (large size) with indentations. According to the scanning electron microscopy (SEM) analysis, the surface of the starch granules was observed to be smooth, with minimal protein fragments or highly hydrated fine fibre fractions adhering to them, and a noticeable fissure in the centre of the granule, which was consistent with previous studies (Berg et al., 2012; Du et al., 2014; Singh et al., 2014). Disordered crystallisation of the double helix arrangement surrounding the hilum region of amylopectin may be responsible for the formation of this crack (Blennow et al., 2003). According to Hoover and Sosulski (1991) and Jane

et al. (1999), the morphological differences in starch granules are largely determined by the biological origin, maturity, amyloplast biochemistry and plant physiology. Moreover, the plastid membrane and physical properties also play a vital role in granule formation, influencing the shape and possibly the arrangement and binding of amylose and amylopectin (Lindeboom et al., 2004). The average granule diameter (Dv50) of navy bean starch was 26.47 µm, with most starch particles measuring 20–35 µm. This result was in agreement with the mean diameter (26.6 µm) reported by Du et al. (2014) and slightly different from the range reported by Berg et al. (2012). Variations in starch granule size are related to plant species and the stage of maturity. There is considerable evidence that the distribution of starch granules changes with the development of storage organs in plants, with A-type granules forming early in development before B-type granules (Chojecki et al., 1986; Hoover & Sosulski, 1991). It has been suggested that starch granules with different particle sizes cause variations in the composition and conformation of amylose and amylopectin, resulting in differences in physicochemical properties, including gelatinisation, enzyme susceptibility and digestive characteristics (Kaur & Sandhu, 2010; Lindeboom et al., 2004).

Figure 3.2 highlights the morphological structure of navy bean cotyledon cell samples obtained by different isolation methods. The cotyledon cells were mainly intact (cell wall integrity) with negligible broken cells, released starch granules or fragments of cell wall visible under the light microscope. These occurred because of mechanical damage during the isolation process. The cells appeared elliptical and elongated, and some were irregularly shaped. Within each cell, starch granules were densely embedded in a protein matrix and surrounded by the cell wall. The starch granules from the acid–alkaline treatment were ungelatinised, with cracks present in the centre, whereas the starch in the cells extracted by heat processing methods (A, B, BRF and BRB) had all absorbed water and swollen, and were partially gelatinised without any

fissures. As a result, the cells appeared to be much fuller and still maintained a compact cellular structure even after boiling or autoclaving. In addition, an interesting phenomenon was that BRB cells appeared in large irregular clusters, which was caused by fluid-bed drying. A disadvantage of fluid-bed drying is that it is challenging to harvest very fine powder, and the operating conditions are difficult to control. The particles will stick together if there is a high flow rate and a large wet particle size. These results are similar to those found in other studies (Edwards et al., 2020; Li et al., 2019; Ray et al., 2016; Rovalino-Cordova et al., 2018; Sivakumar et al., 2016). However, instead of having a normal elliptical shape after swelling by water absorption, the starch in these cells (A, B, BRF, BRB) exhibited deformed granules, as illustrated by the arrow in Fig. 3.2, suggesting that either heat, water or space were restricted, leading to limited gelatinisation. In the SEM images, the cell wall surface of all cotyledon cells was shrunken and folded. This is related to the drying method. In the present study, freeze-drying and fluid-bed drying were used to recover the cell samples, especially for the heat-treated cells, to minimize starch retrogradation. During the drying process, spaces are formed through the removal of water molecules from the cells, but it should be noted that the drying method may also influence the plant tissue by forming ice crystals that puncture and cause cavities (Berg et al., 2012; Lewicki & Pawlak, 2003).

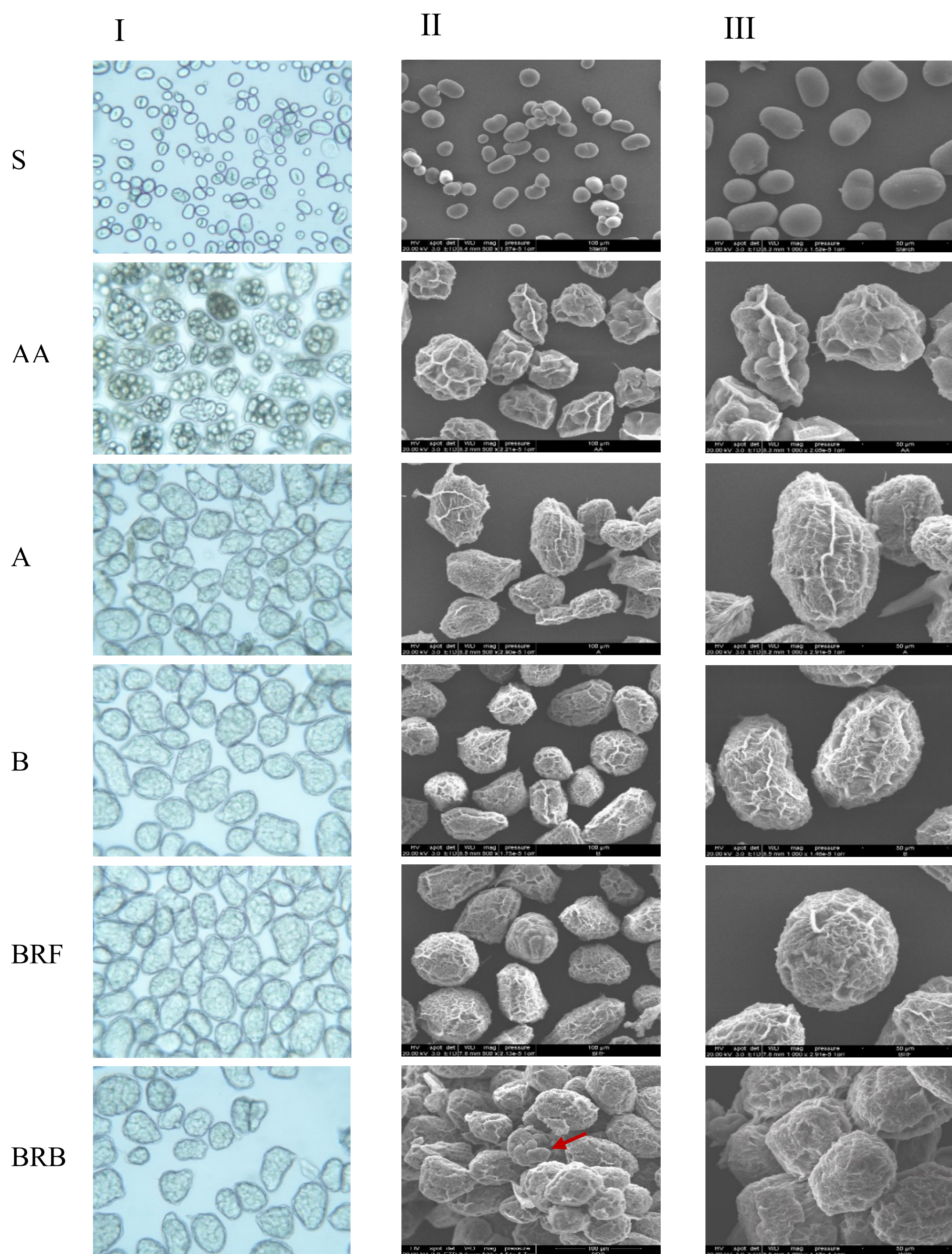

Fig. 3.2. Microscopy images of navy bean starch and cotyledon cells obtained by various extraction techniques. Light micrographs (I), scanning electron micrographs (II) (scale bar = 100 μm) and magnified view of SEM (III) (scale bar = 50 μm). Navy bean starch (S), acid–alkaline treatment (AA), autoclaving (A), hydrothermal processing (B), boiling + 7-day retrogradation + freeze-drying (BRF), and boiling + 7-day retrogradation + fluid bed-drying (BRB).

The particle size distribution of all the cells isolated by the various techniques is exhibited in Fig. 3.3, and the specific sizes are listed in Table 3.1. All samples showed a single peak distribution, with a large difference in the mean diameter between starch (26.47 μm) and cotyledon cells, ranging from 98.30 to 121.23 μm (BRB > BRF > B ≈ AA > A), which was negatively correlated with specific surface area. BRB cells showed the highest granule diameter (121.23 μm) as well as the widest particle size distribution. This may be due to particle clustering, as shown in the BRB image in Fig. 3.2. The size of navy bean cotyledon cells is similar to that reported in other studies (Berg et al., 2012; Do et al., 2019; Singh et al., 2014). The mean cell diameter for A and B did not differ much from that of AA cells because of water absorption and swelling, whereas the internal starch granules swelled significantly. This also reflects the fact that the cell wall restricts the space and further restricts starch gelatinisation.

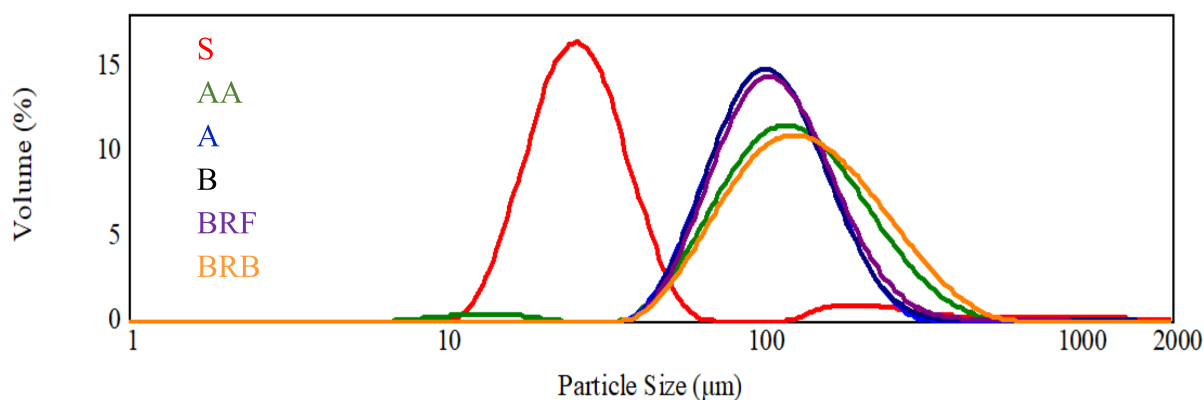

Fig. 3.3. Particle size distribution of navy bean starch and cotyledon cells: starch (S), acid–alkaline treatment (AA), autoclaving(A), hydrothermal processing (B), boiling + 7-day retrogradation + freeze-drying (BRF), and boiling + 7-day retrogradation + fluid bed-drying (BRB).

Table 3.1. The morphological properties of navy bean starch and cotyledon cells.

Sample	Dv10 (μm)	Dv50 (μm)	Dv90 (μm)	Mean diameter (μm)	Specific surface area (m^2/g)	Span
S	17.26	26.95	47.01	26.47[c]	0.227[a]	1.10[b]
AA	64.90	123.81	244.53	99.34[b]	0.0604[b]	1.45[a]
A	63.96	105.06	176.51	98.30[b]	0.061[b]	1.07[b]
B	64.55	105.63	180.54	99.40[b]	0.0604[b]	1.10[b]
BRF	65.91	109.51	191.74	102.65[b]	0.0584[b]	1.15[b]
BRB	70.63	135.27	276.51	121.23[a]	0.0495[c]	1.52[a]

Table shows the mean values of triplicates.

Starch (S), acid–alkaline treatment (AA), autoclaving(A), hydrothermal processing (B), boiling + 7-day retrogradation + freeze-drying (BRF), and boiling + 7-day retrogradation + fluid bed-drying (BRB).

Data in the same column with different superscript letters are significantly different ($p<0.05$).

3.3.2 Yield (%)

The different extraction methods all resulted in relatively intact cotyledon cells in this experiment, but the yield varied depending on the extraction technique. Among all, the acid–alkaline treatment had the lowest yield (AA: 4.80 %), which was significantly different from that of the other methods (above 40 % yield) used in the study; all values are exhibited in Fig. 3.4. The theoretical maximum intact cotyledon cell extraction rate is reported to be about 55%, as the soluble components (mainly from the abaxial cell layer, 30%) are lost during processing (soaking and heating); moreover, the bean coat and embryo account for roughly 5% and 1%, respectively (Edwards et al., 2020). In addition, not all cotyledon cells tend to separate. Hence, the yields, except for that of the acid–alkaline treatment, are reasonable in this experiment. One of the reasons for the low yield of AA cells is that during the

following hydration at room or chilling temperatures, along with externally applied mechanical forces (grinding), the cell walls undergo substantial rupture, making the separation of intact cells very difficult. This is caused by the increased turgor pressure acting outwardly from each granule to the cell membrane and cell wall, causing the tissue to become rigid (De Belie et al., 2000). Given that the acid–alkaline method has not often been considered for future commercial application, future research could consider how to improve and scale up the acid–alkaline technique.

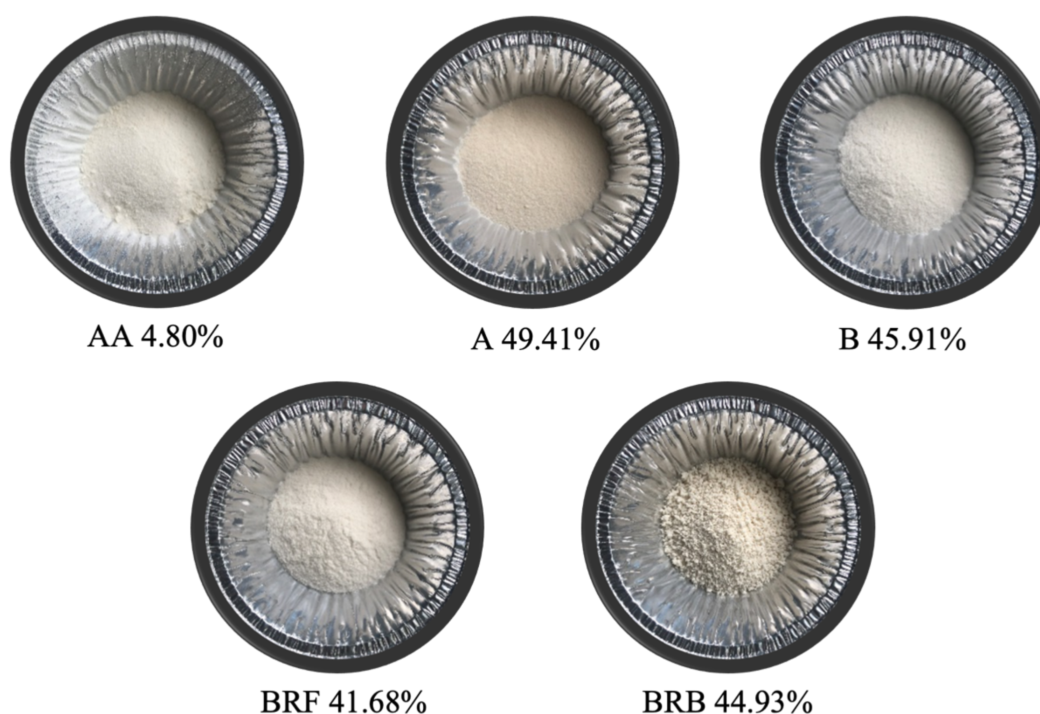

Fig. 3.4. Appearance and yield of intact cells.

The current results displayed higher yields from heat-treated cotyledons than for the acid–alkaline method. As a result of soaking, the cotyledon tissue and cells were sufficiently hydrated to become softer without undergoing drastic changes in their physico-chemical properties. Heat treatment, on the other hand, led to a rupture in the structure, releasing components such as soluble sugars, pectin from the middle lamellae, and protein into the aqueous solution, thus increasing the yields considerably. Moreover, the bean coat of navy beans was not removed during the heat

treatment, and it may also contain some soluble or insoluble fibres (Carpita & Gibeaut, 1993; Hamza et al., 1987; Kutoš et al., 2003). The yield from autoclaving was higher than that from the boiling, indicating that autoclaving broke down more tissue; and thus more of the components (e.g., pectin) were released by dissolution. High pressure affected the cell wall structure through increased porosity and permeability, which can have an impact on the subsequent digestive properties (Li et al., 2019; Siah et al., 2014).

3.4 Conclusion

The intact navy bean cotyledon cells were treated in different ways, including acid–alkaline treatment, hydrothermal processing, and autoclaving. Light microscopy images showed that all cotyledon cells retained their integrity and starch granules were embedded in the intracellular protein matrix. The cells obtained by the acid–alkaline method were ungelatinized. However, the heat-treated cells, all of which had their internal starch swollen by water absorption and partial gelatinisation, were of comparable size, revealing that the unique structure of the cell wall plays an important role in the restricted gelatinisation of starch, which could also presumably be responsible for reducing the enzyme susceptivity. Moreover, the yield of the types of cells (AA, A, B, BRF, BRB) was 4.80%, 49.41%, 45.91%, 41.668%, and 44.93%, respectively, showing that the yield varied considerably among extraction methods. Thus, according to the results of the experiments in this chapter, the process successfully extracted intact cotyledon cells with starch granules remaining inside the cells. It can be assumed that intact cells could be used as a novel ingredient to replace or partially replace traditional wheat flour in starch-based foods, thus lowering the postprandial glycaemic response caused by the digestion of starch. However, the acid–alkaline treatment needs to be improved in terms of yield in the future.

