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High Protein Chinese Steamed Bread: Physico-chemical, Microstructural Characteristics and Gastro – small Intestinal Starch Digestion *in Vitro*

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Abstract

In Asia, high protein low carbohydrate foods are in high demand because their consumption can provide improved nutritional benefits and help maintaining blood glucose levels close to normal. High protein versions of popular, highly consumed food products (staple foods) such as Chinese steamed bread (CSB) can be very useful to improve the health status of our populations. Thus, the objectives of this study were: to develop high protein Chinese steamed bread (HPCSB) using plant protein, dairy protein combinations. The high protein versions of the steamed breads were then compared with control 100% wheat flour based Chinese steamed bread for physico-chemical, microstructural, textural and *in vitro* starch digestion characteristics. In order to develop HPCSB, plant proteins (soy protein isolate) and dairy proteins (rennet casein and milk protein concentrate) were blended into wheat flour at two different levels. The addition of proteins has led to a change in colour characteristics (*L*, a*, b**) and also resulted in a decreased specific volume of the breads. The textural characteristics measured through textural profile analysis of HPCSB showed an increased hardness and gumminess than control. The microstructure of HPCSB was observed to be more compact and had fewer air cells when observed through Scanning Electronic Microscopy. Furthermore, *in vitro* starch digestion of HPCSB depicted that the addition of proteins was capable of lowering the starch hydrolysis (%) and estimated glycaemic index (eGI), especially for RC I and RC II at significant levels. Addition of both proteins influenced the microstructure of HPCSBs, which in turn affected the textural and starch digestion properties. High protein Chinese steamed bread with low glycaemic properties can be prepared by critically selecting the protein sources with minimum changes in their physical and textural characteristics.

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Abbreviations

AACC	American Association of Cereal Chemistry
ANOVA	Analysis of variance
CSB	Chinese steamed bread
DSC	Differential scanning calorimetry
HPCSB	High protein Chinese steamed bread
MPC	Milk protein concentrate
RC	Rennet casein
RVA	Rapid Visco Analyser
SEM	Scanning electronic microscopy
SGF	Simulated gastric fluid
SM	Soy protein isolate + milk protein concentrate
SPI	Soy protein isolate

Chapter 1. Introduction

Bread is one of the most important staple foods around the world (Cauvain, 2003). Over the centuries, conventional bread products have been developed and have attained the desired quality. Chinese steamed bread (CSB, "饅頭") is a fermented wheat flour product which is fermented with yeast and then cooked in a steamer (Huang, 2014). It is a staple food in parts of China for over two millennia and is now gaining popularity in other parts of the world. (Hui & Evranuz, 2012; Zhu, 2014).

Nevertheless, bread contains high carbohydrates, meaning excessive intake can increase blood glucose and the level of insulin in a short duration, which might lead to health issues. Therefore, there is a need for lowering carbohydrate content of foods by the addition of non-starch or high protein ingredients (Guilherme, Virbasius, Puri, & Czech, 2008). Also, nutritional deficiency is a problem in Asian countries. Efforts are being made to counter this problem by increasing the quality of foods consumed. Inadequate levels of dietary protein have prompted public demand for high-protein, low-carb foods (Shao *et al*., 2017).

While the protein content of normal wheat flours ranges from to 10%-14%, such protein levels do not have enough nutritive value due to lack of essential amino acids especially lysine (Huang, 2014). Protein enrichment complements this deficiency in cereals as it leads to an improvement in their amino acid content (Gupta, Batey, & MacRitchie, 1992). One way to supplement the public diet with extra protein is to enrich food products that are already accepted and widely used, such as bread. Protein supplements, for example, soy protein and dairy protein are easily available for fortifying such baked products because of their proven health benefits. By the addition of protein to bread dough, the protein content of the breads and especially the essential amino acid content, can be increased. For a protein supplement to be commercially viable, the added proteins should not significantly change the organoleptic properties of breads.

Supplementation of the protein in wheat flour at higher levels has not been successful so far because of the adverse effect on the sensory quality and shelf-life of the products. Some protein sources have a tendency to interfere with the fermentation process thereby resulting in breads having lower volumes. It has been found that the addition of

any non-wheat protein or high protein wheat-derived product to bread formulation improve its nutritive value but generally results in a loss of bread volume, poorer texture and a general loss of organoleptic properties. For example, soy-fortified bread exhibit undesirable characteristics as diminished loaf volume and poor crumb grain. Also, soy protein has some antinutritional factors such as phytic acid, lectins and hemagglutinins which may inhibit the calcium and iron absorption and may cause diarrhoea (González Pérez & Arellano, 2009). The blending of soy and dairy proteins in an appropriate combination may result in a new product containing both dairy and plant nutritional advantages.

Dairy ingredients can be used in bread for nutritional benefits, including increasing calcium content and protein efficiency ratio; and functional benefits including flavour, texture enhancement, and storage improvement. Dairy ingredients enhance water absorption can also improve dough- handling properties (Cocup & Sanderson, 1987). Caseins are particularly rich in lysine and make excellent nutritional supplements for cereals, which are deficient in lysine (Rollema & Muir, 2009). Milk protein concentrate (MPC) contains significant quantities of calcium and phosphate. MPC can be used for its nutritional and functional properties. The high protein, low lactose ratio makes MPC suitable for protein-fortified beverages and low-carbohydrate foods (O'Kennedy, 2009).

Therefore, the main objectives of this project were to (1) use dairy proteins (rennet casein, milk protein concentrate) and plant protein (soy protein isolate) to develop a high protein Chinese steamed bread (HPCSB) by maintaining an acceptable eating quality and texture; and (2) Study the physico-chemical, textural, thermal, microstructural characteristics and *in vitro* gastro-small intestinal digestion of HPCSB. Microstructural properties of the digests obtained during gastro-small intestinal digestion were also studied.

Chapter 2. Review of Literature

2.1 History of Chinese steamed bread

Chinese steamed bread (CSB) is an ancient product made from wheat flour which is cooked by steaming (Huang, 2014). It has been proved that steamed bread can be traced back since 770-221 BC, as back then Chinese already learned how to use a mill and thus they know how to make food from flour (Huang, 2014). An early agriculture monograph was written during the 6th century and indicated that a steamed product can be made from a fermented dough, which was recognised as the earliest version of steamed bread (Hui & Evranuz, 2012).

CSB is a staple food in some parts of China for over two millennia and is gaining popularity around the world. (Hui & Evranuz, 2012; F. Zhu, 2014). In northern China that steamed bread is a staple food. In southern China, which is a rice-growing area, steamed bread is eaten mostly at breakfast. It is usually eaten hot and can be consumed with all meals. The original steamed bread was made with fillings, but over time it has gradually evolved.

2.2 Classification

There is a debate about how to categorise Chinese steamed bread (CSB). Some authors stand for allocating CSB based on production methods, others stand for ingredients of CSB. Owing to different eating habits in various regions and cultural diversity of people, not only the formulations are slightly varied but also the texture and appearance. The texture of CSB varies from very firm, dense, sticky, cohesive to fluffy and soft, hence, it is tough to set a standard for the classification of CSB (Zhu, 2014; Zhu, 2016). The different classifications of CSB have been summarised below (**Table 2.1**).

Keeratipibul *et al*. (2010) stated that CSBs can be roughly classified into two categories, depending on the leavening agent. The first category is the ones using natural starch dough whereas the second category being the one using yeast. CSB made from the former has a cake-like, dense, form and cohesive texture whereas CSB made from yeast is usually bread-like, elastic and soft. Sourdough is now largely replaced by yeast in

factories as the steamed bread made by the latter one has a higher fermentation rate, and more consistent (Huang & Miskelly, 2016b).

Table 2.1 Different classifications for CSB.

Basis for classified	Formulation type	Reference
Fermentation	1. Sourdough CSB 2. Yeast CSB	(Keeratipibul *et al*., 2010)
Ingredients	1. Southern CSB 2. Northern CSB	(F. Zhu, 2014)
Processing procedure	1. One-step method 2. Two-step method 3. Sourdough method	(C. Wu *et al*., 2012)
Shape	1. Pillow-like 2. Round	(Keeratipibul *et al*., 2010)

On the other hand, F. Zhu (2014) argued that the classification of CSB should base on the basic ingredients' composition and the production process. He stated that CSB should be categorised into two major types: northern and southern style CSBs. Northern style CSB has been described as having a dense and chewy texture. In contrast, southern style CSB has a softer and more open texture. Guangdong style CSB is also a type of dessert, which is popular in Asian countries.

2.3 The production of CSB

The production of CSB is similar to that of western bread, the only difference being that the final cooking procedure is steaming instead of baking in an oven, so their appearances are different. Steamed bread has a soft, shiny crust and fine, white crumb. The common weight of steamed bread varied between 30-120 g depending on different types and the shape is either round or pillow-like (**Figure. 2.1**).