CHAPTER FOUR: Physico-chemical characterisation and *in vitro* digestion of cotyledon cells

4.1 Introduction

Pulses are of interest to researchers and consumers as a major source of inert carbohydrates such as fibre, hemicellulose, RS, and SDS. Carbohydrates are the main component of pulses and consist mainly of starch and non-starch polysaccharides (dietary fibre). The digestive properties of pulse starches directly affect their nutritional digestibility. Englyst et al. (1992) classified starches according to their digestive properties into three types: RDS, SDS, and RS. Navy beans are one of the most popular pulses and are known to have a low glycaemic response.

Numerous studies have demonstrated that the low digestibility of pulse starch is related to the intact cell wall, the intracellular protein matrix, and the presence of its own amylase inhibitors. Do et al. (2019) found that isolated cotyledon cells from adzuki bean, chickpea, lentil, and lima bean obtained by acid–alkaline treatment showed lower starch hydrolysis than free starch granules from the same sources. Not surprisingly, the micrographs of the cells demonstrated the presence of intact cell walls after *in vitro* digestion. Hydrothermal processing and autoclaving play a vital role in cell isolation, but more studies have revealed that the protein matrix may also encapsulate starch, reducing the surface area on which starch granules may bind the enzyme, thus delaying the digestion of the entire system (Rovalino-Cordova et al., 2018; Xiong et al., 2019). Additionally, tannins, phytic acid, lectins, and other amylase inhibitors can reduce the bioavailability of starch by binding enzymes, starch or metal ions to form insoluble complexes, which slow the digestion rate (Jain et al., 2009; Reddy et al., 1985; Yoon et al., 1983).

Therefore, this study set out to assess the physico-chemical properties, functional characteristics, and *in vitro* starch digestion of navy bean starch and cotyledon cells obtained by different extraction methods. It is expected that this research will contribute to a deeper understanding of different extraction and processing methods for cotyledon cells' digestibility, providing a reference for the development and utilisation of starch-based foods.

4.2 Materials and Methods

4.2.1 Materials

All navy bean starch and cotyledon cell powders in this chapter were extracted using the methods described in Chapter 3.

4.2.2 Determination of physico-chemical characterisation

4.2.2.1 Colour

The colour was determined with a Minolta Chroma Meter (CR-400, Konica Minolta Business Technologies, Japan). For all starch and cells, the L*, a* and b* values were measured from different positions with six readings for each.

4.2.2.2 Proximate analysis

Proximate analysis involves determination of the percentages of moisture, fat, protein and ash. Moisture content was tested by the air-oven method. Firstly, about 2 g of a powder sample was placed into a pre-dried and weighted aluminium moisture dish, which was placed in an oven at 108 °C for 4 h. After this, the sample was cooled down to room temperature in the desiccator and weighed accurately. It was returned to the oven for 1 h, then cooled down and reweighed until the weight was constant. The procedure was carried out in triplicate.

The Kjeldahl method was applied to determine the nitrogen content (AOAC, 2006), and the nitrogen to protein conversion factor (6.25) was used for the calculation. A 0.5-g sample was weighed and mixed with 2 Kjeltabs and 15 mL of concentrated

sulfuric acid for 60 minutes at 420 °C. After this process, 70 mL distilled water was added to ensure that all solids had been dissolved. This was followed by distillation (Kjeltec™ 8200, Foss, Denmark), then the final digestion sample was obtained and titrated with 0.1 M HCl to a grey-mauve end point. This was carried out in triplicate.

The crude fat in each powder sample was analysed by the Mojonnier method (AACC 30-10). Around 2 g of powder was mixed with 2 mL ethanol to moisten all particles. The sample was placed in a boiling water bath for 40 min after adding 10 mL HCl. Next, 10 mL ethanol was added, and the mixture was transferred to a Mojonnier tube, then 25 mL diethyl ether was added to rinse the beaker into tube. The mixture was shaken gently for about 1 minute. After the addition of 25 mL petroleum ether, the tube was placed in a centrifuge at a speed of 600 rpm for 2 minutes. 5 mL ethanol was added to prevent an emulsion from forming, then the extraction with 15 mL diethyl ether and 15 mL petroleum ether was repeated. All the extract was added into a pre-weighed flask that was placed on a heating plate to allow the solvent to evaporate completely, then the sample was transferred to a dryer to dry for 90 minutes and cooled to room temperature for weighing again.

Ash content was measured by a dry ashing technique. A 1-g sample of powder was weighed and charred evenly in a pre-heated dry crucible. After that, it was transferred to a muffle furnace (Carbolite™, Thermos Fisher Scientific, China), where it was incinerated at 550 °C for 5 hours. It was then placed in a desiccator to cool to room temperature and weighed.

Carbohydrate content was calculated as 100 minus the moisture content, ash content, crude protein and crude fat, the remaining content is the carbohydrate content.

The total starch content was evaluated with a total starch assay kit (K-TSTA-100A, Megazyme International Ireland Ltd., Ireland). Cell powders were required to be ground to break up the cell walls before testing in order to release the starch granules. A 0.1-g sample was accurately weighed into a 15 mL corning culture tube and

moistened with 0.2 mL of 80% (v/v) aqueous ethanol, and the mixture was stirred and dispersed. Next, 2 mL of dimethyl sulphoxide (DMSO) was added immediately, mixed well, and placed in a boiling water bath for 5 minutes. After that, 3 mL of thermostable α-amylase (3000 U/mL) was added, and the mixture was shaken vigorously and incubated in a boiling water bath for 6 minutes. Amyloglucosidase (3300 U/mL) was added and the mixture was placed in a water bath at 50 °C for 30 minutes, then the volume was fixed to 100 mL. The supernatant was centrifuged, and the glucose content was determined by GOPOD reagent and the absorbance was measured at 510 nm before calculating the total starch content.

4.2.3 Determination of swelling power

Swelling power was assessed via the method developed by Edwards et al. (2020) with slight modifications. In this case, navy bean starch and the cells were determined at three different temperatures (37 °C, 60 °C, and 90 °C). Around 33 mg of the sample was mixed with 1 mL RO water in 1.5 mL Eppendorf® safe-lock™ tubes before vortexing for 5 s. The suspension was placed in a thermomixer (Eppendorf™ ThermoMixer™ C, UK) with circumvolution at 1400 rpm while heating at 37 °C, 60 °C, and 90 °C, respectively. Once the heating phase was complete, the tube was incubated in a cool water bath for 5 min then centrifuged at 13,000 $\times$ g for 10 min. The suspension was divided into two layers after centrifugation, the supernatant was discarded and the lower sediment layer was weighed. Swelling power was calculated via the equation:

$$Swelling\ power\ (\frac{g}{g}) = \frac{Ws}{Wd} \quad (4.1)$$

where W_s is the weight of wet sediment, and W_d is the original weight of the dry sample.

4.2.4 Determination of thermal properties

DSC enthalpy images were collected using a Differential Scanning Calorimeter (TA Q100, TA Instruments, Newcastle, DE) following the method presented by Do et al. (2019). A sample to water ratio of 1:3 (w/v) was applied in this case. Approximately 4 mg of the sample was weighed into a 40-μL aluminium pan and mixed with the required amount of water, then sealed and kept at room temperature overnight to ensure all the powder was moistened before the analysis. An empty pan was used as a reference. The procedure was heated from 20 °C to 120 °C at 10 °C /min. The onset, peak, and conclusion temperature (T_o, T_p, T_c) and enthalpy (ΔH) were recorded.

4.2.5 Gastro-small intestinal starch digestion *in vitro* of starch and cells

4.2.5.1 Cooking samples for *in vitro* digestion

In order to prepare an aqueous solution with around 4 % starch (w/w) for *in vitro* digestion, the required sample was mixed with water in a Schott bottle, followed by incubation at 95 °C in a water bath for 20 min with gentle stirring. This was cooled down in a 37 °C water bath for further testing.

4.2.5.2 *In vitro* digestion procedure

The *in vitro* digestion of navy bean cotyledon cells and starch was simulated according to the two-stage method of Dartois et al. (2010) with minor modifications. Human simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were freshly prepared according to Pharmacopeia (2000). Approximately 170 g of a cooked sample (starch or cotyledon cells) was transferred into a 500 mL jacketed glass reactor and stirred magnetically to prevent the sample from settling on the bottom. The temperature was maintained at 37 ± 1 °C throughout the process. The pH was adjusted to 2 with 3 M HCl, followed by adding 17 mL SGF containing pepsin (enzyme/starch ratio, 1.765:100, w/w), and a 0.5-mL aliquot was taken in absolute ethanol as G0. The pH was then gradually adjusted to 1.2 by adding 0.2 M

hydrochloric acid and kept at 1.2 throughout the gastric digestion phase. The entire gastric phase lasted 30 minutes, with samples taken at 15-minute intervals and recorded as G0, G15, and G30. After 30 minutes, the pH was adjusted to 6.8 with the addition of a NaOH solution (3 M and 0.5 M) to inactivate the pepsin. Next, 22 mL of SIF containing pancreatin (enzyme/starch ratio, 1.3:100, w/w), amyloglucosidase (enzyme/starch ratio, 0.26:1, v/w), and invertase (enzyme/starch ratio, 1:1000, w/w) was added to the reactor and timed. To obtain the intestinal samples, 0.5 mL of the aliquot was withdrawn and transferred into 2 mL of absolute ethanol at various time points between 0 and 120 min to terminate the enzymatic hydrolysis, and recorded as I0, I5, I10, I15, I30, I60, I90 and I120.

All collected samples during the gastric and small intestinal stage were vortexed and centrifuged at 1800 × *g* for 10 min. Next, 0.1 ml of the supernatant was mixed with invertase and amyloglucosidase in a potassium acetate buffer and placed in a 37 °C water bath for 10 minutes to convert all small sugars to glucose, then the absorbance value was measured at 510 nm with GOPOD (Megazyme International Ireland Ltd., Ireland) reagent to calculate the starch hydrolysis rate.

4.2.6 Statistical analysis

All data were analysed by Tukey's test and variance (ANOVA) in Minitab 19 software, and the confidence level of the difference between the means was 95% ($p < 0.05$). The results were expressed as means and standard deviations of triplicates unless otherwise stated.

4.3 Results and Discussion

4.3.1 Physico-chemical characterisation

4.3.1.1 Colour

The data for navy bean starch and cotyledon cells are demonstrated in Table 4.1. Interestingly, the colour of the powders produced by beans of the same origin but

subjected to different treatments varied. The colour difference was caused by the presence of polyphenolic compounds, ascorbic acid and carotenoids during the extraction process. In general, high lightness values (L*) and low chroma values (a* and b*) in the products will be favoured by consumers. The pure starch powder had a lighter colour (highest L*) and lower value of chroma, whereas all cotyledon cells were darker; among them, the BRB cells were the darkest (lowest L*). After being cooked, stored and fluid-bed dried, the beans lost quality, and became brown and darker in colour (Uebersax, 2006). However, the freeze-drying process retained the initial colour of cotyledon cells (BRF). A similar result was found in the previous study (Ceballos et al., 2012). Moreover, the L* value of the A cells (autoclaved) was lower and darker (the change was visible to the naked eye) because the high pressure caused more components to dissolve, such as the pigments (proanthocyanidins) in the bean coat (Edwards et al., 2020).

Contrary to the trend of the L* value results, the cotyledon cell powders exhibited higher a* and b* values than the starch, especially the b* value, which was nearly four times higher for the cell powder than for the starch. This could be attributed to the concentration of chlorophyll, carotenoid, and xanthophyll in cells. Overall, as the navy bean itself is white, this natural characteristic offers ideal opportunities for subsequent commercial applications, and the resulting cotyledon cell powder overcomes the unpleasant colours produced by various other coloured beans, greatly increasing the possibilities and flexibility of application as well as acceptability.

Table 4.1. The colour of navy bean starch and cotyledon cells.

Sample	S	AA	A	B	BRF	BRB
L*	98.53±0.06[a]	90.75±0.05[d]	87.62±0.06[e]	91.24±0.05[b]	90.92±0.10[c]	87.41±0.09[f]
a*	-0.46±0.01[d]	-0.60±0.01[e]	1.75±0.03[a]	0.46±0.01[c]	0.66±0.01[b]	0.46±0.02[c]
b*	3.14±0.06[f]	9.27±0.04[e]	13.51±0.02[a]	9.82±0.02[d]	10.71±0.02[c]	11.77±0.02[b]
White Index	96.5±0.07[a]	86.89±0.06[b]	81.59±0.05[e]	86.83±0.03[b]	85.95±0.08[c]	82.76±0.08[d]

Data are displayed as mean ± SD, n=6.

Starch (S), acid–alkaline treatment (AA), autoclaving(A), hydrothermal processing (B), boiling + 7-day retrogradation + freeze-drying (BRF), and boiling + 7-day retrogradation + fluid bed-drying (BRB).

Data in the same row with different superscripts are significantly different ($p<0.05$).

4.3.1.2 Proximate analysis

Table 4.2 shows the chemical composition of the navy bean starch and cotyledon cell extracts obtained by various techniques. The starch sample had a very high total starch content of 95.34% but an extremely low protein content (0.35%). According to Huang et al. (2007) and Tester et al. (2004), the purity of purified legume starch is generally around 93% and the protein content is below 0.6%, which is comparable to the results in the present study. Likewise, the fat content in the starch was about a quarter of that in the cells, but it was slightly higher than that observed in other studies on navy beans (0.09–0.60%) (Gujska et al., 1994; Hoover et al., 2010; Hoover & Ratnayake, 2002; Zhou et al., 2004). This may be related to cultivar variances, the physiological condition of the beans and the different determination methods.

In terms of the cotyledons, it is apparent that starch and protein were the major components, accounting for 51.61–64.14% and 16.61–25.28%, respectively, with a minor fat content (1.86 –2.44%). These results are within the range of the previous

literature (Do et al., 2019; Edwards et al., 2020). Furthermore, the heat treatment decreased the total starch content while increasing the protein level. Specifically, cells from autoclaving (A 51.61%) showed the greatest decrease in total starch content compared with that from the boiling process (B 53.90%, BRF 53.57%, BRB 53.48%) and the highest protein content. This is mainly because heat treatment inevitably alters the polysaccharide structure of the cell wall, which then increases the porosity of the cell walls to some extent, especially with autoclaving (under high-pressure conditions), releasing starch granules which are discarded after wet-sieving, with a corresponding increase in protein content (Comino et al., 2013). However, the percentage increase in protein content was numerically smaller than the decrease in total starch content, indicating that protein and non-starch polysaccharides may be lost during the extraction process. The components can have an impact on the product's properties, with proteins or lipids forming complexes with starch granules, further altering the digestibility of starch (Singh et al., 2010).

Table 4.2. The chemical composition of navy bean starch and cotyledon cells.

Sample	Moisture (%)	Protein (%)	Fat (%)	Ash (%)	Carbohydrate (%)	Total starch (%)
S	9.23±0.02^{a}	0.35±0.00^{f}	0.68±0.02^{d}	0.14±0.04^{e}	89.60±0.02^{a}	95.34±0.55^{a}
AA	7.3±0.09^{c}	16.61±0.08^{e}	2.30±0.07^{b}	1.15±0.08^{a}	72.64±0.21^{b}	64.14±0.77^{b}
A	4.22±0.06^{d}	25.28±0.09^{a}	1.86±0.05^{c}	1.01±0.04^{b}	67.63±0.10^{e}	51.61±0.68^{d}
B	4.16±0.03^{d}	23.10±0.14^{c}	2.44±0.03^{a}	0.92±0.05^{bc}	69.39±0.18^{d}	53.90±0.94^{c}
BRF	3.52±0.03^{e}	23.68±0.10^{b}	2.20±0.03^{b}	0.78±0.01^{c}	69.83±0.14^{c}	53.57±0.75^{c}
BRB	7.82±0.02^{b}	21.68±0.10^{d}	2.21±0.01^{b}	0.61±0.06^{d}	67.69±0.12^{e}	53.48±0.19^{cd}

Data are displayed as mean ± SD, *n*=3.

Starch (S), acid–alkaline treatment (AA), autoclaving(A), hydrothermal processing (B), boiling + 7-day retrogradation + freeze-drying (BRF), and boiling + 7-day retrogradation + fluid bed-drying (BRB).

Data in the same column with different superscripts are significantly different (p<0.05).

4.3.2 Swelling power

Figure 4.1 shows the performance of navy bean starch and each cotyledon cells in terms of swelling power measured from 37 °C to 95 °C. Cotyledon cells exhibited swelling power of 4.88 to 6.44 g water / g powder throughout their entire temperature range, which was stable and exceeded the gelatinisation temperature. The swelling power of starch ranged from 2.63 to 9.77 g water / g powder under the same conditions. Starches inside the cells had significantly lower swelling power compared with the pure starch granules. Initially, the starch absorbed and swelled much less than the cotyledon cell powder at a low temperature (below the gelatinisation temperature), but once the temperature reached the gelatinisation temperature (for most bean starch, this is above 60 °C) and is hydrated, the starch absorbed water and swelled dramatically, more than the cell powder. Similar results have been found for chickpea, kidney bean, lentil, adzuki bean, mung bean and lima bean (Do et al., 2019; Edwards et al., 2020; Guo et al., 2020). Especially, the cells treated by heating processing (A, B, BRF, and BRB) seemed to have stronger resistance to starch swelling power than the cells from acid–alkaline treatment.

The swelling power of starch granules is generally understood to mean the ability of swollen amylopectin molecules to bind to water and form a swollen gel network after heating and cooling. The hydrogen bonds maintaining the structure of double helices in the crystallites of the starch granules weakened, resulting in the expansion of the starch granules and an increase in the volume (Tester & Karkalas, 1996). In this study, amylose leaked into the starch sample supernatant after centrifugation, which

may have been influenced by the amylose content, the amylopectin structure, the starch chain interaction in the amorphous and crystalline domains, and the lipid–starch complex (Wang, Li, et al., 2014; Zhou et al., 2004). For cotyledon cells, lower swelling power indicates that the cell wall structure had a hindering effect and restricted the starch's swelling power. It has been shown that intact cell walls exert turgor pressure on the water-absorbing and swollen starch granules inside, resulting in reduced swelling power and preventing the leaching of amylose. In addition to this, the cell wall acts as a natural physical barrier, restricting the movement of water molecules as well as the space required for starch granules' expansion, contributing to the reduced swelling power of the cells. This also reduced the solubility of the starch to a certain extent (Do et al., 2019; Rovalino-Cordova et al., 2018). Formation of more crystallites would enhance the stability of starch granules, which, in turn, limits the swelling power. In this case, however, because of starch retrogradation, the retrograded cells (BRF, BRB) did not behave very differently from the A and B cells.

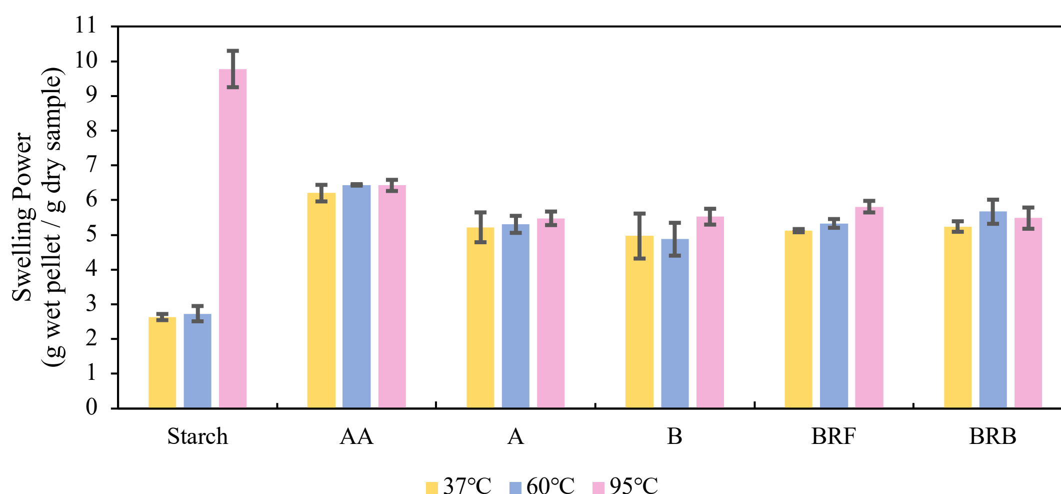

Fig. 4.1. Swelling power of navy bean starch and cells. Values for swelling power are means of cells (n=2). Samples were obtained from the following: starch (S), acid–alkaline treatment (AA), autoclaving(A), hydrothermal processing (B), boiling + 7-day retrogradation + freeze-drying (BRF), and boiling + 7-day retrogradation + fluid bed-drying (BRB).

4.3.3 Thermal properties

The gelatinisation temperature (onset, peak, and conclusion temperature) and enthalpy value (ΔH) of the navy bean starch and various cotyledon cells are presented in Table 4.3. The heating stability of the crystallites is reflected in the gelatinisation temperatures (To, Tp, Tc), whilst the enthalpy value (ΔH) is linked to the dissolution of the starch's double helices. Starch gelatinisation was defined by Cooke and Gidley (1992) to refer to the breakdown of amylopectin double helices from a semi-crystalline structure to an amorphous conformation, which is an endothermic transition. The thermal properties of starch are influenced by the type of starch crystallisation, the degree of crystallinity, the amylopectin structure and the chain length, but not the ratio of amylose to amylopectin (AM-AMP) (Noda et al., 1998; Srichuwong & Jane, 2007). It is generally assumed that the higher the content of longer branch chain amylopectin, the higher the gelatinisation temperature, since more stable double helix structures are formed. Moreover, the heating rate and water content applied in the experiments may also have affected the thermal properties of starch.

As shown in Table 4.3, the onset temperature of gelatinisation and the enthalpy value of navy bean starch were 60.70 °C and 11.97 J/g, respectively, both of which were higher than those of cotyledon cells. These results were consistent with previous research (Chung et al., 2010). Single endotherms with a well-defined peak were seen in the enthalpy images (Fig. 4.2). Chung et al. (2010) found that the starch from navy beans has a higher gelatinisation temperature and a higher crystalline stability than that of peas and lentils, probably caused by the higher bonding between the oxygen and hydrogen atoms on the closely parallel amylose molecules, forming covalent bonds (Hoover & Sosulski, 1985). It is also possible that navy beans contain a large number of long amylopectin chains (degree of polymerisation: ≥37) and a relatively small amount of short amylopectin chains (degree of polymerisation: 6–12).

Theoretically, short amylopectin chains are too short to form a stable double helix structure; hence, unravelling and melting them during gelatinisation does not take much energy (Chung et al., 2009, 2010; Srichuwong & Jane, 2007). In this case, the higher gelatinisation temperature and ΔH of navy bean starch may indicate a higher proportion of ordered crystalline domains, resulting in higher gelatinisation energy.

Table 4.3 summarises the parameters and enthalpy value of navy bean cotyledon cells, and the thermogram is exhibited in Fig. 4.2. The thermograms of cotyledon cells exhibited a wider range of gelatinisation temperature (all above 30 °C, with BRB cells having the widest T_{c-o} 36.02 °C) than the starch (T_{c-o} 21.29 °C), but with a lower onset of melting temperature and enthalpy than starch. These results are comparable with those reported by Edwards et al. (2020). Among all the cotyledon cell samples, the cells obtained by the acid–alkaline treatment had a higher onset, peak, and concluding temperatures (58.64 °C, 76.94 °C, and 93.02 °C, respectively) than the rest of the cells (the average T_o, T_p, T_c values were 45.02 °C, 59.67 °C, and 78.67 °C, respectively). Starch gelatinisation in cells treated by heating occurred over a similar temperature range (44–79 °C), and their enthalpy values were also similar, at approximately 3.64 J/g. DSC analysis revealed the presence of retrograded and ordered starch from cells (A, B, BRF, and BRB) that underwent endothermic transformation at a lower temperature than raw starch cells (AA). Obviously, because the starch in the cells obtained by the acid–alkaline method is ungelatinised, it contains a more ordered crystalline region and therefore has a higher onset melting temperature than the other cells. This demonstrates that boiling and autoclaving caused some damage to the internal structure of the starch. The starch granules absorbed and swelled sufficiently as a result of boiling in excess water, and the double helix of the amylopectin molecules in the crystalline region unwound entirely. These results are consistent with the disappearance of the "Maltese cross" in polarised light microscopy, and the absence of significant X-ray diffraction peaks and low crystallinity in other reports

(Edwards et al., 2020; Li et al., 2019, 2020; Xiong et al., 2019). The enthalpy value of cotyledon cells from pinto bean, garbanzo bean, pea and black-eyed pea extracted by boiling method by Xiong et al. (2018) is as low as 0.4–1.6 J/g. In other studies, however, following boiling at 100 °C, cotyledon cell samples retained roughly 8–9 % of the remaining ordered structure through protection by cell walls (Li et al., 2020).

Interestingly, a second high temperature melting endotherm of the AA cells was also observed between 96 °C and 115 °C, with an enthalpy of around 0.87 J/g (data not shown). Likewise, similar results were obtained in a previous study (Edwards et al., 2020). Protein denaturation is regarded to be the most likely cause of this. According to one study (Ladjal-Ettoumi et al., 2016), proteins isolated from pulses absorb heat at roughly 80 °C to 100 °C and suffer structural damage.

Table 4.3. DSC thermal properties of navy bean starch and cotyledon cells.

Sample	T_o (°C)	T_p (°C)	T_c (°C)	T_{c-o} (°C)	ΔH (J/g)
Starch	60.70±0.21^{a}	67.38±0.22^{b}	81.99±0.89^{b}	21.29±0.83^{c}	11.97±0.74^{a}
AA	58.64±0.45^{b}	76.94±1.64^{a}	93.02±0.21^{a}	34.38±0.41^{a}	2.68±0.16^{c}
A	45.95±0.68^{c}	57.70±0.32^{d}	76.01±0.21^{e}	30.06±0.50^{b}	3.47±0.22^{bc}
B	45.13±1.40^{c}	59.99±0.78^{cd}	79.14±0.21^{d}	34.01±1.58^{a}	3.68±0.25^{b}
BRF	44.52±0.16^{c}	59.58±0.59^{cd}	79.02±0.22^{d}	34.50±0.16^{a}	3.76±0.18^{b}
BRB	44.48±0.44^{c}	61.39±0.64^{c}	80.50±0.17^{bc}	36.02±0.44^{a}	3.64±0.02^{b}

Data are displayed as mean ± SD, *n*=3.

Onset temperature (T_o), peak temperature (T_p), conclusion temperature (T_c), and enthalpy of gelatinisation (ΔH (J/g)). Samples were obtained by acid–alkaline

treatment (AA), autoclaving (A), hydrothermal processing (B), boiling + 7-day retrogradation + freeze-drying (BRF), and boiling + 7-day retrogradation + fluid bed-drying (BRB).

Data in the same column with different superscripts are significantly different ($p<0.05$).

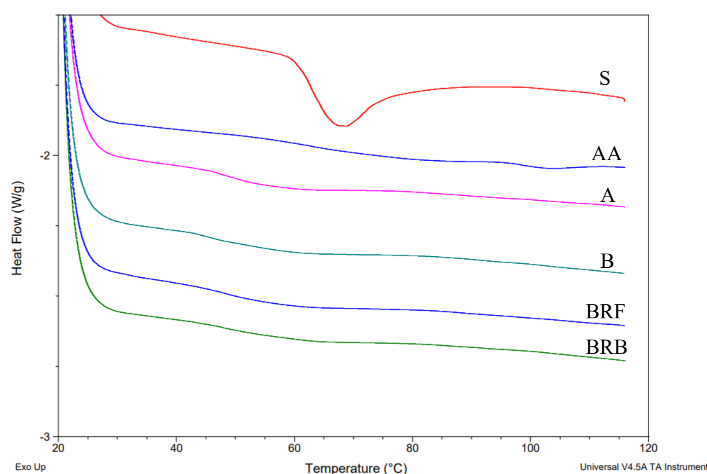

Fig. 4.2. DSC enthalpy images of starch and cells.

4.3.4 *In vitro* starch digestion

In Fig. 4.3, the hydrolysis rate of cooked navy bean starch and various cotyledon cells under two-stage *in vitro* digestion is reported. Interestingly, no significant starch hydrolysis (0.21–4.29%) was observed during the gastric stage. The starch sample was dramatically hydrolysed from 0.69% at G30 to 77.27% at I0 after the addition of SIF containing α-amylase, and then continued to increase steadily until reaching a plateau with a final hydrolysis rate of 91.48% (I120). In contrast to the starch sample, all cotyledon cell samples increased at a slow rate from I0 to I15, then climbed rapidly between I30 and I60 before slowing down to a lower level of starch hydrolysis. At I15, around 19–43% of the starch in the cells had hydrolysed and the process continued until I120. Interestingly, data from the experiments showed that the cells were much less hydrolysed in the final I120 sample (72.84–82.55%) than in the starch samples (91.48%). Specifically, the cell samples revealed intriguing results. Firstly,

the retrograded starch cell samples (BRF, BRB) always had a lower hydrolysis rate than AA, A, and B cells. The hydrolysis rate of BRB cells was the lowest, indicating they were strongly resistant to enzymatic hydrolysis caused by the starch retrogradation forming an ordered structure. Similar results were also found in previous papers (Chen et al., 2020; Chung et al., 2006; Zhou & Lim, 2012). The A and B cells, on the other hand, had a slightly higher hydrolysis rate than that of AA cells, with A cells having the highest hydrolysis rate. Overall, BRB cells had the lowest starch hydrolysis of all the cell samples and were therefore chosen as the raw material to make low-GI bread for *in vivo* (human) study.

The findings of these experiments reveal that cotyledon cells have slower starch digestibility, which is consistent with earlier research (Berg et al., 2012; Dhital et al., 2016; Do et al., 2019; Edwards et al., 2015, 2020; Guo et al., 2020; Li et al., 2019). This low starch digestibility is the consequence of several factors working together. Firstly, the integrity of the cell wall plays a decisive factor in acting as a permeable and physical barrier limiting the access of digestive enzymes. Cellulose, hemicellulose, pectin and glucan are collectively known as non-starch polysaccharides and are the main components of the cell wall. The strength of the cell wall is mainly derived from the cellulose microfibril layer, which is extensively cross-linked with hemicellulose polysaccharide chains. The pectin and hemicellulose network helps to connect adjacent cells acting as a cushion, and glucan interlocks with the cellulose microfibrils to give the cell wall structural rigidity (Selvendran & Robertson, 1990). Additionally, the cell wall structure may limit the degree of starch gelatinisation during cooking (Bhattarai et al., 2017). In contrast, the navy bean starch only contained swollen and gelatinised granules after mechanical wear and heating, with a few broken granules, indicating that the gelatinised starch granules are more likely to bind to α-amylase and be catalytically hydrolysed in the absence of the cell walls' protection. Hence, the starch sample will be digested at a considerably faster

rate than the cellular samples at the beginning of the small intestine phase. Following hydrothermal treatment, the cell wall undergoes hydration and swelling, which results in altered cell permeability. In addition, as the temperature, heating time, or pressure increases, permeability increases accordingly (Li et al., 2019, 2020). It is also possible that a combination of soluble and insoluble components in the cotyledon cell samples may also inhibit starch digestion, though this enzyme inhibition effect decreases with increasing treatment temperature (Li et al., 2020). Consequently, the heat treatments (boiling and autoclaving) boosted the cell wall breakage relative to the acid–alkaline processing, increasing the susceptibility of the starch substrate to amylase. This explains why AA cells had a slower starch digestibility than A and B cells.

Secondly, it is crucial to consider the effect of the cytoplasmic matrix. The unique structure of cotyledon cells, where the starch granules are embedded in a protein matrix, forming a starch–protein complex, provides an additional barrier to prevent the access of α-amylase and somewhat reduces the available surface area for binding contact with the enzyme by coating starch granules, leading to less hydrolysis (Rovalino-Cordova et al., 2018). Likewise, the extra protection of the protein matrix of starch and starch–protein interactions was also observed in other studies (Ezeogu et al., 2008; Rovalino-Córdova et al., 2019; Xiong et al., 2018). Furthermore, apart from the barrier effect of the cell wall (outer layer) and protein matrix (inner layer), non-specific binding of α-amylase has been reported in cell wall components (Bhattarai et al., 2017).

Lower hydrolysis rates were observed for BRF and BRB cells, mainly because starch retrogradation was introduced and applied during the extraction process. This alters the functional and nutritional significance, since retrograded starch is enzymatically digested more slowly and the release of glucose into the bloodstream is delayed. During heating of the starch–water mixture, the starch granules hydrate and swell to form a gel, resulting in the collapse of the structures as the crystallites melt, the

double helices unwind, and hydrogen bonds break. Starch retrogradation occurs when the amylose and amylopectin chains steadily revert to an ordered form that varies from the native starch structure after cooling, causing an increase in the degree of crystallinity degree and strong resistance to enzymatic digestion (Biliaderis et al., 1981; Hoover et al., 2010; Zhou & Lim, 2012). Englyst and Kingman (1990) found that retrograded amylose is not digestible during the small-intestinal stage. The degree of starch retrogradation mainly depends on the extent of gelatinisation, the subsequent cooling time, and the storage conditions (i.e., water content, time, and temperature). Amylose will retrograde rapidly first, followed by amylopectin, which will retrograde more slowly, causing a further reduction in starch digestibility as the storage time increases. In particular, the content of RDS decreases and the content of SDS increases (Wang et al., 2015). The reduced digestibility of BRB cells is most likely related to the various drying techniques used. Figure 3.2 shows that the BRB cells are present in clusters, which greatly increases the particle size and, to some degree, restricts the amount of starch granules and the number of cells accessible to the enzyme, limiting the mobility of water and thus further reducing the starch's digestibility. In this case, the drying temperature was 30 °C, which is lower than the gelatinisation temperature. In a study on waxy rice, Jaiboon et al. (2011) found that the lower the drying temperature, the lower the starch digestibility.

In general, the binding or accessibility of enzymes to starch, the presence of the intracellular protein matrix and the effect of amylase inhibitors within the cell primarily affect the *in vitro* starch digestibility.