Originally, Chinese steamed bread was made by hand. Currently, because of the industrialisation of steamed bread and its convenience, it has been mass-produced. There three main production methods commonly used: 1) sourdough method, 2) one-step method, 3) two-step method.

Figure 2.1 Round shape steamed bread (Matson, 2017)

2.3.1 Sourdough method

In the sourdough method, flour is mixed with water and 10% sourdough starter then waited for 15 min. after that the dough is fermented for 3 hours at 38°C. After the dough ferments, it becomes sour due to the acids produced by *Lactobacillus spp.* thus fermented dough is mixed with remaining flour and water because of its low pH, the dough needs to be neutralised back to pH 6.2-6.7. Afterwards, the dough is sheeted to let gluten network developed. then the dough is rolled into a cylindrical shape and divided into even pieces and rounded into shape as desired. Finally, the doughs are proofed for about 0.5h (32-36°C) and at last steamed for about 15min in a steamer (Wu *et al*., 2012).

Sourdough starter is usually obtained from the fermented dough batch from the previous day. Therefore, this method preferred in small scale production because sourdough is easy to prepare and has less cost and it can be used without controlling temperature. However, the processing time is so long that it is tough to control, and it might trigger some microbiology contamination (Huang & Miskelly, 2016c).

2.3.2 One-step method

In terms of the one-step method, all ingredients are mixed with flour and all other ingredients are mixed together to make a dough. After about 30min of fermentation at 32℃, the dough is kneaded for some time depending on the weight of the dough. Then the dough is sheeted, divided and moulded to produce dough. After that, the dough is proofed for about 20min at 32-37℃, then steamed for 15min. The one-step method is simplified therefore widely used in industry. However, the drawbacks of products made this method are they have less flavour and it stales quicker (Hou & Popper, 2006).

2.3.3 Two-step method

The two-step method is a traditional steamed bread preparation procedure. firstly, 80% of the flour is mixed with water and yeast. After fermentation for 1 hour at 32 ℃, remaining flour is added and remixed, the rest processes to make into steamed bread as previously described. Compared to the products made by one-step procedure method, the final product using this method can possess larger volume, better texture and better flavour. However, this method needs labour work, more space to produce, and much longer production time.

2.4 Comparison between baked bread and steamed bread

There are numerous similarities between Western bread and steamed bread. Both of these are fermented wheaten products and have similar organoleptic properties. Their differences are described below.

2.4.1 Ingredients

In respect of making steamed bread, it requires wheat flour, yeast or sourdough and water, whereas, in western bread making, other ingredients are included such as salt, sugar, fat, milk, emulsifier, and bread improvers. Higher protein quality is required when making western bread compared to steamed bread. However, the colour of flour for steamed bread is more critical Compare to western bread, since the crumb and crust of steamed bread should be both soft and white. Additionally, western bread dough has 10-

15% more water addition than Chinese steamed bread dough. which results in a typically fine, chewy texture of the steamed bread (Rubenthaler, Pomeranz, & Huang, 1992).

2.4.2 Processing methods

The processing methods of CSB and baked bread have their similarities as well as their differences. The manufacturing process is quite similar where the mixing of wheat flour, water and yeast take place after which it is fermented, moulded and proofed. Their cooking methods differ in terms of the final product required (Zhu, 2014). Steamed bread dough is cooked by steaming at about 100°C at atmospheric pressure whereas western bread is baked in an oven at over 200°C. Bread dough after baking forms a hard and brown crust having a larger specific volume. Western bread has a prominent, baked flavour than steamed bread due to baking at high temperatures which triggers Millard reaction, forming a golden yellow crust. In contrast, steamed bread has a thin, white soft and moist crust without a browning effect, it has comparatively denser crumb (Hou & Popper, 2006).

2.4.3 Shelf life

When making steamed bread, the water activity was raised by steaming process, which means the condition for bacteria and mould growth is appropriate, subsequently, the shelf life of steamed bread is only 2 days during winter and even 1 day in summer. On the other hand, during the baking process, the moisture of bread dough loses, and a hard crust is formed. Additionally, the additives like vinegar, fat and emulsifier to western bread can act as preservatives, which result in a longer storage time for up to 5 days (Choi, Chung, & Lee, 2007; Huang & Miskelly, 2016a).

2.4.4 Formation of Lysine availability and acrylamide

It is well known that baking causes browning of the crust slightly darkens the crumb. This is attributed to the Maillard reaction, that is, amino groups interact with reducing sugars.

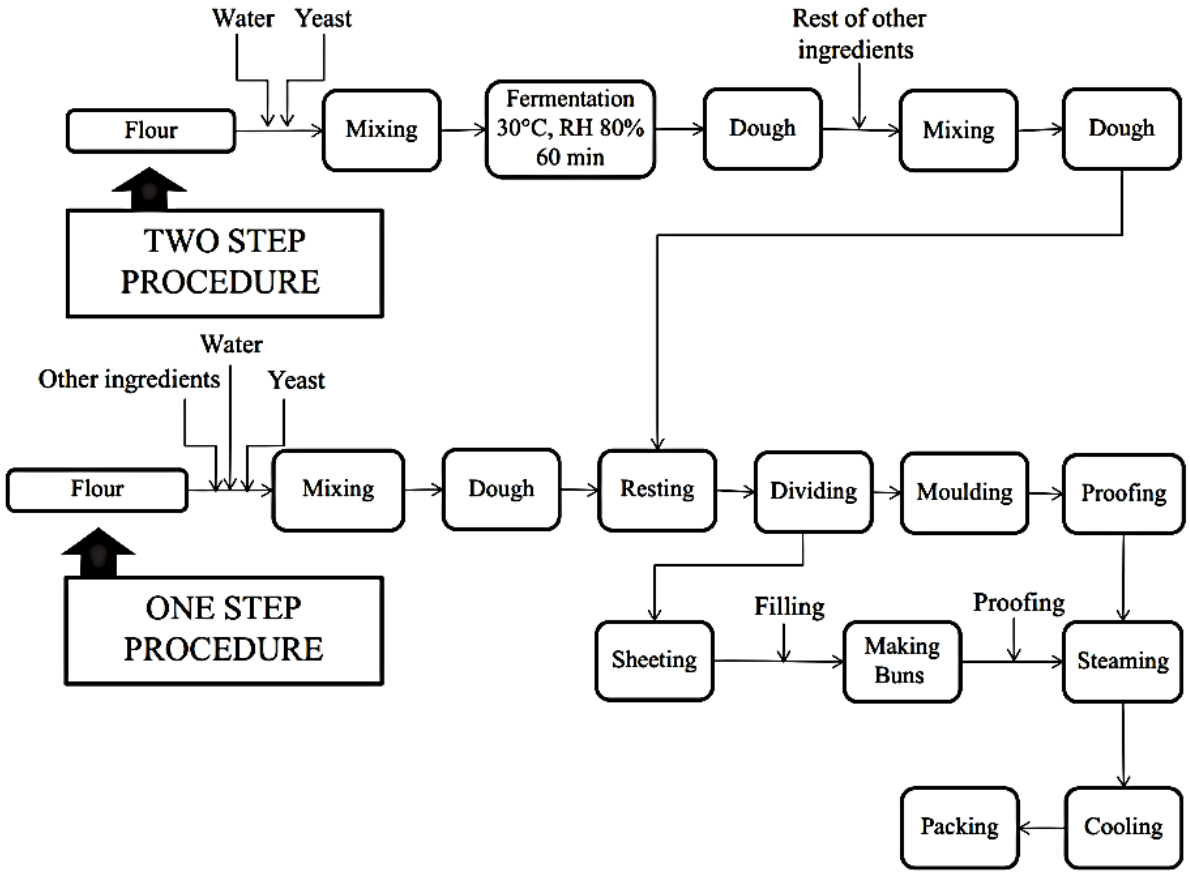

Figure 2.2 Flow diagram for making steamed bread using one-step and two-step method (Huang & Miskelly, 2016c).

This reaction decreased the nutritional value of the bread as it results in the loss of availability of soluble amino acids (mainly lysine). Tsen, Reddy, and Gehrke (1977) indicated that lysine availability in steamed bread is higher than baked bread. Thus, steamed bread is nutritionally richer than baked bread. Acrylamide is a by-product of the Maillard reaction during high-temperature cooking such as baking, it is a carcinogenic, neurotoxin, in mammals, and probably also a human carcinogen (Hou & Popper, 2006; Lingnert *et al*., 2002). Surdyk, Rosén, Andersson, and Åman (2004) found the existence of acrylamide in the crust of a baked bread while it was not detected in steamed bread. They explained that is because most of the acrylamide comes from the crust.

2.5 Nutritional enhancement of CSB

Despite rapid development and availability of western foods, preference for locally sourced and manufactured as well as nutritionally better alternatives to processed food are being sought-after by consumers (Huang & Miskelly, 2016a). CSB is nutritionally inferior as it is a high glycaemic index, has a high carbohydrate, a lower amount of resistant starch and dietary fibre. Given the various lifestyle associated diseases, the consumer demand for high protein products is on the rise for numerous reasons, including nutrition, health and weight control concerns. They have become more and more sensitive to the connection between health and their selection of food recently. Therefore, the food industry has demanded considerable investment in nutritional and functional food (Liu, Brennan, Serventi, & Brennan, 2017). Various functional ingredients sourced from plant and dairy can be added to CSB to achieve desired nutritional and/or eating properties (Zhu, 2014).

Use of plant and dairy-based functional ingredients, either alone or in combination can be used to uplift the nutritional quality of popular processed foods (Dhinda, Prakash, & Dasappa, 2012). A simple way to include functional ingredients is during the bread making process. There is extensive literature on how numerous functional additives have been incorporated into wheat bread with the aim to augment their nutritional properties (Dhinda *et al*., 2012; Rosell, 2003).

2.5.1 Protein enrichment of foods

Food proteins are composed of amino acids, and they are complicated biological macromolecules. (Shang, Chaplot, & Wu, 2018). It is fundamental for human health and development to consume proper protein amount daily. Food proteins support the fundamental metabolism of cellular and subsequently, human bodies, they also maintain human growth, hence are significant in our diet. There are numbers of reports indicated that increasing the protein intake maintains a healthy immune system and anabolic reactions (Hartmann & Meisel, 2007; Tieland, BorgonjenVan den Berg, van Loon, & de Groot, 2012).

Along with the skyrocketing growth of the population. The urge for nutritious foods such as protein is rising dramatically. Furthermore, given the economical upgrade of developing countries, there is a trend that people tend to shift their diet from carbohydrate primarily to protein primarily diet or protein-based functional foods because they are now more concerned on a healthy diet (Shang *et al*., 2018).

In respect of carbohydrates, the excessive intake can raise blood glucose and insulin levels in a short period, which might cause health issues including high blood pressure, heart disease, stroke, kidney failure and sometimes blindness and the peripheral nerves of the legs and feet will get worsening (Guilherme *et al*., 2008). Therefore, with the aim of reducing the occurrence of diabetes, it is necessary to take food consumption controlling blood glucose and insulin levels into consideration, so that to maintain them steadily. Moreover, when wheat is milled into wheat flour for bread making, the protein and amino acid content are decreased by about 1%. To be more specific, there is a requirement for modifying high carbohydrate foods into lower with high protein ones.

Shang *et al*. (2018) indicated that 0.8 g/kg daily is suggested for It is recommended to intake /day proteins for healthy ordinary adults. Proteins are vital for sustaining a healthy life as they are structural nutrients, which in a proportion of 75% of the dry basis of total body weight Currently, plant and animal proteins are the primary source of consumption. When proteins are digested in the human body, proteins are fragmented into amino acids continuously which are recycled to synthesize muscles, proteins with

immune functions, bone matrix enzymes, and other essential compounds like hormones, enzymes (Henley, Taylor, & Obukosia, 2010).

2.5.1.1 Protein enrichment for bone and cardiovascular health

Bone is a dynamic connective tissue, it acts primarily supporting and protecting soft tissues, and is fundamental in mineral homeostasis (Morgan, Barnes, & Einhorn, 2013). Unfortunately, however, according to Marcus and Majumder (2001), half of the female over 50 years old may have osteoporosis, this occupancy is higher than the summation of stroke, breast cancer and heart attack occurrence. Because ageing leads to less estrogen releasing, female has higher morbidity than male.

Protein as one of the most vital and main nutrients in the human body, it has been regarded as a novel plan for osteoporosis prevention. Protein has been considered as a new strategy for the prevention of as it is the most important and major nutrient for the human body. Nevertheless, whether a high-protein diet can help retard the is still under debate. Heaney and Layman (2008) claimed that high-protein diet can be considered as a side effect of osteoporosis, as it increases the calcium in urinary, which they concluded high protein causes the acidosis in the body system, consequently, cause a bone fracture. Even so, Bonjour (2005) and Calvez, Poupin, Chesneau, Lassale, and Tomé (2012) argued that no proof can confirm that the rising calcium in urine holds responsible for the osteoporosis. Moreover, Heaney and Layman (2008) conduct an *in vivo* experiment on hundreds of nuns aimed at studying whether protein consumption has an effect on the efficiency of calcium assimilation, and they reported that the connection failed to be found. Nowadays, protein is considered as one of the crucial nutrients to the good condition of the bone. First of all, it provides the context of the structure where the bone grows and maintains as it stimulates the bone cells differentiation, proliferation and mineralization and impedes bone resorption. in addition, it increases calcium assimilation (Fernandes, Lawrence, & Sun, 2003; Heaney & Layman, 2008). Protein like collagen can work as the preservation and matrix formation of bone. As for non- collagen proteins, they can give strength to the matrix made by collagen and help to bind mineral (Heaney & Layman, 2008). Therefore, it is important for bone health to consume a sufficient amount of protein daily. Therefore, high protein diets can be beneficial to prevent osteoporosis.

Singh, Dubey, Paliwal, Saraogi, and Singhai (2012) and Marsh, Straub, Villalobos, and Hong (2011) reported that there is almost one-third of death globally is due to cardiovascular which is one of the major elements attribute to mortality.

2.5.1.2 Protein enrichment for elderly population

The ageing problem draws our attention because its population is soaring out of expectation. Thus, it is another angle for healthcare that needs to be taken into consideration. Losing the mass of muscle is one the distinctive feature during the ageing process and this may lead to the dysfunction of muscle and more severely, abilities in terms of stairs climbing, walking rate are under restrictions, which might result in a worse living quality (Fielding, 2013). All nutrition elements are consequential to human especially for the elderly, and protein is a top priority among them. Baum and Wolfe (2015) noted that for older adults, amount of taking protein daily should more than required as it can guard against cardiovascular function, prolong the energy consuming, improve bone health and fasten wound healing.

2.5.1.3 Protein enrichment for weight management and satiety

Nowadays, not merely the ageing population is boosting but people with obesity. Along with the urbanisation and economic growth, people tend to have less time paying attention to their diet but turn to fast food so that imbalanced nutrition and sugar addiction become commonplace. Correspondingly, obesity and overweight have become one of the most concerned health issues. Obesity as a hidden danger can result in many other diseases such as cardiovascular disease, type 2 diabetes, hypertension, cancer, respiratory problems and osteoarthritis (Jones & Rasmussen, 2009).

However, evidence has already been proved that increasing the consumption of the proper amount of protein in substitution of the original diet, can help obesity people manage their total daily energy intake so that they can maintain their body weight by raising the satiety (Gosby, Conigrave, Raubenheimer, & Simpson, 2013; Plantenga & Lejeune, 2005). Because satiety is one of the essential components for losing weight, in other words preventing obesity, and it was reported that most, compare to both carbohydrate diet and fat diet with the same amount of calorie, protein diet is more satiating (Eisenstein, Roberts, Dallal, & Saltzman, 2002). Additionally, Layman *et al*.

(2005) found that raising protein intake can also make fat loss easier. Skov, Toubro, Rønn, Holm, and Astrup (1999) further suggested that weight loss can be achieved by substituting proteins for carbohydrates.

Furthermore, Plantenga and Lejeune (2005) demonstrated that the rate of sleeping metabolization, fat oxidation rate as well as thermogenesis can be raised by taking a high-protein diet. Their findings were matched with Plantenga and Lejeune (2005) ideas, which is diet-induced thermogenesis has positive correlations. That is, satiety feeling can be reached by increasing oxygen uptake, energy spending, and body temperature.

Meanwhile, Crovetti, Porrini, Santangelo, and Testolin (1998) conducted a comprehensive aimed at studying the connection between energy spending and satiating effect by offering mixed high protein meal, they stated that high protein diet elevates the rate of thermic effect and protein oxidation, which brings about the sensation of satiety. By the same token, Raben, Larsen, Flint, Holst, and Astrup (2003) reported that a high protein diet can arouse more thermic effect than other diets. It has been further studied by Leidy, Lepping, Savage, and Harris (2011), and they agreed that dietary protein boosts the satiety in teenage overweight girls.