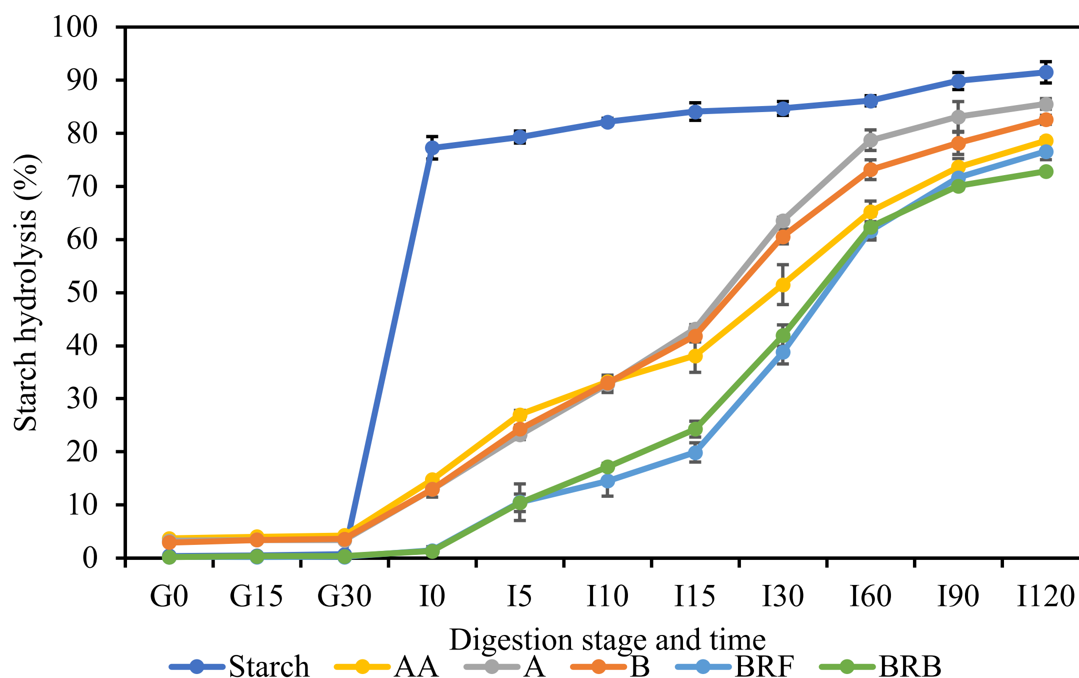

Fig. 4.3. Starch hydrolysis (%) during *in vitro* simulated gastric (G, 30 min) and small intestinal (I,120 min) digestion of cooked navy bean starch and various cotyledon cells. Samples were obtained from the following: wheat flour (WF), acid–alkaline treatment (AA), autoclaving (A), hydrothermal processing (B), boiling + 7-day retrogradation + freeze-drying (BRF), and boiling + 7-day retrogradation + fluid bed-drying (BRB).

4.4 Conclusion

The present study was designed to determine the changes in the physico-chemical properties and *in vitro* digestion kinetics of navy bean cotyledon cells extracted by different extraction methods (acid–alkaline treatment, hydrothermal processing and autoclaving) and different drying methods (freeze-drying and fluid-bed drying), and to compare their properties with their counterpart navy bean starch. This study shows that carbohydrates and proteins are the main components of the cotyledon cells and that the swelling power of the intracellular starch granules is more stable with increasing temperature due to the presence of the cell wall. The thermal performance results are consistent with the trend. Simultaneously, the *in vitro* digestibility of

cotyledon cells was much lower than that of pure starch due to the physical barrier effect of the cell wall, which hindered the accessibility and binding of starch and amylase. The digestibility of cells extracted by different methods was variable. Heat treatment and autoclaving increased the permeability of the cell wall to some extent, leading to higher digestibility, but the nature of starch retrogradation after heating allowed the amorphous structure to reform crystalline domains and reduce the *in vitro* starch digestibility. The findings of this research provide an insight into the application of cotyledon cells as a novel raw material of SDS in a starch-based product with a low glycaemic response. However, an issue that was not addressed in this study was that the fluid-bed drying affected the texture of the cotyledon cells, which were not as fine as that of wheat flour, which may affect the sensory aspects when subsequently applied to the product. Further research is needed to find an optimised process that is suitable for commercial production of cotyledon cells from legumes and their application to develop low glycaemic baked products such as bread.

CHAPTER FIVE: Navy bean cotyledon cells bread and its characteristics

5.1 Introduction

The prevalence of type 2 diabetes, obesity and other cardiometabolic diseases is increasing dramatically worldwide, posing a serious threat to human health. Thus there is a growing demand for nutritional and functional foods. The glycaemic index (GI) refers to the ability of food to elevate blood glucose levels compared with glucose or white bread. In clinical nutrition, the concept of GI is mainly used to guide the daily diet of diabetic patients. Low-GI foods are those with a GI value of less than 55, whereas foods with a GI value greater than 70 are considered high-GI foods (Jenkins et al., 1981). Foods with a low GI value can help prevent, mitigate and manage diabetes. There is now convincing evidence that GI is positively associated with the risk of diabetes, the dietary fibre intake is negatively correlated with the risk of diabetes, and that long-term consumption of fibre-rich foods such as legumes is effective in reducing the risk of diabetes (Livesey et al., 2008; Salas-Salvadó et al., 2011).

Legumes such as navy beans have low GI and generally promote a slow postprandial blood sugar release to manage diabetes (Sievenpiper et al., 2009). Several factors are responsible for the low glycaemic response, such as the integrity of the cell structure, in which the starch granules are tightly embedded in a protein matrix surrounded by the cell walls, limiting the enzyme susceptibility (Boukid et al., 2019; Rovalino-Cordova et al., 2018). Furthermore, the presence of a high proportion of non-digestible components (e.g., dietary fibre and resistant starch) contributes to the reduced glucose availability (Hoover & Zhou, 2003; Lehmann & Robin, 2007; McDougall et al., 1996). However, conventional processing by milling will disrupt the cell walls and release the starch granules, resulting in highly digestible starch

flour, leading to a high glycaemic response. Hence, retaining the integrity of the cell wall after processing is a challenge.

A new and practical extraction method has been developed, as described in Chapter 4. The novel cell powders that preserve an intact structure contain high dietary fibre and resistant starch, limiting starch gelatinisation and acting as a physical barrier to restrict α-amylase accessibility (Boukid et al., 2019; Dhital et al., 2016; Rovalino-Cordova et al., 2018).

Bread is a popular staple food and is consumed widely. However, bread made from wheat flour has a greater glycaemic response and is generally classified as a high-GI food (Belderok et al., 2000). Surprisingly, several studies have reported that low- or medium-GI bread can be achieved by incorporating cereals, whole grains, fibre, and various legume flours (Bajka et al., 2021; Boukid et al., 2019; Goñi & Valentín-Gamazo, 2003; Hsieh et al., 2017; Wolter et al., 2013; Xu et al., 2019; Zabidi & Aziz, 2009).

Thus, we hypothesise that the cotyledon cells can be a novel ingredient as a substitute for wheat flour retaining an intact structure during breadmaking processes, leading to a low glycaemic response.

The main aim of the present study was to verify the effects of cotyledon cells as an alternative ingredient to flour on the nutrition and quality of bread. The secondary aims were to investigate the influence of cell structure and resistant starch on bread starch digestibility via *in vitro* and *in vivo* digestion studies to develop a low glycaemic index bread. This research has vital implications for a novel cellular ingredient for application in starch-rich food products to assist in diet management.

5.2 Materials and Methods

5.2.1 Materials

In this experiment, the intact cotyledon cells obtained by the BRB treatment were selected and applied for making bread. The BRB cells used in this chapter contained 53.48% total starch, 21.68% protein, 2.21% fat, and 67.69% carbohydrate, evaluated according to the methods shown in Section 4.2.2.

Other ingredients used for making bread were high-grade wheat flour (Champion Professional, Auckland, New Zealand), caster sugar (Chelsea, Auckland, New Zealand), iodised table salt (Cerebos Ltd., Seven Hills, Australia), dry baker's yeast (Tasti Products Ltd., Auckland, New Zealand), butter (Anchor, Fonterra Co-operative Group, Palmerston North, New Zealand), whole milk powder (Pams, Auckland, New Zealand), fresh egg white (separated manually, purchased from a local supermarket) and wheat flour gluten (Davis Trading Co Ltd., Auckland, New Zealand). The wheat flour used in the formula contained approximately 11.5 g protein, 1.4 g fat, 76.8 g carbohydrate, 3.5 g dietary fibre, and 1550 kJ per 100 g. The wheat flour gluten applied contained 75 g protein, 6 g total fat, and 9 g carbohydrate per 100 g. The amount of gluten added was equivalent to the amount of wheat flour lost.

5.2.2 Water holding capacity

Water holding capacity (WHC) was determined by a previous method with some modifications (Edwards et al., 2020). For this, suspensions with a 1:5 (w/v) ratio of the sample to RO water suspensions were vortexed for 1 min and held for 2 h before centrifugation at 1000 × g for 15 min. The supernatant was discarded and the wet sediment was weighed. The WHC was calculated as the weight of water absorbed divided by the sample weight, as follows:

$$Water\ holding\ capacity\ \left(\frac{g}{g}\right) = \frac{Ww}{Wd} \quad (5.1)$$

where W_w is the mass of water absorbed and W_d is the mass of the dry sample.

5.2.3 Bread preparation

The three different bread recipes are shown in Table 5.1, where 100% wheat flour bread was used as a reference. The procedures for laboratory-scale bread manufacture (i.e., mixing, kneading, proofing, moulding, second proofing, and baking) are shown in Fig. 5.1. and Table 5.2.

Table 5.1. Recipes for breadmaking.

Ingredients (g)	Control	25% BRB	50% BRB
Wheat flour	250.0	187.5	125.0
BRB	0.0	62.5	125.0
Gluten	0.0	10.0	20.0
Yeast	3.0	3.0	3.0
Caster sugar	15.0	15.0	15.0
Table salt	4.0	4.0	4.0
Butter	25.0	25.0	25.0
Milk powder	24.8	24.8	24.8
Egg white	28.0	28.0	28.0
Water	140.0	205.0	290.0

Control: 100% wheat flour bread; 25% BRB: 25% of flour replaced with BRB cells; 50% BRB: 50% of flour replaced with BRB cells.

Dry yeast was added into warm water (around 30 °C) to reconstitute for 10 min. Meanwhile, all the dry ingredients were poured into a kitchen machine (Kenwood, Ltd, Havant, UK) and mixed well at low speed, then the reconstituted yeast solution was added and kneaded until a ball of dough was formed. The dough was covered with plastic film to prevent moisture loss and transferred to an incubator at a temperature of 30 °C for the first proofing. After 1 hour of fermentation, the dough volume had increased to roughly twice its original size, and it was filled with pores, as illustrated in Fig. 5.2. The dough was divided into three portions, which were sheeted

and rolled, then the shaped loaves were placed into pans and allowed to prove for around 40 min before baking. The loaves were baked at 176 °C for 25 min in an oven (Turbofan EC40D7, Auckland, New Zealand). After cooling down to room temperature, the bread was sealed in a thick plastic bag for further testing.

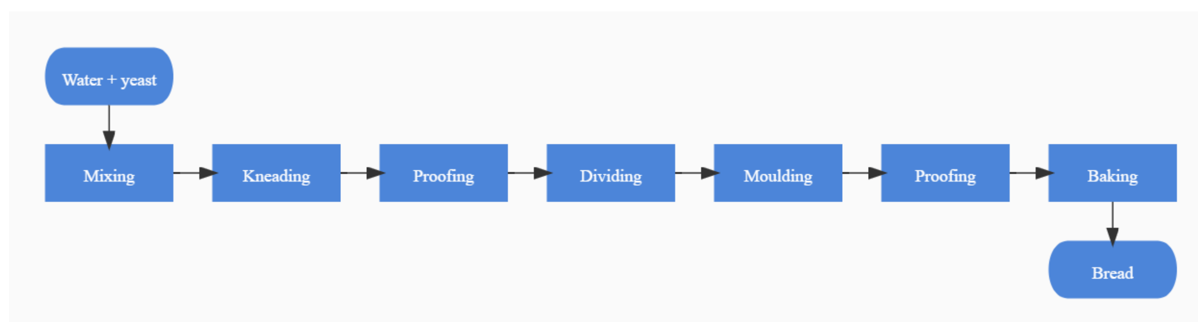

Fig. 5.1. Breadmaking procedure.

Table 5.2. Processes of breadmaking.

Time (min)	Control	25% BRB	50% BRB
Mixing (dry materials)	3	5	5
Kneading	10	20	25
First proofing	60	60	60
Dividing & Moulding	20	20	20
Second proofing	60	60	60
Baking	25	25	25

Control: 100% wheat flour bread; 25% BRB: 25% of flour replaced with BRB cells; 50% BRB: 50% of flour replaced with BRB cells.

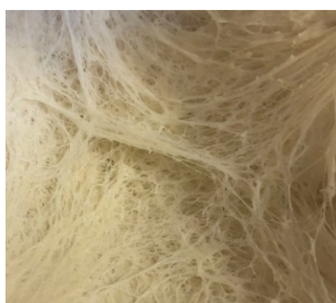

Fig. 5.2. Gluten network

5.2.4 Nutrition composition

Proximate analysis of the bread was completed by Massey University Nutrition Laboratory. Moisture (weight loss by drying, AOAC 925.10), crude protein (N-P=6.25, AOAC 968.06), fat (Mojonnier, AOAC 922.06), total dietary fibre (Megazyme, AOAC 991.43), ash (AOAC 925.10), available carbohydrate (by difference), starch (α-amylase Megazyme kit, AOAC 996.11) and resistant starch (Megazyme kit) were analysed.

5.2.5 Bread quality assessment

5.2.5.1 Bread volume, specific volume, water loss, and pH

After baking, the bread was allowed to cool to ambient temperature for about 1 hour before the weight and volume were recorded, with the volume being determined by the rapeseed displacement method (AACC 10-05.01). The specific volume (mL/g) was calculated by dividing the volume by the weight.

The water loss was the difference in weight before and after baking.

The pH test was carried out by adding 1 g of breadcrumbs to 9 mL of distilled water, then the mixture was homogenised for 1 minute and measured with a pH meter.

5.2.5.2 Colour

Colour was determined using a Minolta Chroma Meter (CR-400, Konica Minolta Business Technologies, Japan). For each loaf, L*, a*, and b* values were measured from different positions with six readings for the crumb and crust respectively, which indicate the lightness–darkness, redness–greenness, and yellowness–blueness scales. The white index (*WI*) of bread was calculated by the equation below:

$$WI = 100 - ((100 - L^*)^2 + a^{*2} + b^{*2})^{1/2} \quad (5.2)$$

5.2.5.3 Texture

The texture profile analysis (TPA) of bread was carried out according to the methods of Liu et al. (2018) with some modifications. All bread samples were fresh (after

cooling for 1 hour), and were measured with a texture analyser (TA-XT2, United Kingdom) and analysed with Texture Exponent software version 6.1.15.0, equipped with a flat probe 51 mm in diameter and a 5-kg loading cell. The bread crust was removed and the crumb was cut into cubes measuring 3×3×2.5 cm. In this experiment, the pre- and post-test speeds were 2 mm/s, and the test speed was 5 mm/s to mimic the human oral speed. Hardness, cohesiveness, gumminess, springiness, chewiness and resilience were analysed.

5.2.6 Microscopy

The microstructure of the dough and bread was observed by SEM (Philips Electron Optics, Eindhoven, The Netherlands). Prior to observation, fresh dough and bread were freeze-dried, then the dehydrated samples were glued to stubs for gold spray-coating (Baltec SCD 050 sputter coater). The voltage and spot were set to 20 kV and 3, respectively. The samples were broken off, and the naturally broken parts were selected for observation in order to check the gluten skeleton and the different gas-holding capacities of the dough.

5.2.7 *In vitro* starch digestibility

In line with previous *in vitro* gastro-small intestine digestion, an oral simulation phase was added to this experiment (i.e., a three-stage model system was used). The bread for *in vitro* starch digestion was baked a day beforehand to diminish the effect of starch retrogradation. The bread was accurately weighed to obtain a 4% concentration of starch content in 170 g total weight, followed by cutting the sample into small pieces (1×1×0.5 cm^3).

The simulated saliva fluid (SSF) contained α-amylase (2.0 g/L) in a buffer ($NaHCO_3$, NaCl and KCl), and the bread was mixed and pounded with SSF at a ratio of 1:1 for 1 min and incubated for 2 min before being transferred to a net in the reactor with gentle stirring. A circulating water bath was connected to the reactor to maintain a constant temperature of 37±1 °C. The pH was adjusted to 2 with 3M hydrochloric

acid, then 17 mL of simulated gastric fluid (SGF) containing pepsin was added and a 0.5mL aliquot was taken as G0. Then pH was adjusted and maintained at 1.2 throughout gastric digestion, and samples were taken at 15 minutes and 30 minutes as G15 and G30. Immediately after the 30-minute gastric digestion process, the pepsin reaction was stopped by adding sodium hydroxide to the reactor to bring the pH to 6.8, then 22 mL of simulated intestinal fluid (SIF, containing pancreatin, amyloglucosidase and invertase) was added. A 0.5-ml sample aliquot mixed with 2 mL absolute ethanol was taken, denoted as I0. Samples were taken at 0, 5, 10, 15, 30, 60, 90, and 120 min during the small intestine digestion stage. All the mixed ethanol samples were centrifuged at 1800 × g for 10 minutes, then mixed with invertase and amyloglucosidase and incubated in a 37 °C water bath for 10 minutes, followed by a further 10 minutes of centrifugation under the same conditions. Samples were analysed by the D-glucose assay (Megazyme International Ireland Ltd., Ireland). The absorption was measured at 510 nm to calculate the starch hydrolysis, and obtain a hydrolysis plot to measure the eGI value.

The starch digestion kinetic profiles were fitted to the first-order rate equation proposed by Zhang et al. (2013):

$$C = 1 - e^{-kt} \quad (5.3)$$

where C is the percentage of digested starch at digestion time t, and k is the first-order rate constant (min^{-1}).

For each bread, the hydrolysis AUC (0–180 min) was computed via Equation 5.4. The hydrolysis index (HI) was determined as a percentage of the difference in the AUC between a food and a reference food (white bread) (Goñi et al., 1997). In this case, the control bread (white bread) was the reference food with HI=100. The HI of 25% BRB and 50% BRB bread was calculated by the following equation:

$$HI = AUC_{sample}/AUC_{reference} \times 100 \quad (5.4)$$

Thus, from the known HI, the eGI could be calculated according to the following formula (Goñi et al., 1997):

$$eGI = 39.71 + 0.549HI \quad (5.5)$$

5.2.8 Glycaemic Index (GI) (*In vivo* human studies)

5.2.8.1 Characterisation of the bread samples, the reference and the glucose standard

The nutritional analysis of the bread prototypes (control and 50% BRB bread) by Massey University Nutrition Laboratory determined the available carbohydrate per 100 g of each test bread (Appendix A). These values were used to determine the amount of carbohydrates needed to feed the participants for GI testing. Each test bread serving was weighed out with electronic scales. The amount of bread per serving per participant was the amount required to provide 50 g of available carbohydrates (Table 5.3). Weighed portions of each test bread were kept in a sealed container until the time of consumption. The bread was consumed in two morning sessions. Water (250 mL) was provided and consumed alongside each test bread. The reference solution was 50 g of D-glucose anhydrous (Laboratory Unilab Reagent, Ajax Finechem Pty Ltd) dissolved in 250 mL of still water. This was consumed on three separate mornings.

Table 5.3. Portion size of test bread containing 50 g of available carbohydrate.

Test bread	Amount (g)
Control bread	118.2
25% BRB bread	181.2

5.2.8.2 Subjects and study design

The University of Otago Ethics Committee has approved GI testing, and informed consent was obtained from all 12 subjects (7 female and 5 male) at the University of

Otago, Dunedin, New Zealand. The mean age of the study group was 23 years (range: 20–25 years). Height (calibrated stadiometer) and weight (Seca digital platform scales, model 770; Alpha, CMS Weighting Equipment Ltd, London, United Kingdom) were measured and used to calculate the body mass index (BMI). The mean BMI of the study group was 23 kg/m^2 (range: 19–26 kg/m^2). Screening criteria included healthy individuals with no allergies or intolerances, with no diabetes or chronic illness, and who were not taking any medications known to affect glucose tolerance.

The subjects attended the clinic on five occasions (after an overnight fast of 10 hours) for three glucose standard tests and two test bread products. Two fasting blood samples were collected by the finger prick method within 5 minutes of each other, and the mean result was taken as the baseline blood glucose concentration. Participants then consumed the test bread at a steady pace. Further blood samples were taken at 15, 30, 45, 60, 90, and 120 minutes. Capillary blood was analysed immediately for blood glucose concentration using a HemoCue Glucose 201+ analyser (Hemocue, Angelholm, Sweden). The precision and accuracy of the performance of each Hemocue analyser used during testing were verified by running a test with commercially prepared EuroTrol GlucoTrol-NG Levels 1, 2, & 3 Quality Control Solutions and a quality control cuvette. The control results were within +/- 2 standard deviations of the reference ranges. The coefficient of variation (CV) for the control cuvette was 0% (n=12). The CV for duplicate measurements of fasting glucose was 4.1%.

5.2.8.3 Statistical analysis

The GI value was determined by the method outlined in ISO 26642:2010E (1) and AS 4694-2007 (2). This involved calculating the incremental area under the blood glucose response curve (IAUC) of a 50 g carbohydrate portion of a test bread and expressing it as a percentage of the mean response to 50 g of carbohydrate from the reference

food (glucose) taken by the same participant. The area under the curve was determined as the area of those increments above baseline only. The GI value for the test bread was the mean value based on GI values from all the volunteers tested.

$$GI = \frac{Incremental\ blood\ glucose\ area\ of\ test\ food}{Incremental\ blood\ glucose\ area\ of\ glucose} \times 100 \quad (5.6)$$

5.2.9 Statistical analysis

All data were analysed by Tukey's test and variance (ANOVA) in Minitab 19 software, and the confidence level of the difference between the means was 95% ($p < 0.05$). The results were expressed as means and standard deviations of triplicates unless otherwise stated.

5.3 Results and Discussion

5.3.1 Properties of the ingredients

Table 5.4 exhibits the properties of main ingredient (flour) used for breadmaking. Obviously, the protein (21.68%) and fat (2.21%) content of the BRB cells was much higher than that of wheat flour (11.50%, and 1.4%, respectively), but BRB cells had a slightly lower carbohydrate content (67.69%) than wheat flour (76.80%). Hence, the addition of BRB powder to bread will significantly improve the protein content of bread and is recommended as a promising ingredient to produce high-protein products. Moreover, the high lipid content could affect the food texture and flavour.

The ability of a protein matrix to absorb and retain bound, hydrodynamic, capillary and physically trapped water against gravity is commonly referred to as the WHC, which is a crucial property of food ingredients in processing that affects food quality (Damodaran, 2017; Traynham et al., 2007). The WHC of wheat flour and BRB cells are summarised in Table 5.4, and were 84.40% and 287.13%, respectively. Apparently, the WHC of BRB cells was about three times higher than that of wheat flour. This is a result of the high protein content (21.68%) of the BRB cells compared with that in wheat flour (11.5%), which increased the WHC to a certain extent. The

protein concentration, surface polarity or hydrophilicity, conformation, and amino acid conformation affect WHC, and the interaction of protein and carbohydrate contributes to high WHC (Guldiken et al., 2021; Qiu et al., 2017; Yu et al., 2007).

Table 5.4 displays the colour of the flour and BRB cells. The lightness (L*) decreased, whereas the a* (redness/greenness) and b* (yellowness/blueness) values rose with an increase in the proportion of BRB cells, indicating that a greater replacement of BRB cells resulted in much darker flours. These results are similar to those found in Hallén et al. (2004).

Table 5.4. The properties of ingredients.

	Moisture	Protein	Lip	Carb	WHC	Colour			White Index
	%	%	%	%	%	L*	a*	b*	
WF	12.11	11.50	1.40	76.80	84.40	93.95±0.06[a]	-0.34±0.02[b]	10.78±0.09[b]	87.64±0.10[a]
BRB	7.82	21.68	2.21	67.69	287.13	87.41±0.09[b]	0.46±0.02[a]	11.77±0.02[a]	82.76±0.08[b]

Colour data are displayed as mean ± SD, n=6.

Lip, lipids; Carb, carbohydrates; WHC, water holding capacity; WF, wheat flour; BRB, navy bean BRB cells.

Data in the same column with different superscripts are significantly different ($p<0.05$).

5.3.2 Nutrient composition

Table 5.5 exhibits the nutrient composition of the control, 25% BRB and 50% BRB bread. During baking, the bread produced with wheat-BRB cells lost slightly less water than the control bread. With an increase in the proportion of BRB cells, the moisture content rose: 50% BRB (44.4%) > 25% BRB (40.1%) > control bread (34.2%). This might have been caused by the greater WHC of the cotyledon cells, as stated above (Table 5.4), which is attributable to the higher protein content in the cells

than in wheat flour, which increased the dough's hydration qualities during processing.

In terms of protein content, adding extra gluten in the place of wheat flour resulted in the same amount of wheat protein per serving across all bread types. However, because the BRB cells have a higher protein content, fat content and dietary fibre content than wheat flour (Table 5.4), there was a significant increase in protein content for bread from 9.1% (control bread) to 11.0% (25% BRB) and 12.3% (50% BRB). With the increase in protein content, the carbohydrate and total starch content of the control, 25% BRB and 50% BRB bread decreased by 42.3%, 31.3% and 27.6%, and 33.7%, 28.6% and 22.1%, respectively. It is worth noting that the proteins and fats from the cotyledon cells are present intracellularly and hence have a minimal effect on the glycaemic response during the early digestion phase (Bajka et al., 2021). Another notable difference is that 25% BRB and 50% BRB bread had approximately double the amount of dietary fibre compared with the control bread. This was caused by the presence of cell walls, which greatly increased the content of dietary fibre. Numerous studies have demonstrated that dietary fibre has a major influence on postprandial metabolism (Grundy et al., 2016; Harris & Smith, 2006; Holland et al., 2020; McDougall et al., 1996).

As expected, the amount of resistant starch content in the bread increased with the addition of BRB cells, with 0.5% (control bread), 1.1% (25% BRB) and 1.6% (50% BRB), respectively. The addition of BRB cotyledon cells increased the RS content by ~3 times. This data indicates that the resistant starch in the cotyledon cells was retained during the breadmaking process. Moreover, it was evident that the presence of cell wall structures had a significant impact on the level of RS, and hence the starch digestibility (Rovalino-Cordova et al., 2018).

After heating, the starch granules were gelatinised, followed by cooling, resulting in the formation of retrograded starch (Niba, 2003). As a result of the repeated heating

and cooling throughout the breadmaking process, the legume bread contained much more RS than the control bread.

The formation of resistant starch is connected to the retrogradation of starch, particularly amylose. Generally, the amylose level is considered to be the main factor influencing the development of RS and is positively correlated with RS yield. Onyango et al. (2005) discovered that this process led to enzyme-resistant amylose–amylose linkages. Apart from amylose, linear amylopectin molecules may be involved in starch retrogradation, thus resulting in higher RS content in bread (Leeman et al., 2006; Tian & Sun, 2020). Moreover, other factors such as interactions with other chemical components and processing conditions also affect the RS content. A hydrogen bond can be formed between proteins and amylopectin molecules to bind other starch molecules, preventing amylopectin from ageing and lowering the amount of RS. Additionally, metal ions play a similar role in reducing the RS content (Krolikowska et al., 2017). Furthermore, the process of the heating–cooling cycle, temperature and time are also factors that affect RS (Kaur et al., 2018).

Table 5.5. Nutrient composition of bread.

	Control	25% BRB	50% BRB
Moisture	34.2	40.1	44.4
Protein	9.1	11.0	12.3
Fat	7.3	7.1	5.9
Carbohydrate	42.3	31.3	27.6
Dietary fibre	5.5	9.0	8.5
Ash	1.6	1.5	1.3
Total starch	33.7	28.6	22.1
Resistant starch	0.5	1.1	1.6

Nutrient analysis provided by Massey University Nutrition Laboratory. Results are on an as fresh basis. Control: 100% wheat flour bread; 25% BRB: 25% of flour replaced with BRB cells; 50% BRB: 50% of flour replaced with BRB cells.

5.3.3 Effects on bread quality

5.3.3.1 Bread weight, volume, specific volume, water loss, and pH

Bread volume is one of the most visual indicators of bread quality. To the naked eye, all bread loaves had a similar appearance and size, whether or not BRB cells were added (Fig. 5.3). However, higher substitution levels, as reflected in Table 5.6, may lead to a considerable increase in weight with a further reduction in the volume of bread containing BRB cells, but the difference in volume between the 25% BRB and 50% BRB bread was not statistically significant. Hence, the specific volume decreased progressively as the percentage of BRB cells increased (1.53±0.01, 1.14±0.01, and 0.97±0.01 mL/g for control, 25% BRB and 50% BRB bread, respectively). These findings are consistent with prior studies (Bajka et al., 2021), which revealed that adding cotyledon cells enhanced the bulk density (g/mL).

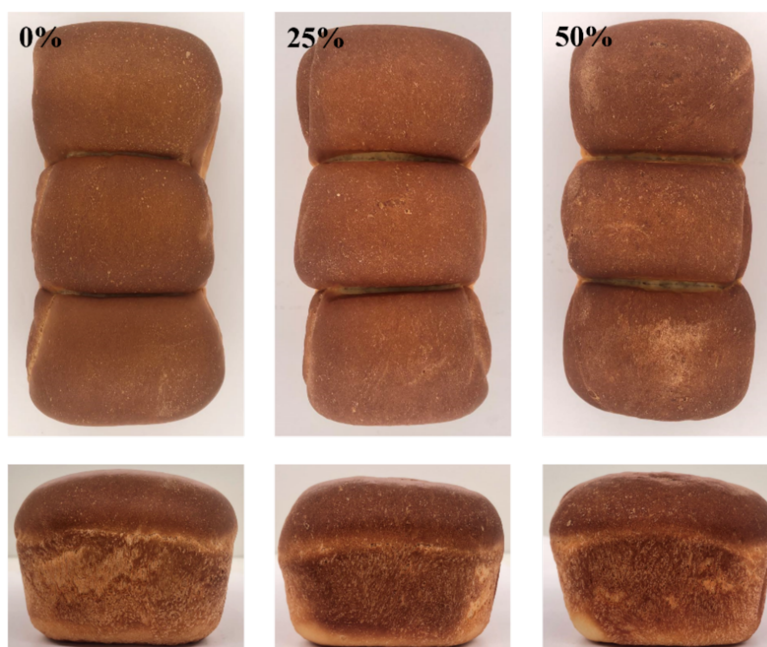

Fig. 5.3. Appearance of bread with 0%, 25% and 50% of the wheat flour replaced with BRB cotyledon cells.

The specific volume of bread is determined by various factors, including the proportions of gluten, starch, and fermented CO_2. Skendi et al. (2010) discovered that the addition of β-glucan to wheat flour contributed to a reduction in the specific volume of a loaf, with the amount of reduction depending on the amount of β-glucan. Baking volume is positively correlated with gas production and gas retention, which is formed during the fermentation period (Belderok et al., 2000; Gobbetti et al., 1995). Gluten forms a skeleton around the starch granules and traps carbon dioxide (CO_2 In general, the stronger the extensibility and elasticity of the gluten, the better it will hold the CO_2 trapped in the dough and, as a result, the dough will rise more. However, the addition of BRB cells might destroy the structure of the protein–starch complex required for gas retention in the dough structure, resulting in a reduction in bread volume, as shown by the microstructure images (Fig. 5.4 and Fig. 5.6), which revealed discontinuous, uneven and rough structures for the 25% BRB and 50% BRB bread. It is also possible that the high water absorption capacity of BRB cells reduced the volume of steam created, resulting in a lower loaf volume. Interestingly, as the proportion of BRB cells increased, the numbers of the cavity in bread also increased, which resulted in the production of more individual large gas coalescences. Similar results were observed by Dhinda et al. (2012), Li et al. (2015), Kamaljit et al. (2010) and Ahmed et al. (2013), who reported that the addition of grain or insoluble dietary fibres would disrupt the gluten network for holding gas and further decrease the bread volume.

Table 5.6. Bread weight, volume and specific volume.

Product	Bread weight (g)	Volume (cm^3)	Specific volume (mL/g)
Control	368.24±0.25^{c}	562.33±2.52^{a}	1.53±0.01^{a}
25% BRB	436.88±0.88^{b}	496.67±5.77^{b}	1.14±0.01^{b}
50% BRB	510.79±0.84^{a}	493.33±7.64^{b}	0.97±0.01^{c}

Data are displayed as mean ± SD, n=3. Data in the same column with different superscripts are significantly different (p<0.05).

Bread mouthfeel is influenced by the moisture content in the bread crumb, whereas crumb pH reflects the sourness of the bread, which is a crucial characteristic. The water loss and crumb pH are exhibited in Table 5.7. The control bread lost much more water than the wheat–BRB cells bread during baking. This is consistent with the previous results regarding the higher WHC of the BRB cells (Table 5.4). S. Liu et al. (2018) discovered a similar trend, claiming that as the proportion of legume flour increased, the moisture loss reduced. There was no significant difference between 25% BRB and 50% BRB bread. Another significant aspect of the quality of the crumb is the pH. The pH of the control bread (5.9) was slightly higher than that of legume bread (5.86–5.87), which could be related to the changes in protein structure caused by the addition of BRB cells, thus affecting the yeast fermentation process.

Table 5.7. Water loss during baking and crumb pH.

Product	Water loss during baking (%)	Crumb pH
Control	11.09±0.46[a]	5.90±0.01[a]
25% BRB	8.52±0.16[b]	5.86±0.01[b]
50% BRB	8.10±0.06[b]	5.87±0.01[b]

Data are displayed as mean ± SD, n=3. Data in the same column with different superscripts are significantly different (p<0.05).

5.3.3.2 Colour

Colour and appearance are important characteristics for sensory evaluations of a product and are also increasingly common and direct communication tools for consumers. Colour has the ability to convey an intuitive impression to customers, so it is frequently used as a physical indicator of bread quality (Rubenthaler et al., 1990).

Compared with other indicators, consumers will pay more attention to colour and base purchasing decisions on this (Eddy et al., 2007).

The physical appearance of bread with 0%, 25% and 50% wheat flour replaced by BRB cells is demonstrated in Fig. 5.3 and Fig. 5.4. When the amount of BRB cells increased from 0% to 50%, the shape of the bread changed to being uneven with a tendency to collapse. The colour of the bread crumb altered from creamy white to brownish white. Table 5.8 summarises the effects of BRB cells on the colour characteristics of the crumb and crust. Notably, the BRB cells in bread formulations resulted in colour changes in the crumb and crust. As predicted, the crumb's white indexes (76.80–79.45) were substantially higher than that of the crust (49.15–51.06), and the colour of the crumbs varied more than that of the crusts.