Increase satiety is not the only strategy protein working for weight management, on the report of many studies, the protein comprises a potential to manage body weight, more specifically, protein also helps fat loss. It was found that, after being offered with a high protein diet for 10 weeks, overweight adult women showed a more fat loss compared to a carbohydrate diet (Layman *et al*., 2003). Similarly, Johnston, Tjonn, and Swan (2004) conducted an investigation on 20 healthy adults for the efficiency of losing weight on a high-protein diet and low-carbohydrate diet, and they found that the former diet had more satisfied fat loss. However, Paddon Jones (2008) still reported some side effects of consuming high protein diet, such as adding burden on kidney and ultimately blood pressure. Nevertheless, overall, high protein diet with appropriate portion is still a highly promising grand plan for weight management.

2.5.1.4 Protein enrichment for sports nutrition

Protein is also very important in executing physical activity. In general, resistance exercise requires amino acids to support muscle become hypertrophied, while as for

endurance training, the amino acids are more needed for being oxidised (Tarnopolsky, 2004). Compared to ordinary people with basic daily activity, athletes require numerous nutrients to maintain the daily training, besides, nutrition is more vital to athletes as it influences not only the usual physiological maintenance but the final competitive state (Campbell, 2007; Crittenden *et al*., 2009). As stated by Kerksick *et al*. (2007), a higher protein supplement is for recharging the glycogen stores and to assist in repairing muscle and growth. They also pointed out that it is vital to consume nutritional protein supplement before or after training since it blocks muscle damage, improves muscle recovery and more importantly, promoting its growth.

An elevating number of published papers affirmed that food proteins are fundamental for human health and growth, they also contain numerous bioactivities strength that they might are advantageous to cardiovascular and bone health, and they are particularly beneficial to the athletes. Moreover, the dietary protein market has expanded dramatically especially in health enhancement and nutrition subdivision, a number of high-protein products have been launched on the market victoriously and this trend is expected to flourish in the coming future. Different proteins such as vegetable or dairy origin can be used as a source of proteins and added to wheat flour to supplement the protein content of the original wheat four. Other than wheat flour, the major sources of protein found in baked products are eggs, milk and soy (O'Kennedy, 2009; Rosell, 2003).

2.5.2 Dairy proteins

Milk proteins such as whey and casein are widely used in the manufacture of bakery products. These are generally used in their dried form in most baked foods. Improving the nutritional, organoleptic, and functional properties of bread using by dairy products have been reported in the literature (Erdogduarnoczky, Czuchajowska, & Pomeranz, 1996).

In terms of bread baking quality, dairy ingredient addition leads to increased water absorption, a decrease in the staling rate, and increased in the crust colour (Dubois and Dreese 1984). In a food system, these ingredients behave as emulsifiers that bind fat, absorb water and stabilize air in the food matrix making them important ingredients to modify the textural and rheological properties of formulated food products. They also

contribute to enhancing product stability and sensory appeal (Cocup & Sanderson, 1987; O'Regan, Ennis, & Mulvihill, 2009).

It was shown that after resistance training, compared with other protein, intaking milk increases lean mass and protein synthesis in muscle, especially whey protein. (Tang, Moore, Kujbida, Tarnopolsky, & Phillips, 2009). In bread baking, increased water absorption, reduced staling rate are some of the advantages of dairy ingredients (O'Regan *et al*., 2009). Nevertheless, some milk fraction has been described as loaf volume-depressing (Erdogduarnoczky *et al*., 1996).

2.5.2.1 Rennet casein (RC)

Caseins are the principal component of milk proteins, accounting for the 80% majority of the total proteins in milk. Rennet casein is obtained by allowing casein micelles to proteolytically destabilise by addition of chymosin like proteinases derived from the bovine or microbial origin (Rollema & Muir, 2009). Also, caseins are particularly rich in lysine and so make excellent nutritional supplements for cereals, which are deficient in lysine (Huppertz, Fox, & Kelly, 2018a). Therefore, casein/caseinates are practical to add to breakfast cereals, milk biscuits, protein-enriched bread. Moreover, Boirie *et al*. (1997) demonstrated that casein can hinder the protein collapse in the human body by 34%. Owing to the ease with rennet casein can be produced by rennet-induced coagulation, it became a much more valuable product and is now one of the principal functional food proteins (O'Regan *et al*., 2009).

Rennet casein has a multitude of applications in the food industry. They are mainly used in products such as cheese analogues, bakery, beverages, meat, and confectionery products since it plays a major role in the structural and functional properties of these products (Augustin, Oliver, & Hemar, 2011; Bisson, Bussiere, Fournet, & Jacquenod, 1988; Kaneko, Yokoyama, & Tsuruoka, 1989; Namdari, 1994; Remment, 1963; Rollema & Muir, 2009; Varnam & Sutherland, 2001). It also reacts with gastric acid and become precipitates during digestion. The gastric emptying time gets prolonged might induced by this coagulation as well as the postprandial plasma amino acid might get slightly increase (Plantenga & Lejeune, 2005).

As compared to acid casein and caseinates, rennet casein has an overall higher mineral content which is because rennet casein production does not involve any acidification step (Ennis *et al*., 1998; Mizuno & Lucey, 2005). Current research aims to completely substitute gluten with a functional casein-based ingredient (Stathopoulos and Kennedy, 2008). The principle behind this approach is that by increasing the calcium concentration to an optimum level in the casein/caseinate ingredient, it will be possible, under the correct pH and ionic strength conditions, to replace the highly functional (covalent) S–S bonds in a gluten-based dough with calcium-induced casein–casein complexes (O'Kennedy, 2009).

2.5.2.2 Milk protein concentrate (MPC)

The functional properties of MPCs are generally attributed to their milk proteins (O'Regan *et al*., 2009). MPC has a protein content of ~80%. MPCs are typically aggregated proteins and represent a concentrated form of milk proteins that contains caseins and the whey proteins in the same amounts as the whole milk (Ye, 2011). MPC can be tailored to many food formulations either for their nutritive value or for their role as textural improvers (Augustin *et al*., 2011; Havea, 2006; Rollema & Muir, 2009).

Milk proteins have an ability to bind small hydrophobic vitamins such as vitamin A, vitamin B12 and folate. This binding may improve the absorption of these vitamins in the intestinal tract as it may protect the vitamin against the possibly detrimental environmental conditions during passage through the digestive system thus improving the bioavailability of these vitamins (Ford, Salter, & Scott, 1969; O'Regan *et al*., 2009; Sandberg, Begley, & Hall, 1981). Several studies have shown how MPC addition has improved food product characteristics (Uluko, Liu, Lv, & Zhang, 2016).

2.5.3 Plant proteins

Plant proteins are highly related to human protein nutrition, they are a major determinant of the lysine content of diets worldwide. Plant protein can serve as a comprehensive source of amino acids (Pellett & Young, 1994).

2.5.3.1 Soy protein isolate (SPI)

Soybean is the most important legume due to its high protein content and relatively low price. They are also good sources of minerals and vitamins. legume proteins are a relatively rich source of lysine (higher than wheat flour), tryptophan and threonine. Soybean is the most important legume due to its high protein content and relatively low price. The consumption of soy foods can have beneficial effects on nutrition and health, such as lowering plasma cholesterol, which are the main pathogenic factors leading to coronary heart disease (Hasler, 2002), preventing cancer, diabetes and obesity and protecting against bowel and kidney diseases. Isoflavones from soybean improve bone strength and are potent antioxidants and free radical scavengers. The addition of soy proteins to bread products could, therefore, improve their nutritional quality (Nishinari, Fang, Nagano, Guo, & Wang, 2018).

Several studies claim that the consumption of legumes has healthy effects, preventing some common health problems (Nishinari *et al*., 2018). Therefore, legume protein preparations are gaining importance in the food industry, representing an attractive alternative in the development of new foods. it is widely recognized that protein digestibility-corrected amino acid score of soy protein is much higher than that of other plant proteins such as wheat protein (Nishinari *et al*., 2018). Torres, Torre Villalvazo, and Tovar (2006) agreed that soy protein consumption lessened not only total cholesterol and low-density lipoprotein cholesterol but the triglycerides and raised high-density lipoprotein cholesterol. these findings have helped patients with hypercholesterolemia as developing dietary strategies. They also indicated that the plasma levels were also reduced by consuming soy proteins compared to dairy proteins and carbohydrates.

Furthermore, since soy foods contain a high amount of isoflavones it can also help women with postmenopausal to guard against osteoporosis. Hence, soy proteins have gathered plenty of notice. It has been further confirmed that by using ovariectomized rats, the bone loss could be prevented through consuming soy protein (Arjmandi *et al*., 1996). Additionally, Fernandes *et al*. (2003) explained that is because soy proteins have anti-inflammatory property.