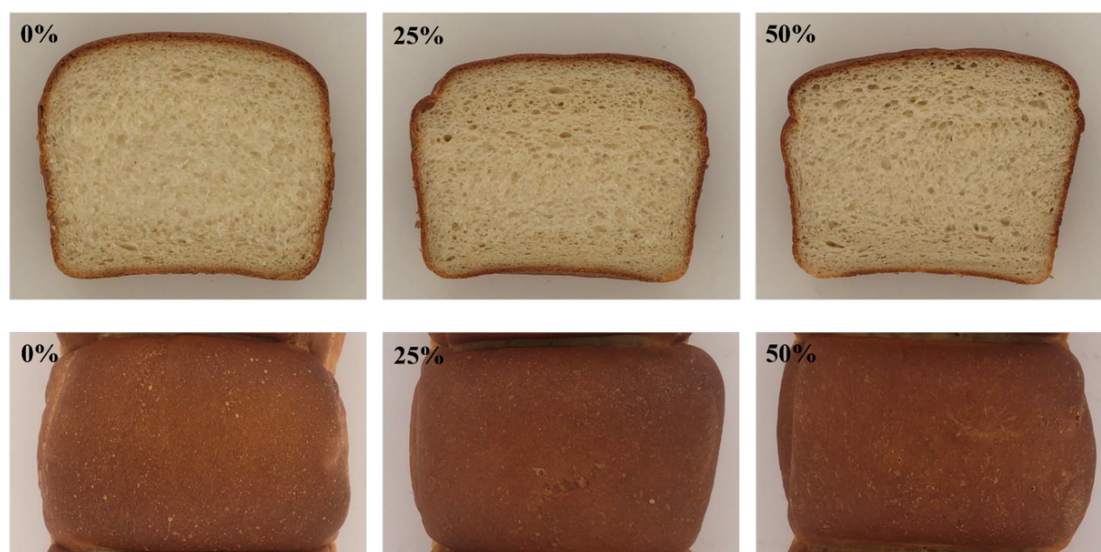

Fig. 5.4. Crumb and crust colour of bread with 0%, 25% and 50% of the wheat flour replaced with BRB cotyledon cells.

Generally, all the bread samples had a light colour; nevertheless, as shown in Table 5.8, BRB cells reduced the degree of L* in the crumb from 85.13 to 82.91 and reduced the white index from 79.45 to 76.80. The lower L* value and white index indicated that BRB cells were incorporated into bread to produce a darker crumb. In similar observations by Bajka et al. (2021), the addition of legume cells from

chickpea reduced the whiteness of bread. Increased fibre content in cells with a lot of darker components may contribute to the darkness of bread (Liu et al., 2015). For the crust, the L* values ranged from 53.27 to 55.25, which were much lower than the L* values for the crumb. Different Maillard and caramelisation reactions are responsible for the crust's darker colour (Rizzello et al., 2016). Another reason might be the higher protein content present in BRB cells. The higher the protein level, the greater the amino acid content (lysine), and hence more Maillard reactions and, as a result, the greater the generation of brown compounds. Carbohydrates interact with the free amino acid side chains of proteins (mainly lysine) to form amino acid–sugar reaction products through the Maillard process (polymeric proteins and brown pigments). Through the inhibition and degradation of key amino elements, this process may reduce the nutritious value of bread (Hurrell, 1990). Similar results were found by Mancebo et al. (2016), who stated that the incorporation of protein decreased the L* value but increased the a* and b* of cookies, resulting in products that looked redder or more yellow.

Unlike the crumb, for which the L* values showed a downward trend, the a* and b* values for the crumb both showed a considerable increase compared with the control bread. However, there was no significant difference in the a* and b* values for the crusts. Likewise, Olojede et al. (2020) reported that the addition of chickpea flour changed the colour properties, resulting in higher a* and b* values.

Table 5.8. Colour of bread crumb and crust.

Product	Crumb			White index	Crust			White index
	L*	a*	b*		L*	a*	b*	
Control	85.13±0.96[a]	-1.70±0.07[c]	14.06±0.34[b]	79.45±0.62[a]	54.96±1.2[a]	11.66±0.33[b]	15.66±051[a]	50.91±1.23[a]
25% BRB	83.19±0.68[b]	-1.51±0.07[b]	14.95±0.50[a]	77.44±0.24[b]	55.25±0.68[a]	11.84±0.21[b]	15.86±0.82[a]	51.06±0.49[a]
50% BRB	82.91±0.57[b]	-1.19±0.08[a]	15.64±0.70[a]	76.80±0.66[b]	53.27±0.83[b]	12.61±0.55[a]	15.55±0.83[a]	49.15±0.97[b]

Data are displayed as mean ± SD, n=5. Data in the same column with different superscripts are significantly different ($p<0.05$).

5.3.3.3 Texture

Texture profile analysis (TPA) gives an objective assessment of the textural characteristics, which are an important determinant in a food's acceptance. Incorporation of BRB cells had a considerable influence on breadcrumb texture (Table 5.9), in terms of hardness, cohesiveness, gumminess, springiness, chewiness and resilience. Additionally, commercial wholegrain bread was purchased from the local supermarket to evaluate and validate the acceptability of the BRB loaves. Fig. 5.5 reveals the trend in the properties as the amount of BRB cells increased. Hardness, gumminess and chewiness all increased compared with the control bread, whereas springiness, resilience and cohesiveness reduced as the proportion of BRB cells rose.

The breadcrumb hardness, corresponding to the peak force of the first compression of the bread, was considerably higher for bread with BRB cells (4.71–5.47 N) than for the control bread (3.36 N), and the greater the proportion of BRB cells, the harder the bread, though the hardness was lower than in commercial wholegrain bread (9.71 N). This may be caused by the high WHC of BRB powder (Table 5.4), which is in line with Aprodu et al. (2016), who reported that there was a negative correlation between WHC and the hardness of breadcrumbs. Similar trends were observed for gumminess

and chewiness (Fig. 5.5A). The results were consistent with a previous study (Bajka et al., 2021) that obtained high hardness, gumminess and chewiness in the crumb structure of bread produced with intact chickpea cotyledon cell flour. Likewise, some studies have shown that the addition of legume flour or cellular legume powder to bread increases the hardness, gumminess and chewiness (S. Liu et al., 2018; Rizzello et al., 2014).

On the other hand, the springiness, resilience and cohesiveness (Fig. 5.5B) of legume bread (25% BRB, 50% BRB) decreased slightly as the proportion of BRB cells increased. The lowest value of springiness was found for 50% BRB bread (1.00%). However, there was no significant difference between the breads in terms of springiness. The springiness of a product refers to how effectively it physically springs back after being deformed. For resilience and cohesiveness, 50% BRB bread also presented the lowest values (0.38% and 0.74%, respectively). Cohesiveness and resilience are two parameters that reflect how well a product can resist a second deformation (after a second compression cycle) and how well it can return to the initial position.

It is well known that cellular legume powder has a marked impact on the texture of baked products (Dhinda et al., 2012; Mohammed et al., 2012). Indeed, legume flours are consistently related to a weak dough structure and baking quality, as well as a reduction in bread volume and porosity, and an increase in loaf hardness. There is a high possibility that BRB cells contain very strong water absorptive properties compared with wheat flour, leading to a denser crumb structure. Additionally, the presence of gluten might have an impact on softness in the control bread by developing an extensible protein network that inhibits the movement of water. As a result of the lack of gluten in BRB cells, the flow of water from the crumb to the crust will be accelerated, leading to a harder crumb (Kohajdová et al., 2011; Roach & Hoseney, 1995). In the same way, the addition of BRB cells thickened the crumb

walls around the air cells inside the crumb and the protein particles strengthened the crumb structure (Mohammed et al., 2012).

Table 5.9. Texture profile analysis of bread.

Sample	Hardness (N)	Cohesiveness (%)	Gumminess (AU)	Springiness (%)	Chewiness (N/s)	Resilience (%)
Control	3.36±0.44^{c}	0.80±0.02^{a}	2.52±0.25^{c}	1.21±0.31^{a}	2.99±0.44^{c}	0.57±0.07^{a}
25%l BRB	4.71±0.34^{b}	0.81±0.03^{a}	3.60±0.07^{b}	1.17±0.17^{a}	4.24±0.70^{b}	0.47±0.01^{ab}
50%l BRB	5.47±0.82^{b}	0.74±0.06^{a}	3.67±0.30^{b}	1.00±0.01^{a}	3.69±0.30^{bc}	0.38±0.03^{b}
CB	9.71±0.13^{a}	0.73±0.01^{a}	6.42±0.13^{a}	0.93±0.01^{a}	5.99±0.20^{a}	0.41±0.02^{b}

Data are displayed as mean ± SD, n=3. Data in the same column with different superscripts are significantly different (p<0.05). CB is commercial bread from the local supermarket.

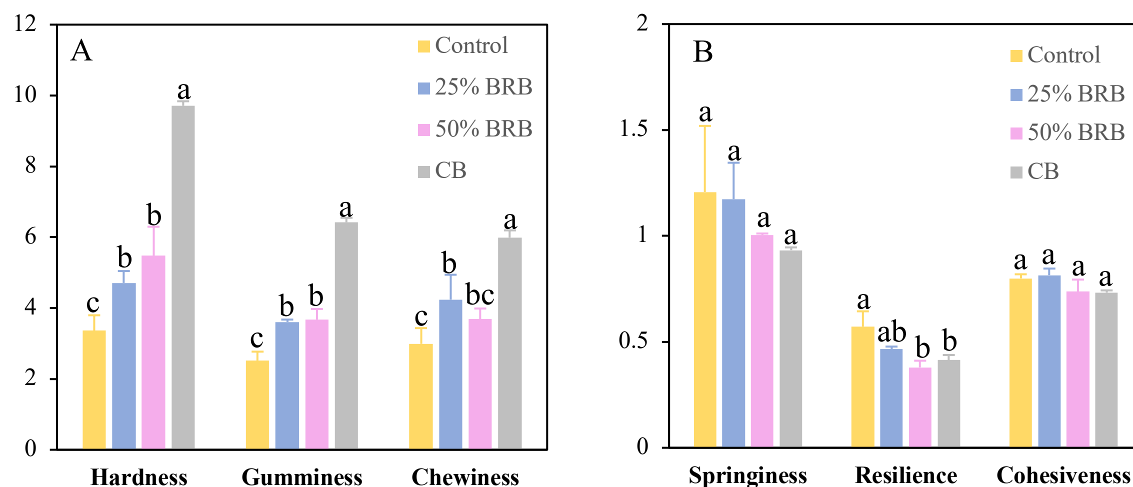

Fig. 5.5. Texture analysis of bread. (A) hardness and gumminess of bread, and (B) springiness and resilience of bread. Values are shown as mean, and bar with different letters (a, b) are significantly different (p<0.05).

5.3.4 Microstructure characteristics of dough and bread

Figure 5.6 displays representative SEM micrographs of dough and fresh bread with 0%, 25% and 50% of the wheat flour replaced by BRB cotyledon cells (control, 25% BRB and 50% BRB). A matrix of protein strands provides a structure for the dough (Bechtel et al., 1978). This gluten protein matrix is interpenetrated by starch granules from wheat flour and BRB cotyledon cells was observed in micrographs of the three types of dough.

It was evident that in the control dough, the crumb pores were small and round and homogeneous; the gluten network was continuous and well developed; and large and small wheat starch granules appeared to be more evenly embedded, forming a smoother gluten structure than in the legume doughs. This finding is in line with prior research, which found that starch granules were virtually completely incorporated into the gluten network in wheat bread (X. Liu et al., 2018). The structure of the control dough revealed a significant level of crosslinking between the thin gluten filaments, as well as the production of gluten sheets.

On the other hand, there were no substantial structural differences between 25% BRB and 50% BRB doughs. The pores looked stretched (larger and coarse). The structures found in the legume doughs showed that the intact BRB cotyledon cells were embedded in the gluten matrix, but the gluten network was less organised, especially in the 50% BRB dough, where a coarse network was present and there was no tight crosslinking between the fine fibres to facilitate gluten sheet development. The addition of BRB cells, a non-gluten component, meant that the concentration of glutenins and gliadins that form the network was diluted. Furthermore, BRB cotyledon cells contain insoluble components such as dietary fibre, which may also promote the disruption of the network during dough processing, leading to a weak gas retention ability. Likewise, similar findings were reported by Bigne et al. (2016) and Nawrocka et al. (2016), who revealed that mesquite flour and dietary fibre as an

ingredient of wheat flour would modify and cause aggregation of the gluten matrix. The resistance and extensibility of dough were interrupted, which, in turn, may affect bread texture characteristics such as hardness (Angioloni & Collar, 2012). This result is consistent with the trend shown by the TPA (Fig. 5.5).

Therefore, the control dough (wheat flour) exhibited an extensible and continuous gluten network, as opposed to the dough prepared from BRB cells, which displayed an uneven and interrupted protein matrix studded with starch granules and intact cotyledon cells. In this study, the addition of BRB cells to dough affected its physico-chemical properties because of its diverse functional and structural properties.

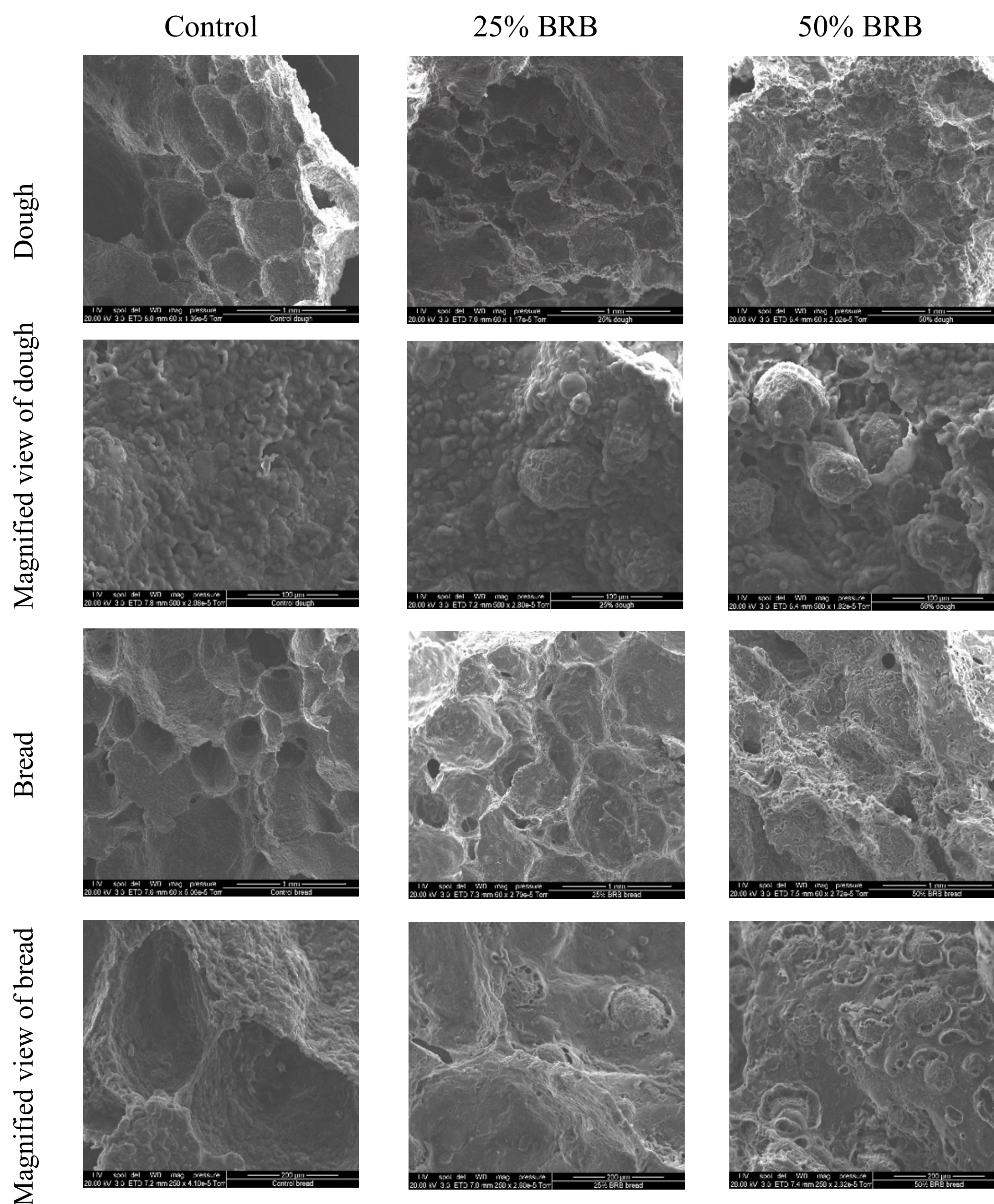

Fig. 5.6. Microstructure of dough and bread.

Regarding the microstructure of fresh bread, there was a clear difference between the control and legume recipes, as shown in Fig. 5.6. The pores in the control bread were small, compact and dome-shaped with a flat surface. There were more air cells than in the legume bread. The crumb was more elastic and uniform with a gluten network, and its structure was more homogeneous. The starch granules in the gluten matrix were gelatinised after baking. The characteristics of the control white bread are

comparable with those in previous studies (Bird et al., 2017; Indrani et al., 2010; Sadowska et al., 2003).