In addition to its high nutritional values, some physiological functions of soy isoflavone such as lowering cholesterol, inhibiting bone resorption and stimulating bone formation, preventing breast and prostate cancer have been reported. Also, the presence of soybean in some foods has been known to cause flatulence (Rackis, Sessa, & Honig, 1979). There is literature also reported that the quality of CSB was degraded when incorporating soy protein with wheat flour (Du *et al*., 2016; Xiao Liu *et al*., 2016).

Overall, soy proteins are prone to benefit cardiovascular, and heart conditions, while milk proteins are broadly utilised to support bone health, build muscle mass, and improve sports performance. A combination of soy protein and milk protein may be more beneficial.

2.5.4 Dietary-fibre and antioxidant enrichment

There is an extensive literature reported on the incorporation of dietary fibre and antioxidant elements in foods in order to enhance their nutritional value without any negative effect on their physical quality (Chang, Li, & Shiau, 2015; Fu, Chang, & Shiau, 2015; Gao, Liu, & Zheng, 2018; Liu, Luo, Chen, Xu, & Liu, 2016; W. Liu, M. A. Brennan, L. Serventi, & C. S. Brennan, 2017; Luo, Liang, Xu, Li, *et al*., 2017; Sy-Yu, Ming-Yin, & Yao-Ling, 2015; Xu, Gao, Ma, Guo, & Wang, 2014).

2.6 Characterisation and evaluation of flour and bread

2.6.1 Colorimeter

The principle quality attributes that the determine the food acceptance are colour, flavour and texture. However, colour has a much greater influence on food judgment. Colour is also used to determine the quality of a particular food product (Nielsen, 2017).

Consumers generally prefer steamed bread with a white, shiny appearance, colour of steamed bread is generally attributed to flour colour and processing parameters such as setting and steaming rate (Huang, 2014; Zhu, 2014). The colourimeter is a useful technique for precise measurement of surface colour. Results are usually in the form of $L^*a^*b^*$ colour coordinate system. L^* values are indicative of darkness or lightness where

a^* indicates values between red/green and b^* for balance between yellow/blue (Nielsen, 2017).

Differences in colour are calculated by subtracting L^* a^* b^* values for the sample from the reference (**Figure 2.3**).

The total colour difference (WI) is calculated by the following equation:

$$WI = (\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2})^{\frac{1}{2}}$$

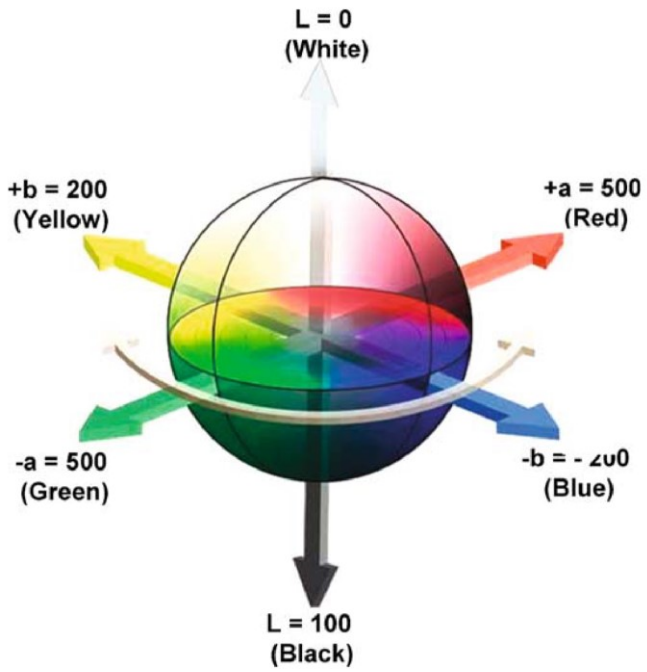

Figure 2.3 The CIE Lab colour space (B. Singh, V Parwate, & Shukla, 2009)

For baked product, importance is given to the red-yellow part of the colour spectrum for crust colour while for the crumb region interest is paid to the yellow to white spectrum. Measurement of crumb brightness is of high importance as the product crumb is the main factor during the judgment of consumers. Addition of protein rich ingredients such as soy protein to steamed bread has shown to give a darker colour compared to white steamed bread (Du *et al*., 2016; Liu *et al*., 2016; Ribotta, Arnulphi, León, & Añón, 2005).

2.6.2 Rapid Visco Analyser (RVA)

RVA is a cooking viscometer and is designed for testing starch pasting properties speedily. It is specially configured for testing starch-based products since it can heat and cool samples speedily in a controlled manner. The RVA has developed into an instrument with a much wider application. It is used extensively in the gains, food and starch in research, product development, process control and quality assurance.

RVA basically analyse the transformation of starch during heating and cooling in the presence of water. At room temperature, starch can swell 50% bigger than its own size and this stage is reversible.

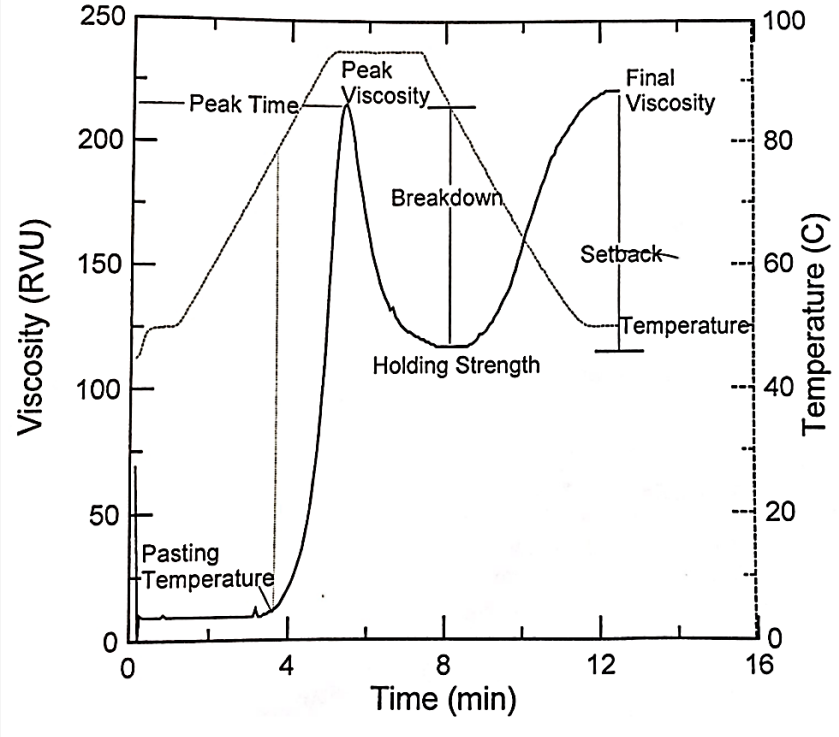

Figure 2.4 Typical RVA pasting curve showing the commonly measured parameters (Lab manual 2007)

Gelatinization is an irreversible process and often occurs more slowly with intermediate water content. Crystallites also lose the molecule order. In our study, it was

employed for testing starch pasting in HPCSB formulations. **Figure 2.4** shows a typical RVA pasting curve showing the commonly measured parameters.

Starch pasting involves post-pasting, swelling, granule disruption, amylose leaching, three-dimensional network formation of leached molecules and interactions between granule remnants and leached material. Starch pasting refers to starch gelatinisation, meaning starch granules when heated in the presence of water at about 80℃, leads to the breakage of the crystalline pattern in starch granules, and they start to absorb water, inflating them to many times their original size, some of the linear amylose chains can leach out of the swollen granule into the solution when shear force is applied to the swollen granules and they rupture, spilling their contents into the solution. It is both the granule swelling and the leaching of amylose that forms a tangled mesh network which thickens the solution of gelatinised starch. In our study, during cooking, doughs are gelatinized and forming a semi-gelatinized structure which is called amorphous structure. During cooling, starch crystalline recovers limitedly, which change mechanical properties significantly (Manohar, Devi, Bhattacharya, & Rao, 2011; Yadav, Yadav, Kumari, & Khatkar, 2014).

Starch gelatinisation is a complex phenomenon that occurs when the internal crystalline structure of the starch granule is lost by heating in the presence of an excess of water. Gelatinization can cause granule swelling and distortion increasing as well as water absorption. Furthermore, it also causes limited starch solubilisation (amylose) leaching, leading to viscosity increment. Amylose leaching can lead to phase separation of amylose and amylopectin (Diamantino *et al*., 2019).

2.6.3 Texture profile analysis (TPA)

Crumb structure is very important, and it is emphasized by all authors. Northern style steamed bread should have a fine and even cell distribution. Steamed bread softness, cohesiveness, and elasticity are also important in eating quality items. If bread sticks to the teeth when chewed, it is disliked by most consumers. Food texture is fundamentally a human experience that is observed during our interaction with food and its behaviour and structure when treated. It is very essential to understand the response to a structure of food and its collapse, which is related to physical and chemical studies of the structure

and composition of food materials and their functioning when compressed or deformed (Rosenthal, 1999).

Table 2.2 List of TPA parameters (Bourne, 2002)

Parameter	Definition
Hardness	By compressing The peak force happens when products are compressed for the first time.
Cohesiveness	The ratio of two positive forces under the two compressions.
Springiness	The significant break in the curve on the first bite.
Adhesiveness	The negative force area of the first bite
Gumminess	Hardness * Cohesiveness
Chewiness	Hardness * Cohesiveness* Springiness
Resilience	It is measured on the withdrawal of the first penetration before the waiting period is started.

Texture profile analysis is a double compression test for testing the textural properties of foods. During a TPA experiment, samples are compressed twice by a texture analyser, in order to provide an understanding of how samples act when chewed. Results of TPA are presented as a curve (**Figure 2.5**). Typical textural parameters (**Table 2.2**) can be calculated (Meullenet, Lyon, Carpenter, & Lyon, 1998). When it comes to bread, hardness, gumminess, chewiness and resilience and are important parameters. Table 1 shows the list of typical parameters of TPA:

2.6.4 Penetration analysis

Puncture test is one of the most broadly utilised instrumental technique used for characterising food texture especially crust hardness. The firmness of bread crust is as important as crumb firmness. Its application for texture evaluation has also been inclusively studied (Georget, Parker, & Smith, 1995; Hecke, Allaf, & Bouvier J, 2007).

Puncture analysis is measuring the force which is demanded to push down a probe or a punch into a food sample. The test is characterised by (a) a force testing instrument, (b) irreversible crushing caused by penetration of the probe into the food. (c) The penetration depth is constantly held (Bourne, 2002). When a punch starts to penetrate into the commodity, there is a sudden change in slope called yield point, and it marks the

irreversible crushing of the concealed tissues, which is the greatest significance in penetrating testing.

2.6.5 Tensile test

The tensile test is the methods that stretch or pull the sample, so that can quantify the eventual strength and the elasticity of the commodity (Bourne, 2002). Grips and clamps are used to conduct tensile tests on samples in a wide variety of shapes, materials, and sizes.

Although there is a less widely used method, for testing food itself is very practical since they can mimic the handling of the final product. Since there are several ways of consuming steamed bread. One of the fundamental reasons for conducting this study is to simulate how samples are stretched and pulled by hands to measuring burst strength or adhesion and stickiness. However, this measurement has been stated that it is hard to standardize.

Figure 2.5 Texture profile analysis (TPA) curve (Kortei *et al*., 2015)

2.6.5 Differential scanning calorimetry (DSC)

Physical or chemical decomposition changes that the accompanies by endothermic or exothermic reactions are easily detected using DSC. These changes are assessed by differences in temperature and heat flow between the sample and an inert reference (H. Liu & Lelievre, 1992). The most commonly used DSC is a heat flux DSC where both sample and reference are heated in a block, and the energy required to keep both the sample and the reference at the same temperature while they are heated/cooled is calculated. The energy (heat flow) is indicative of the heat capacity of the sample and is reported versus temperature or time in a DSC thermogram (Nielsen, 2017; Zhang, 2004).

A DSC reflects phase transitions that occur in starch-containing systems. Changes in endothermic and exothermic peaks that occur when conditions are varied reflect molecular transitions in mixed starch systems. DSC also provides an insight into the quality characterisations and structural modifications of finished products and raw ingredients. Therefore, it is a good tool to assess properties like textural change and storage stability (Nielsen, 2017; Raemy, 2003).

The results of the thermal analysis provide an insight into the quality and structure of both finished products and starting ingredients. Therefore, it can define the final properties such as storage stability and texture. Areas of application include quality assurance product development, and research into new materials, formulations and processing conditions (Nielsen, 2017; Raemy, 2003).

DSC can be used to measure the properties and structure of both HPCSB formulations and fresh HPCSB samples. The interaction between water and starch are of considerable scientific and commercial interest, due to the role of water in the stability and functional properties of the main constituents of the majority of food and pharmaceutical systems.

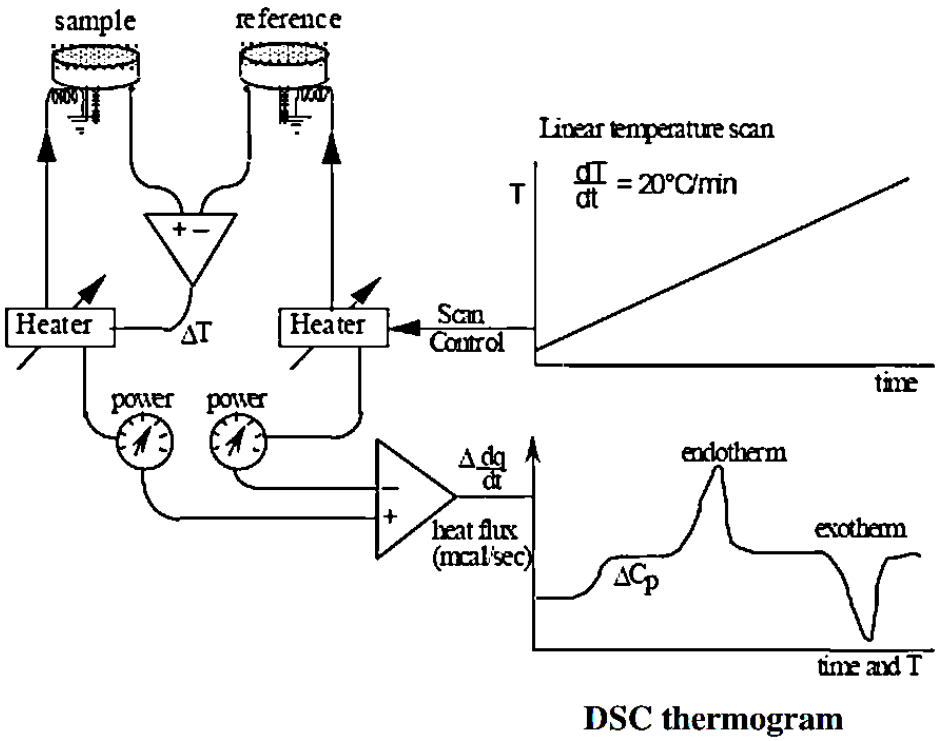

Figure 2.6 Schematic of differential scanning calorimeter (DSC) instrumentation (Zhang, 2004).

2.6.6 Scanning electronic microscopy (SEM)

Scanning electron microscopy (SEM) has been extensively used to study the structure of dough and starch-based samples. In comparison to standard light microscopy, SEM is preferred for starch granule morphology and imaging as it provides a detailed insight with a clearer depiction of starch granule morphology and allows for a more accurate analysis of granule shape and size (Lindeboom, Chang, & Tyler, 2004).

For carrying our SEM, it should be ensured that samples are dried and distributed evenly on a thin layer prior to coating with a layer of conductive material such as gold or platinum. Thus, this limits the use of SEM as only low moisture samples can be used. Also, if starch is present in a high moisture system, the drying process can alter the usual appearance of the sample. however, it is still a useful tool to assess changes in microstructure as a result of various treatments (Jane, Kasemsuwan, Leas, Zobel, & Robyt, 1994).

Food is a complex and often heterogeneous system. Both fresh and processed food contain structures that cannot be seen with naked eyes. Microscopy is one of the most frequently used family of instrumental techniques for analysing foods and food microstructures. The analysis of food microscopic structures is fundamental for the flavour, aroma and texture and nutrition of food products as well as shelf life and health benefits. It not only determines the morphology of the food of the microstructure elucidation but also helps identify the distribution of ingredients (Russ, 2012).

2.6.7 Gastro-small intestinal digestion i*n vitro*

When any type of food is eaten, it undergoes a wide range of physical (e.g. mastication) and chemical (e.g. enzymatic effects) changes as it passes from the mouth to the gastrointestinal tract (**Figure 2.7**). The intestine is the state where food digestion and absorption occurs. Food digestion is a complex process and many processes are still not fully understood (Guo, 2015).

When food containing starch is consumed, the starch present may be in its native conformation or be gelatinised. During oral processing, the food is mixed with salivary α-amylase, and forms a cohesive bolus. Salivary amylase has an optimum pH 6.5-7 and is usually not active once the bolus reaches the stomach. A large piece of food >1mm is left behind while the starchy liquid part gets separated by the sieving action of the stomach and moves to the small intestine where it mixes with pancreatic α-amylase. Thus, hydrolyses the α-1,4-glycosidic bonds in the starch but does not cleave the α-1,6 cross-links in amylopectin. Digestion end products in the lumen consist of molecules like maltose, malt triose. The de-polymerisation of starch to glucose is completed by oligosaccharides which happens in the region close to enterocytes (Berg, Singh, Hardacre, & Boland, 2012; Lehmann & Robin, 2007; Smith & Morton, 2001).