In the legume bread, the crumb looked denser and its pores looked stretched (large and short), with a coarse surface. The gluten network exhibited discontinuity and was destroyed. The disruption was attributed to the addition of BRB cells with increased protein and dietary fibre (Pomeranz et al., 1984). This leads to the crumb looking not as elastic as that of control bread. A similar result was reported by Rizzello et al. (2014), who stated that in comparison with white bread, legume bread had a denser-looking structure.

The size and distribution of crumb pores differed slightly between the 25% BRB and 50% BRB bread, suggesting that the higher the BRB cell levels, the poorer the gas retention ability. The 50% BRB bread showed much bigger holes than the 25% BRB bread, as well as a more disrupted starch–protein network. There were some holes within the pores, especially around the cells. This was probably the result of gas escaping during baking rather than being entangled in the gluten network. As a result of the BRB cells and these holes, an excessive amount of air was allowed to escape from the bread, resulting in a small volume and hard loaf texture.

5.3.5 *In vitro* starch digestibility

5.3.5.1 Kinetics of starch hydrolysis and *in vitro* starch digestibility

The kinetics of *in vitro* starch hydrolysis for the control bread, 25% BRB and 50% BRB bread are exhibited in Table 5.10 and Fig. 5.7. In this case, the digestion fitted the first-order kinetic equation (Goñi et al., 1997), and the larger the hydrolysis rate constant (k), the faster the starch was hydrolysed. The 50% BRB bread had the smallest k value (0.0080) followed by the 25% BRB bread (0.0137) and the control bread (0.0251), which means that the 50% BRB bread exhibited the slowest rate of starch hydrolysis during the small intestine phase. This low starch digestibility might be attributable to its unique internal structure, which needs to be investigated further

on a microstructural level. Bread is highly susceptible to access to α-amylase and has a high glycaemic response due to its spongy structure (Cavallero et al., 2002).

Regarding the starch digestibility, the starch hydrolysis of all bread samples was low (control, 25% BRB and 50% BRB: ∼10%, ∼6% and ∼5%, respectively) during a 2-min *in vitro* oral phase. A significant difference was observed between the control bread and bread with BRB cells. These results were similar to those in previous studies (Butterworth et al., 2011; Woolnough et al., 2010), which stated that around 5% and 13% of starch was hydrolysed during *in vitro* mastication of native starch and white bread. Starch hydrolysis substantially occurs during the *in vitro* oral digestion of bread, although it is a very brief and largely overlooked process in many studies. Additionally, according to Freitas and Le Feunteun (2019), and Gao et al. (2021), the structure of a bread bolus affected the oral stage since α-amylase from the saliva leached through the food bolus after chewing and was able to maintain its activity during the gastric phase. Likewise, Tamura et al. (2017) stated that the small particle size of rice grains would promote starch hydrolysis.

In terms of starch hydrolysis throughout the gastric phase, approximately 3% of the starch was hydrolysed in all bread samples, resulting in a total of 13% (control bread) and 8% (BRB bread) starch hydrolysis by the end of gastric digestion. The small extent of hydrolysis might be related to the absence of starch-hydrolysing enzymes and acid hydrolysis at low pH (Bordoloi et al., 2012; Dartois et al., 2010). The salivary amylase present in saliva could still hydrolyse starch before it is completely inactivated. For example, salivary amylase remained 50% activity at pH 4 and was not completely inactivated until the pH dropped to 3.5 (Freitas & Le Feunteun, 2019; Freitas et al., 2018). Moreover, porcine pepsin was activated at a low pH; as a result, the breakdown of the protein network that envelops the residual starch granules could be considered to occur only during gastric digestion, but had little effect. On the other

hand, the SGF buffer solution and the matrix structure of the bread bolus also slowed down the inactivation of salivary amylase to some extent (Rosenblum et al., 1988).

The addition of SIF during small intestine digestion resulted in a dramatic increase in starch hydrolysis in all bread samples (Fig. 5.7). The presence of pancreatic enzymes might be responsible for the increase in small intestine digestion. Pancreatic α-amylase accelerates the amylolytic process by taking advantage of the breakdown of the protein network by pepsin, promoting starch accessibility. Specifically, for the control bread, there was a notable rise in starch hydrolysis during the first 5 min of the small intestine phase, reaching roughly 80%, which was significantly higher than that of the other bread samples, then gradually increasing to 95% (I120). However, for the BRB cell bread samples, with the addition of cotyledon cells, the digestibility of bread increased moderately during the first 5 min (I5) in the small intestine stage, with ~33% and ~30% for 25% BRB and 50% BRB bread, respectively. There was no considerable difference between BRB cell bread in I5. Interestingly, after 5 minutes, the hydrolysis of the 25% BRB bread began to gradually accelerate compared with the 50% BRB bread. Compared with the 25% BRB bread (80%) and control bread (95%), the 50% BRB bread had the lowest starch hydrolysis levels (61%) on average, in keeping with the trend observed for gastric digestion. Hence, BRB cells significantly impact starch hydrolysis, especially during the small intestine phase, where a 15% decrease in starch digestibility was obtained for 25% BRB bread and 34% for 50% BRB bread compared with the control bread. These results support the idea that the intact cells might contribute to the lower digestibility and beneficial GI. The dense structure also has a lower digestibility than a fluffy and porous structure. This is consistent with Lau et al. (2015), who reported that steam bread with a denser structure prevented the access of amylase to the starch substrate, leading to lower hydrolysis and glycaemic response.

The eGI_G of all bread samples was within the range of 48–67 (Fig. 5.7), and the 50% BRB bread prototype was categorised as low-GI bread (GI $\leq$ 55). As expected, the 25% BRB and 50% BRB bread samples showed lower GI values than the control bread.

Several factors influenced the extent and rate of starch hydrolysis. First, and most obviously, the addition of intact BRB cotyledon cells to the bread recipe as an alternative ingredient to wheat flour led to a change in the functional properties. The BRB cells preserved their structural integrity, SDS, RS, high concentration of fibre, and low glycaemic properties after secondary processing into a baked product.

The intact cell wall provides a physical barrier to enzyme diffusion, which is the rate-limiting step in the digestibility of entrapped starch, which, in turn, affects the binding of enzymes to starch substrates and thus determines the digestion kinetics. Moreover, the cellular structure could also impose restrictions on the degree of starch gelatinisation within the cells, restricting enzyme susceptibility. According to Li et al. (2020), starch granules within pulse cells retained a certain ordered crystalline structure, despite being subjected to boiling treatment. Furthermore, intracellular protein surrounds the starch granules, drastically reducing the exposed surface area of starch substrates, limiting the enzyme accessibility and controlling starch digestibility. It is noteworthy that the intracellular protein is not bioavailable, implying that it cannot activate gut incretins and affect postprandial glucose (Pais et al., 2016).

Besides the integrity of the cell structure, RS and fibre content also contributed to the low starch digestibility and hypoglycaemic effect of bread with BRB cells (Table 5.5). High RS content provides the inhibitory effect that reduces the rate of starch hydrolysis (Yamada et al., 2005). Similarly, RS is a non-digestible compound that is resistant to enzymes, impacting starch digestion and delaying insulinemic responses (Goñi & Valentín-Gamazo, 2003). Hence, RS tends to be a potential aid for treating diabetes. In addition, the presence of RS increases the indigestible carbohydrate

fraction, and its interaction with the colonic microflora in the large intestine can be beneficial to humans by altering the intestinal ecosystem (Menezes et al., 2010). Furthermore, from a molecular point of view, it has been revealed that all α-amylases are able to hydrolyse only the amorphous regions of starch, not in the crystalline regions that contain the double helix structure, which, in turn, reduces starch hydrolysis (Colussi et al., 2018). On the other hand, dietary fibre content is also closely related to lower starch digestibility. During digestion, soluble fibre forms a viscous gel in the small intestine, which decreases the absorption of dietary carbohydrates and thereby reduces postprandial blood sugar levels. However, insoluble dietary fibre is considered to be more important in lowering postprandial blood sugar (Dhingra et al., 2012; Grundy et al., 2016). As well as the RS and dietary fibre content, the lipids in the bread formed complexes by interacting with amylose, which reduced the swelling properties of the starch granules and increased the starch gelatinisation temperature. The heating effect during the baking process also enhanced the binding strength of the amylose and lipids (Pokorny & Kolakowska, 2002). Besides these factors, other components, such as added oil and emulsifiers that significantly affect starch bioavailability, have an inhibitory impact on amylase activity, reducing the glycaemic response (Marques et al., 2007; Rizzello et al., 2014).

Additionally, starch hydrolysis and glycaemic response are related not only to the properties of the ingredients but also to the texture and processing of the bread. The application of BRB cells markedly influenced the texture of the bread. Specifically, BRB cells are associated with a breakdown of the protein network, a decrease in bread volume and an increase in the hardness of bread, according to the results of the present study. The dense structure led to a reduction in amylase accessibility and a corresponding limit in the hydrolysis rate, resulting in a lower GI value, similar to the results of previous studies (Bajka et al., 2021; Kamaljit et al., 2010; Mohammed et al., 2012). During processing, mixing time and fermentation play a crucial role in limiting

starch hydrolysis. For instance, Rizzello et al. (2014) discovered that organic acids were synthesised during fermentation, thus lowering starch hydrolysis.

Table 5.10. Digestion rate constant (k, min-1) after 120 min (I120) of bread samples.

Sample	k (min^{-1})	C_{120} (%)
Control	0.0251	95.01±1.01
25% BRB	0.0137	80.65±1.98
50% BRB	0.0080	61.39±4.29

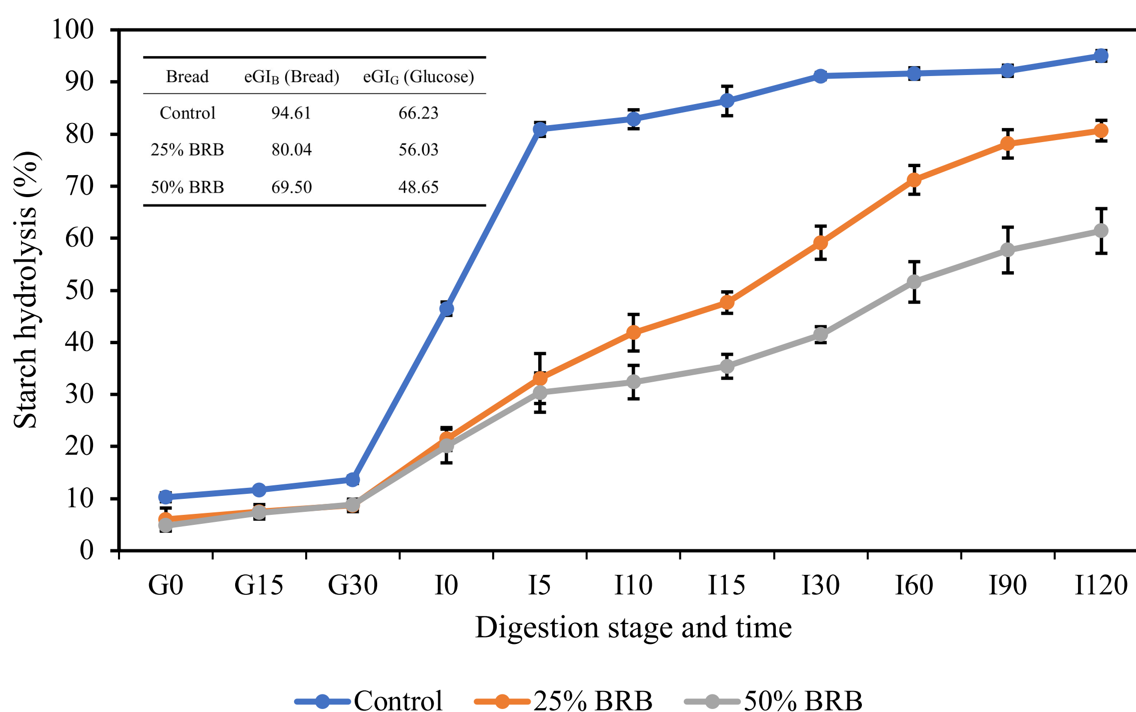

Fig. 5.7. Starch hydrolysis (%) during *in vitro* simulated gastric (G, 30 min) and small intestinal (I,120 min) digestion of bread with 0%, 25% and 50% of the wheat flour replaced by BRB cotyledon cells. Values are displayed as mean, with standard deviation. The estimated glycaemic index (eGI) of bread samples was calculated following the equation:

$eGI_B = 39.71 + 0.549HI$, with white bread as the reference. $eGI_G = eGI_B \times 0.7$, with a glucose solution as reference (Foster-Powell et al., 2002).

5.3.5.2 Microstructural characteristics of bread digesta *in vitro*

Micrographs of the bread digesta after *in vitro* digestion are shown in Fig. 5.8. It is obvious that the microstructure of the digesta particles of the BRB bread was much less disintegrated than that of control bread.

After oral digestion (O), the microstructure was a combined result of mimetic mastication and saliva impregnation. A continuous residual gluten structure was observed in all bread samples, but generally, all bread structures became more porous after oral digestion than fresh undigested bread (Fig. 5.6). This is probably caused by the action of saliva, which degrades the internal structure of the bread matrix and releases α-amylase to trigger starch hydrolysis (Jourdren et al., 2016). The control bread, because of its porous and soft structure, led to greater SSF absorption, in contrast to the bread with BRB cells, which had lower SSF absorption because of their harder and denser texture. As expected, intact BRB cotyledon cells were observed and embedded in the protein network. However, *in vitro* oral digestion was for a very short period and therefore the α-amylase was not able to fully hydrolyse the gelatinised starch granules. This, combined with changes in the pH conditions during the following gastric phase, inhibited the enzyme activity. The difference was present after the oral phase but was more pronounced after the gastric phase.

Massive hydrolysis of the protein network of the control bread residue occurred during the gastric stage (G30), presenting a discontinuous and rough gluten network, and allowing the starch granules to be freely distributed throughout the sample. Through the action of the magnetic stirring bar, the free gelatinised starch granules were washed out into the digestive juice. However, a majority of the intact cotyledon cells (BRB cells) encasing ungelatinised and semi-gelatinised starch granules remained tightly bound to the protein network (in 25% BRB and 50% BRB bread).

During gastric digestion, there were two main actions: surface erosion and fragmentation (Kong & Singh, 2009). Since SGF contains pepsin and acids, the structural disintegration happened quickly within 30 min of gastric digestion, especially for the protein network in the control bread, resulting in a less continuous and irregular structure with larger holes. As a result, this facilitated the exposure of more free starch granules. However, the compact structure of BRB cell bread samples (25%, 50% bread) basically maintained the original structure with the added BRB cells, which might have contributed to the higher WHC of the bolus. Therefore, it took a longer time for the SGF to break the bolus structure and for fragmentation to proceed. It also suggests that starch might be more resistant to SGF because of encapsulation by the cell walls (Bornhorst & Singh, 2013).

Upon the start of the intestinal phase (I5), the gluten network observed during the oral and gastric phases mostly dissolved, exposing many starch granules and exhibiting more open spaces. In the control bread, there were more holes in the protein network, resulting in a looser structure as a result of significant dilution by the SIF and enzymatic hydrolysis. However, a fibrous and thin protein network could still be noticed for the 25% BRB and 50% BRB bread, where the BRB cells still maintained their integrity, but with a shrunken surface and some of the broken protein network remaining. Specifically, in the 25% BRB bread, the connection between the BRB cells and the protein network was disrupted more (see the red arrows for I5 in Fig. 5.8, 25% BRB), and more intact BRB cells were exposed than during the gastric digestion. For the 50% BRB bread, it was evident that the BRB cells were clustered together and embedded in a protein network (see the red arrow for I5 in Fig. 5.8, 50% BRB). These microstructural differences among the control bread, 25% and 50% BRB bread suggest that BRB cells play a crucial role in contributing to a more compact structure, which affects the starch digestibility.

As the digestion time increased, at 30 minutes (I30), the structure of the chyme became more porous. It was particularly noticeable in the control bread, which had very small amounts of semi-gelatinised starch granules attached to the protein network. This might be related to the lower efficiency of enzymes in hydrolysing semi-gelatinised or ungelatinised starch (Slaughter et al., 2001). As expected, the BRB cells were more completely exposed and only partially attached to the protein matrix, which was noted in the 25% BRB chyme. Interestingly, the 50% BRB chyme remained mainly intact as a network with bigger holes, and the BRB cells appeared to be mostly embedded (see the red arrows for I30 in Fig. 5.8, 50% BRB). There is a high possibility that the more compact structure acted as a physical barrier to restrict enzyme accessibility in 50% BRB bread, as well as forming an envelope for BRB cells with starch granules, leading to low starch digestibility.