A small amount of starch still remains resistant to the digestion by enzymes that is ferment in the colon. Resistant starch is classified based on their rate of in vitro enzymatic digestion, rapidly digesting starch (RDS 0-20 min), slowly digesting starch (20-120 min) and resistant to digestion (RS) (K. N. Englyst, Englyst, Hudson, Cole, & Cummings, 1999).

Accurate determination of bioactive carbohydrate in a given product enables the manufacturer to communicate the glycaemic response per serving of a food, especially in the case of diabetes therapeutic food, management of diabetes and disorders of carbohydrate metabolism. The concept of the glycaemic index has been introduced to classify foods on the basis of their postprandial blood glucose response. The glycaemic index is defined as the postprandial incremental glycaemic are after a test meal, expressed as the percentage of the corresponding area after an equi-carbohydrate portion of a reference food such as glucose or white bread (Goñi, Garcia-Alonso, & Saura-Calixto, 1997).

In vitro digestion is used in our project to achieve an insight into the breakdown of CSB made with HPCSB formulations. *In vitro* experiments are advantageous over in vivo because there are no ethical constraints and are often simplistic and economical (Woda *et al*., 2010). The physical characteristics that affect the rate at which starch is digested are 1) extracellular structures inherently present (e.g. cell wall). 2) Barrier at the surface of the granule. 3) the molecular orientation of different starch polymers within the starch granule. These characteristics are greatly affected or disrupted by external mechanical forces such as mastication, processing conditions or by the contraction of intestine. Therefore, starch digestion is affected by the structural form in which it enters the small intestine, and also on the rate of gastric emptying. Any physical or chemical modification done to the food system containing starch will greatly affect the rate at which the starch is digested (Lentle & Janssen, 2011).

In the mixed-starch system, the food matrix plays an important role in the digestion of starch. The addition of protein in the food system may have an influence on the rate of starch digestion. It has been reported that the presence of protein in cereals and other foods affected starch digestibility (Ezeogu, Duodu, Emmambux, & Taylor, 2008). The presence of globulins glutenins and albumin help in fixing the protein bodies in a network surrounding starch granules (Hamaker & Bugusu, 2003).

Also, the presence of protein during cooling or heat processing has been shown to reduce the starch digestibility as conformational transitions in proteins occur that facilitate formation of disulphide-linked polymer chains (Oria, Hamaker, & Schull, 1995), Jenkins *et al*. (1987) reported that starch-protein interactions in wheat flour may be the

reason for a lower glycaemic response and reduced starch digestibility. Also, it has been shown that addition of protease to hydrolyse the protein surrounding starch granules enhances *in vitro* starch digestibility due to the facilitation of amylase and amyloglucosidase into the food matrix alter the clearance of protein which previously acted as a barrier (Rooney & Pflugfelder, 1986). Singh, Dartois, and Kaur (2010) speculated that the addition of extra protein might lead to an increment in cohesiveness between starch and proteins of dough, which may reduce the starch accessibility to a-amylase which leads to less digestibility.

Figure 2.7 Region specificity of the human gastrointestinal tract (Guerra *et al*., 2012).

2.6.7.1 Microscopy during digestion

Microstructure characteristics of starch-based foods such as bread are crucial for understanding how the food is digested during digestion. Also, the starch and protein interaction before and after digestion along with the structure variation during digestion.

In the mixed-starch system, the food matrix plays an important role in the digestion of starch. The addition of protein in the food system may have an influence on the rate of starch digestion. It has been reported that the presence of protein in cereals and other foods affected starch digestibility (Ezeogu *et al*., 2008). The presence of globulins

glutenins and albumin help in fixing the protein bodies in a network surrounding starch granules (Hamaker & Bugusu, 2003).

2.8 Purpose of the current study

Conventional Chinese steamed bread contains high amount of carbohydrates and excessive consumption of digestible carbohydrates may lead to the high blood glucose levels High levels of insulin can lead to obesity and many other health problems.

Consumers especially elderly, athletes, obese people and middle-aged female who suffered from osteoporosis are highly keen to take high protein food. The growth-promoting effects of soy protein and milk protein supplements in bread are related primarily to their contributions of lysine which increase the proteins in bread and make them nearly comparable in quality with proteins in milk and meat. Consequently, food products and dietary management systems are important to help control and maintain blood glucose levels close to normal one. More specifically, there is a need for high protein versions of popular, highly consumed staple foods. However, for centuries, the staple food for Asians especially Chinese is wheat and rice. Thus, it is not easy for them to instantly accept high protein diet from new sources.

Therefore, an “ideal” nutritionally improved HPCSB will be a suitable product, which requires no special formulations and equipment for producing at commercial level and also be acceptable to the consumer with regard to overall eating qualities.

Currently, little information is available in the literature on the fortification of CSB by adding dairy protein and plant proteins, the microstructural characteristics of CSB during, and after *in vitro* gastric-small intestine starch digestion have also not been reported so far.

The aims of the present study were to

1. To develop a high protein, low carb Chinese steamed bread using plant and dairy proteins.

2. To characterise the physico-chemical, microstructural, thermal, textural properties and the starch digestion *in vitro* of HPCSB.

Chapter 3. Materials and Methods

3.1 Materials

Wheat flour, sugar, and baking soda and soy protein isolate (SPI) were purchased from Davis Trading (Palmerston North, New Zealand), while yeast was obtained from a city supermarket (Pak'nsave, Palmerston North, New Zealand). Rennet casein (RC) and milk protein concentrate were purchased from Fonterra Ltd. (Palmerston North, New Zealand).

Enzymes used for the *in vitro* Gastro-intestinal digestive enzymes such as Amyl glucosidase (3260 U/mL) was procured from Megazyme International Ireland Ltd. (Ireland), pancreatin (hog pancreas, 4×USP), invertase (Invertase, Grade VII from bakers' yeast, 401 U/mg solid) and pepsin (porcine gastric mucosa, 800–2500 U/mg protein), were purchased from Sigma-Aldrich Ltd. (St Louis, USA) whereas.

All the chemicals and reagents used in this study were of analytical grade.

3.2 Methods

The ingredients in each formulation used to make HPCSB in four formulations were selected on the basis of their functionality and nutritional properties as discussed in section 2.5.3.

The study design is provided in **Figure 3.1**. The study is devided into three parts:

Part I: To formulate a high protein, low carb steamed bread that has lower starch digestibility and eGI than commercialised Chinese steamed bread using optimal levels of plant and dairy proteins.

Part II: To study physico-chemical, thermal, textural and microstructural characteristics of HPCSB and its *in vitro* starch digestibility using oral and gastro-small intestinal digestion models.