At the end of the small intestine digestion (I120), barely any starch granules could be seen, leaving only a honeycomb-like structure in the control bread, which was a denatured cross-linked protein network. Similarly, in the 25% and 50% BRB chyme, the gluten structure also became more loosely attached to intact BRB cells. It was interesting to note that the gluten skeleton of the 50% BRB chyme was more complete and continuous than that of the 25% BRB chyme. Undoubtedly, the intact BRB cells were greatly exposed through continual enzymatic hydrolysis with increasing time. Therefore, it is clear from the different microstructures that the changes in bread structure caused by the addition of BRB cells is an important factor affecting starch hydrolysis. Different amounts of BRB cells influence the bread quality: the higher proportion, the denser the structure of the bread. The addition of insoluble fibres such as cell walls can reduce the strength of the gluten network, resulting in a discontinuous and uneven network structure, and even network disruption, which increases the density and decreases the volume. Similar trends were also discovered by Xu et al. (2019). Correspondingly, the compact structure inhibits starch

digestibility to a certain extent by acting as an initial physical barrier, restricting the contact between the enzyme and the substrate. It also leads to low absorption of digestive juices, which considerably reduces the enzyme concentration in the bread matrix (Jourdren et al., 2016; Wolter et al., 2013). On the other hand, cotyledon cells retain intact cell walls during and after *in vitro* digestion, which act as a second barrier, limiting enzyme accessibility and thus reducing starch digestibility (Bajka et al., 2021).

In general, the control bread and the BRB cell bread (25% BRB and 50% BRB) showed different structures in their digesta after *in vitro* digestion. The control bread was digested quickly because of its porous structure and high enzyme impregnation, whereas the BRB cell breads took more time to be digested due to their dense structure and the starch granules were protected by an intact cell wall, which greatly limited the accessibility of the enzymes, resulting in low starch digestibility. However, the starch granules and BRB cells washed down by the digestive juice were not detected microstructurally, and further observations of the changes in starch granule morphology and the degree of BRB cell breakage are required.

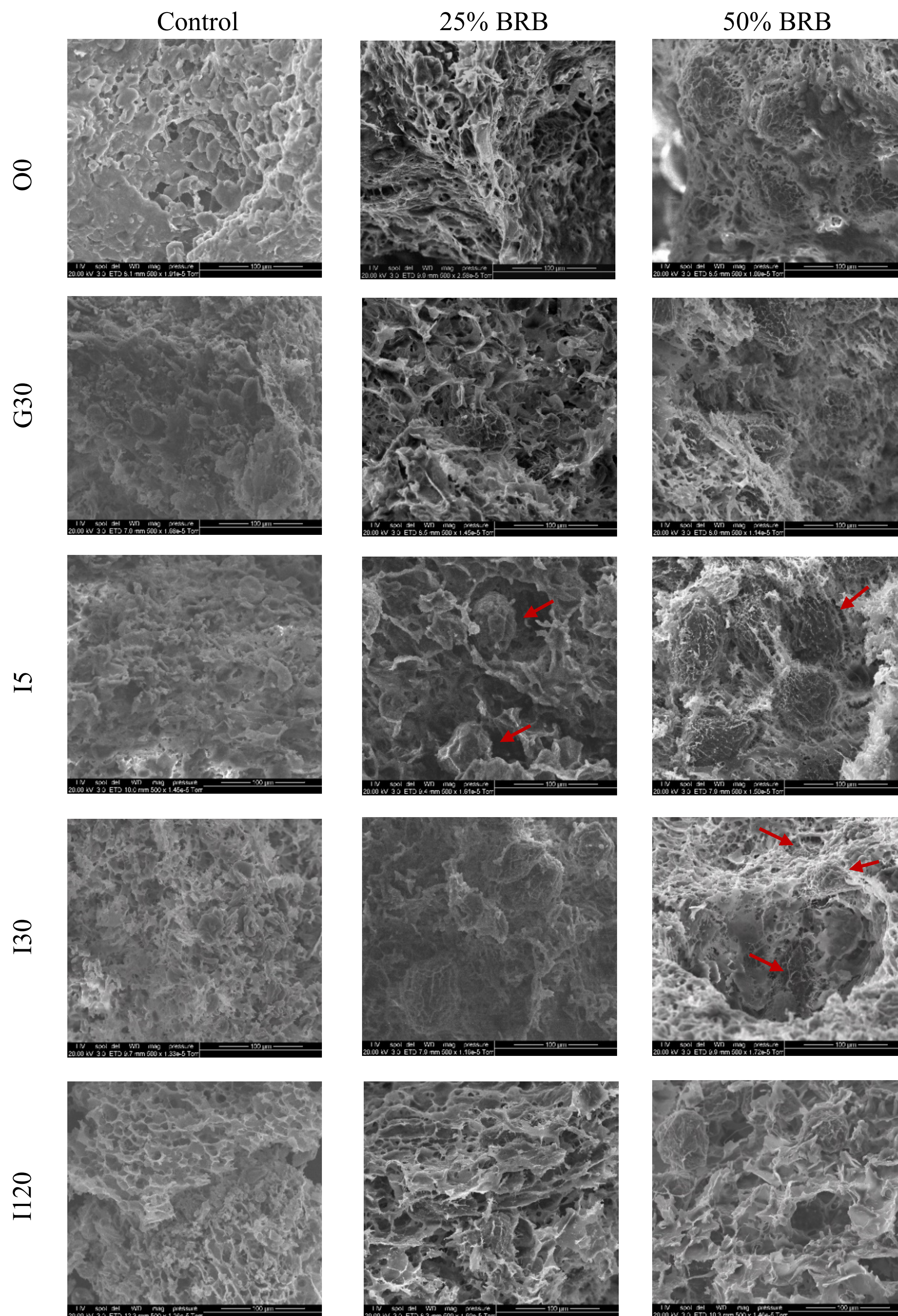

Fig. 5.8. Scanning electron micrographs of bread (control, 25% BRB and 50% BRB) during *in vitro* gastric and small intestinal digestion: after the oral phase (O), gastric digestion for 30 min (G30), and small intestine digestion after 5 min (I5), 30 min (I30) and 120 min (I120).

5.3.6 Glycaemic Index (GI) (*In vivo* human studies)

The incremental glycaemic response to the control bread and 50% BRB bread selected for *in vivo* GI testing is shown in Fig. 5.9. Postprandial blood glucose concentrations peaked at around 30 min and decreased until 120 min for both the standard glucose solution and the 50% BRB bread, and that of the control bread peaked at 45 min and then began to reduce. The 50% BRB bread had a lower peak (glucose concentration) after ingestion than the control bread and the reference. As expected, the mean AUC of the two bread samples was calculated and presented the following order: glucose reference AUC = 219 > control AUC = 168 > 50% BRB AUC = 102. The attenuated glycaemic response to 50% BRB bread is consistent with the *in vitro* data indicating the delayed release of starch. The control bread was categorised as a medium-GI food, and the 50% BRB bread was a low-GI food.

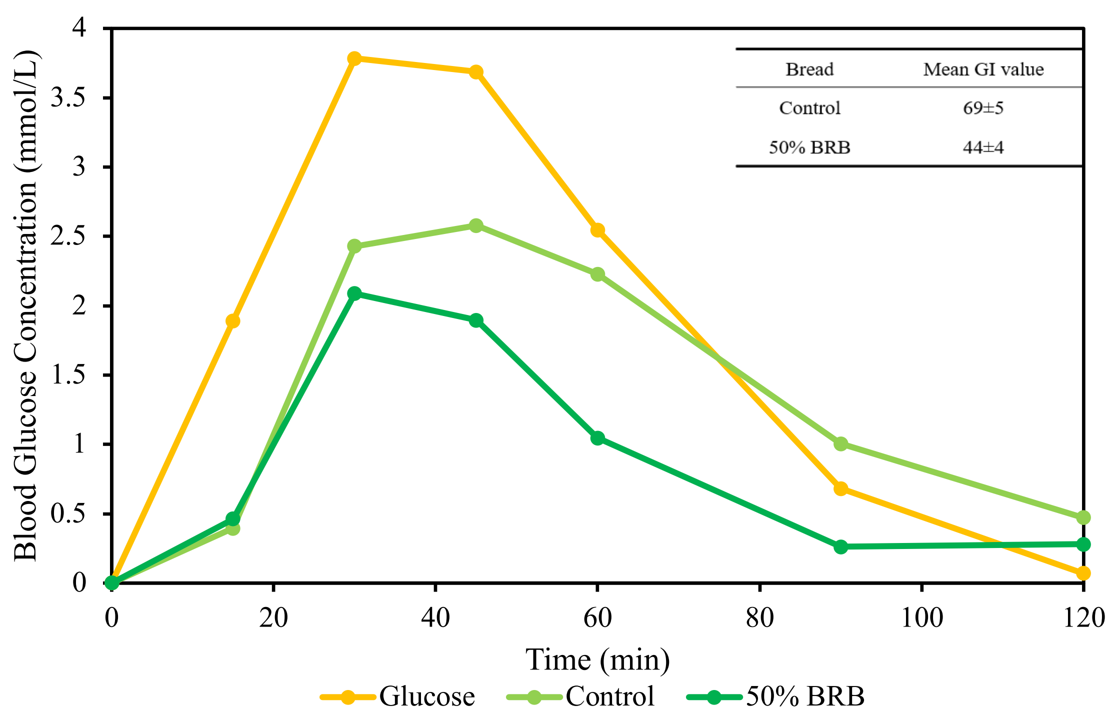

Fig. 5.9. Incremental blood glucose response of control bread and 50% BRB bread versus the glucose reference in healthy subjects (*n*=12). Values are displayed as mean.

The main objective of this study was to determine whether intact cotyledon cells could be applied to develop a low-GI bread based on their slow starch digestibility after secondary processing. The significant differences in postprandial glycaemic response between the control and 50% BRB bread samples can be attributed to the lower bioavailability of BRB starch (i.e., low digestibility *in vitro*). Previous studies have obtained similar reductions in the rate of glucose release and blood sugar by incorporating legume flour or whole grains into bread (Bajka et al., 2021; Björck et al., 1986; Goletzke et al., 2016; Hefni et al., 2021). The glucose peak reflects the high digestibility of wheat starch, and then slowly decreases with the slower availability of starch granules from the BRB cells. This also suggests a positive correlation between the slow release of glucose and the prolongation of satiety (Chung et al., 2017).

Apart from the addition of BRB cells, several factors have been demonstrated to impact the GI value, such as starch content and type, protein and fat content, food texture and processing steps (Cavallero et al., 2002; Marques et al., 2007; Marsh et al., 2011; Tovar et al., 2003). Concerning these parameters, the physiological mechanisms driving the postprandial glycaemic response are not entirely clear. Regarding the reduced GI of 50% BRB bread, the hypothesised explanation is that starch bioavailability was diminished by limited enzyme accessibility and the interaction of the food matrix and the components with starch (Östman et al., 2002). Specifically, total dietary fibre possibly slows the rate of starch digestibility and glucose absorption (Behall & Scholfield, 2005; Brennan et al., 1996). Moreover, the RS content in 50% BRB bread is responsible for the GI value, with a similar impact to some dietary fibres through adding indigestible carbohydrates (Yamada et al., 2005). Some other potentially hypoglycaemic components such as oligosaccharides, phytic acid and tannins have been suggested to have a role in reducing starch digestibility (Madar & Stark, 2002). Hence, further research is required on their levels and the

extent of the effects on digestibility, whether they are present in pulses and their mechanism of lowering GI values.

The reliability of the GI values is an issue to be considered. There was a high level of individual variability among participants related to digestion, and even within the subjects themselves, as they do not remain in a constant state. Individual differences in insulin function, the degree to which food is chewed, the transit and residence time of chyme in the digestive system, and the concentration of amylase and other components associated with delayed hydrolysis at different phases all have an impact on GI values (Brouns et al., 2005; Wolever, 2013).

The *in vitro* and *in vivo* findings showed a similar trend. *In vitro* digestion simulates the oral, gastric and small intestine phases; however, the concentration and types of enzymes involved, as well as the physical form of the food sample (texture, bolus size, fresh or frozen bread) will alter the eGI value throughout each stage of digestion (Read et al., 1986). Thus, the eGI from *in vitro* digestion can be taken as an indicator.

Overall, incorporating cotyledon cells as a novel ingredient in starch-based products to reduce the glycaemic response is a sustainable alternative approach. Both fundamental and applied research has been carried out to develop low glycaemic foods to aid dietary management in diabetic patients.

5.4 Conclusion

In this study, *in vitro* and *in vivo* starch hydrolysis of bread samples was significantly affected by the level of intact cotyledon cells. The 50% BRB bread can be categorised as low-GI bread. The most obvious finding to emerge from this study is the resistance of cotyledon cells to secondary processing and enzymatic hydrolysis, and their huge contribution to reducing the glycaemic response, as a result of cell wall integrity. Thus, this study revealed that retaining cell integrity is the key to reducing starch hydrolysis in a complex matrix such as bread. Meanwhile, incorporation of this

cellular powder into bread improved the dietary fibre content, and RS was increased threefold compared with the control bread. Furthermore, the addition of BRB cells increased the levels of hardness, gumminess and chewiness, and decreased the degree of resilience and the volume of bread by disrupting the gluten network. In general, these findings have significant implications for understanding the role of legume cotyledon cells applied as a functional ingredient to limit starch digestibility and develop low glycaemic baked products such as bread. However, sensory evaluation is an essential part of product development, so further investigation into and experimentation on the acceptance of products are strongly recommended.

CHAPTER SIX: General conclusion, discussion and recommendations

6.1 Conclusion and discussion

In recent years there has been considerable evidence of a link between the structure and health-related functionality of pulses, with the potential to become a novel healthy ingredient for the low GI food industry. The objective of the present thesis was (1) to investigate the relationship between pulse microstructure and *in vitro* starch digestibility, and (2) the role of pulse cotyledon cells in the design of low GI foods.

Firstly, navy beans were selected as the object of study, and their cotyledon cells were isolated using different processing conditions (i.e., acid–alkali treatments, autoclaving and hydrothermal processing) individually and their properties were compared to their counterpart navy bean starch (Chapter 3 & 4). Light micrographs of the cells showed the presence of hexagonal or angular shaped intact cells containing starch granules embedded in the protein matrix. Furthermore, analysis of the starch swelling power and DSC results showed that the starch granules within the cells treated in different ways were not completely gelatinised and still retained some crystalline structure, further confirming the limiting role of cell walls in the starch gelatinisation. Next, the results of *in vitro* starch digestion identified visually that the various treatments exhibit significant differences in starch hydrolysis kinetics. Heat and high-pressure treatments had higher starch hydrolysis, but were still lower than that of pure starch, while cells extracted by applying the heating–cooling–storage conditions had the lowest hydrolysis rate and extent of amylolysis. Based on the morphological features, pasting properties, and digestibility results of isolated cotyledon cells, it was suggested that intact cell walls impede starch gelatinisation, which in turn lowers the enzyme susceptivity. Specifically, the cell walls act as a physical barrier to restrict water mobility, space for starch gelatinisation, and enzyme attack. These findings

provide a new possibility for the development of novel ingredients with similar plant cellular structures and their application to reduce starch digestion. An issue that was not explored in this study was the effect of different temperatures on the changes in the inhibition of enzyme activity by soluble and insoluble inhibitors and cell wall permeability. Also, another uncontrolled factor is the particle size of cells under different drying conditions, which might impact the mouth feel of the developed food.

Furthermore, taking inspiration from the fundamental understanding of the role of cotyledon cells in limiting starch digestion, the possibility of application in low GI bread was explored in Chapter 5. This chapter has compared the effects of partial substitution of wheat flour with cells on the physico-chemical characteristics, quality (volume, texture, and colour), and starch hydrolysis kinetics of the bread. Results showed that the protein, dietary fibre, and resistant starch content of bread containing cotyledon cells have increased, as well as hardness, gumminess, and chewiness, whereas the extent of resilience and volume generally decreased due to gluten network disruption. As the level of cells substitution rose, the rate of starch hydrolysis dropped. A similar trend was observed during *in vivo* (human) studies, showing a delayed glycaemic response. Taken together, these findings suggest that the application of cells to food changes their quality, but at the same time retains their integrity and reduces starch digestibility. An implication of this is the possibility that this new ingredient can be used in low GI foods development for diabetes, obesity, and other areas. However, a limitation of this study is that all loaves of bread were produced at laboratory scale rather than at a commercial scale. In addition, the scope of this study was limited in terms of the sensory study of bread, so it is not possible to know about the level of comparability in terms of texture and taste with commercial bread.

6.2 Recommendations

The food matrix has a range of effects on the human body, mainly due to digestion of starch. Using different isolation methods and isolated cotyledon cells as a model, this study systematically reveals the effect of cell wall on the structure and digestibility of intracellular starch, demonstrating the limiting role of the cell wall during starch hydrolysis, and it can successfully reduce GI values when applied to bread. More broadly, this would be a fruitful area for further work that is briefly discussed below.

(i) The presence of the cell wall significantly reduces the *in vitro* starch digestibility, but the specific structural changes in the cell wall (pectin) need to be further investigated. This will guide how to reduce the cell wall damage during their extraction to improve the yield and extract cells with lower digestibility.

(ii) The role of intracellular proteins in starch digestion should not be overlooked. More information on intracellular protein would help to examine more closely the links between protein and starch.

(iii) Future work could explore more economical and suitable drying methods to achieve cell powders with intact cell walls for various types of food applications.

(iv) Future studies regarding sensory testing would be worthwhile in order to validate the competitiveness of products containing this ingredient in the marketplace.

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Appendix

Appendix A. The nutrient analysis of Control bread and 50% BRB bread

Results are on an as received basis

NutLab ID	Sample Name	Moisture %	Ash %	Crude Protein %	Fat %	Carb %	Starch %	TDF %	Resistant Starch %
TN21-678-01	Bread Control made 21/7	34.2	1.6	9.1	7.3	42.3	33.7	5.5	0.5
TN21-678-02	Bread 50% CC made 21/7	44.4	1.3	12.3	5.9	27.6	22.1	8.5	1.6

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