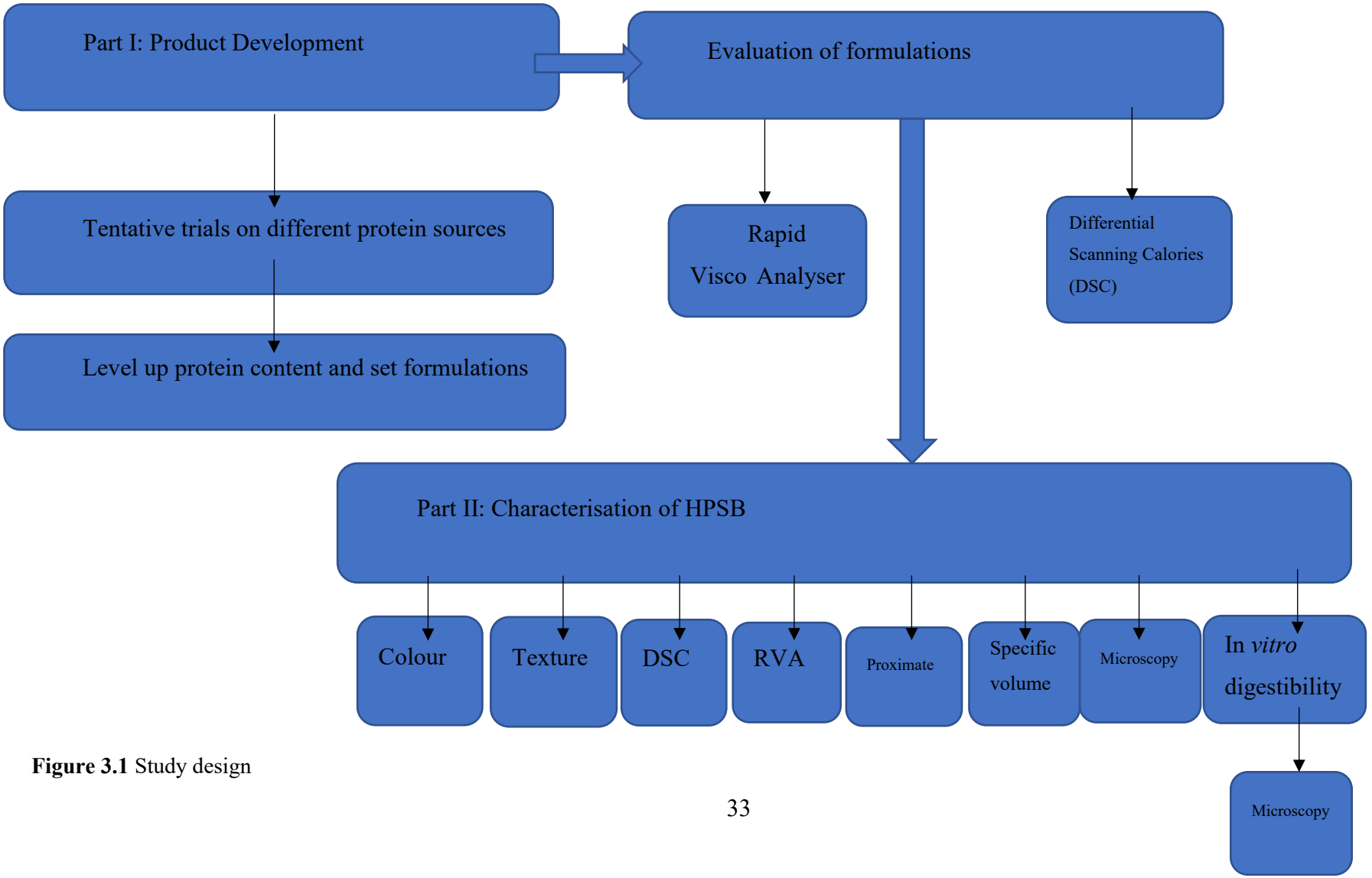

Figure 3.1 Study design

3.2.1 Product development of HPCSB

The production of HPCSB was adapted from the method developed by Huang and Miskelly (2016d) with slight modifications. The flow chart of laboratory scale production of HPCSB is presented in **Figure 3.1.**

Developed formulation and production parameters are presented in **Table 3.1 and Table 3.2.**

Since the use of soy protein isolate can have an unfavourable influence on the sensory profile of bread, it was blended with milk protein concentrate to give it a pleasant aroma and dairy taste. This blend was mixed and added to the wheat flour to be used in bread making.

3.2.2 Physico-chemical characterisation of HPCSB

3.2.2.1 Colour characteristics

The crust and crumb colour of steamed breads was determined using a Minolta Chroma Meter CR-200 (Chemiplas NA Ltd., AU), following the method of (Hsieh, Weng, Yu, & Wang, 2017), both crust and crumb colour of each sample were evaluated at three different points on four slices of breads from the same cooking process.

Data were reported in the form of CIE L^*, a^*, and b^* colour space. The white index of (WI) of HPCSB and the control was calculated using the equation:

$$WI = (\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2})^{\frac{1}{2}}$$

3.2.2.2 Specific volume

Samples were analysed for a specific volume by the rapeseed displacement method as previously reported by Nwosu, Elochukwu, and Onwurah (2014).

$$\text{Specific volume (ml/g)} = \frac{V_{bread}}{m_{bread}}$$

3.2.2.3 Proximate analysis

Proximate analysis is the fundamental characterisation for food development as it provides basic information for food products require studies. Specific volume was calculated using the equation below:

3.2.2.3.1 Protein content

The protein content of the HPCSB is by Kjeldahl method (APAC 2006). This method is based on the determination of reduced nitrogen content,

The protein content is obtained by multiplying a protein conversion factor to the nitrogen content.

0.5 g sample was accurately weighed and added to a digestion tube. After that, two Kjeltabs (containing K_2SO_4 and Se) and 17mL concentrated H_2SO_4 were added to the tube. Meanwhile, a blank digestion was set up as a control. Then, the digestor unit with tubes containing samples was set up and was digested at 420°C until no solid was observed and became transparent in each tube. The tubes were taken off from the digestor and waited till cooled down, each tube was added proximate 100ml water. On the other hand, 4 % of 25ml boric acid was added to a conical flask. Each sample was in a Kjeltec 2100 System (Tecator, Sweden) after placing the receiver conical flask with boric acid, and the distillation was started. When distillation is complete, the sample was titrated with 0.01M HCl when a grey-violet solution was noticed.

The calculation of % nitrogen (N%) in samples are:

$$\%Nitrogen = \frac{(A \times B) \times 14 \times 100}{1000 \times C}$$

Where, A= volume(mL) of HCl used

B= exact molarity HCl

C= weight (g) of original sample used.

The nitrogen to protein conversion factors used in this study was based on the different proteins in the various proportion of each formulation. The conversion factors after calculation are shown below:

Table 3.3 Conversion factors for different HPCSB formulations and control

Sample	Protein content (*N%)
Control	5.70
RC I	6.04
RC II	6.15
SM I	5.82
SM II	5.83

Control: pure wheat Chinese steamed bread; RC I: rennet casein%:14%(w/w); RC II: rennet casein%: 33% (w/w); SM I: SPI%:7%(w/w), MPC%:5% (w/w); SM I: SPI%:7% (w/w), MPC%:8% (w/w).

3.2.2.3.2 Moisture content

The moisture content of the samples was measured according to the standard AOAC method (AOAC, 1990). Bread samples were weighed accurately before dried at 108℃ for 24h in an air dryer (Oven 8150, Labserv, Ireland) after which, the bread was weighed again. The moisture content was the difference before and after drying.

3.2.2.3.3 Total starch content

The estimation of total starch concentration in HPCSB was determined by using method developed by Bordoloi, Singh, and Kaur (2012) and I. Goñi, García Diz, Mañas, and Saura Calixto (1996). A total starch assay kit (K-TSTA, Megazyme International Ireland Ltd., Ireland) was used to determine the total starch content of the ground freeze-dried HPCSB samples.

Table 3.1 Formulations for HPCSB

	Control (g)	RC I(g)	RC II (g)	SM I (g)	SM II (g)
Gluten	0	3.4	6.8	3.3	4.2
Wheat flour	250	218.99	187.87	220	212
RC	0	31.01	62.13	0	0
SPI+MPC	0	0	0	17.6+12.5	18+20
Sugar	8	8	8	8	8
Baking soda	0.5	0.5	0.5	0.5	0.5
Yeast	2.5	2.5	2.5	2.5	2.5
Water	135	160	165	155	160

Control: 100% wheat flour Chinese steamed bread; RC I: Replaced with rennet casein (14% w/w); RC II: Replaced with rennet casein (33% w/w); SM I: Replaced with SPI (7% w/w) + MPC (5% w/w); SM II: Replaced with SPI (7% w/w) + MPC (8% w/w).

Table 3.2 Production parameters for different HPCSB

Time(min)	Control	RC I	RC II	SM I	SM II
Mixing	7	5	5	4	2
Fermentation	30	30	30	30	30
Kneading	3	1	1	0.5	0.5
Proofing	15	15	15	15	15
Steaming	15	15	15	15	15

3.2.3 Pasting properties

3.2.3.1 Formulations of HPCSB and control

The pasting properties of HPCSB different formulations were obtained using Rapid Visco Analyser (Perten RVA 4800, Australia), and RVA data were analysed using Thermocline for Windows 10 (version 3.11) provided by the instrument manufacturer. The Approved Method 76-21.01A (AACC International, 2000) was used for formulations analysis (4.0g sample and 14% moisture basis).

Control (wheat flour) and four formulations (**Table3.1)** without yeast and water and freeze-dried HPCSB samples and a control CSB sample were prepared. 4g wheat flour or freeze dried HPCSB powdered formulations were transferred into an aluminium canister and 25 ± 0.1ml distilled water was added (corrected to compensate to according to a 14% moisture basis). Before measurement, each flour suspension was stirred manually by rotating the plastic paddle of the RVA for 30 to 40 secs to disperse the sample uniformly and to avoid huge lumps. Each formulation with water was held at 50℃ for 1min and then heated to 95℃ in 3.7min, subsequently held at 95℃ for 2.5 min followed by cooling to 50℃ in the next 3.8 min, and finally, samples were held at 50℃ for 2 min. Paddle speed was at 960rpm for the first 10 seconds followed by 150rpm for the remaining duration of the analysis. Pasting temperature, final viscosity, peak viscosity, setback, holding strength viscosity and peak time were evaluated.

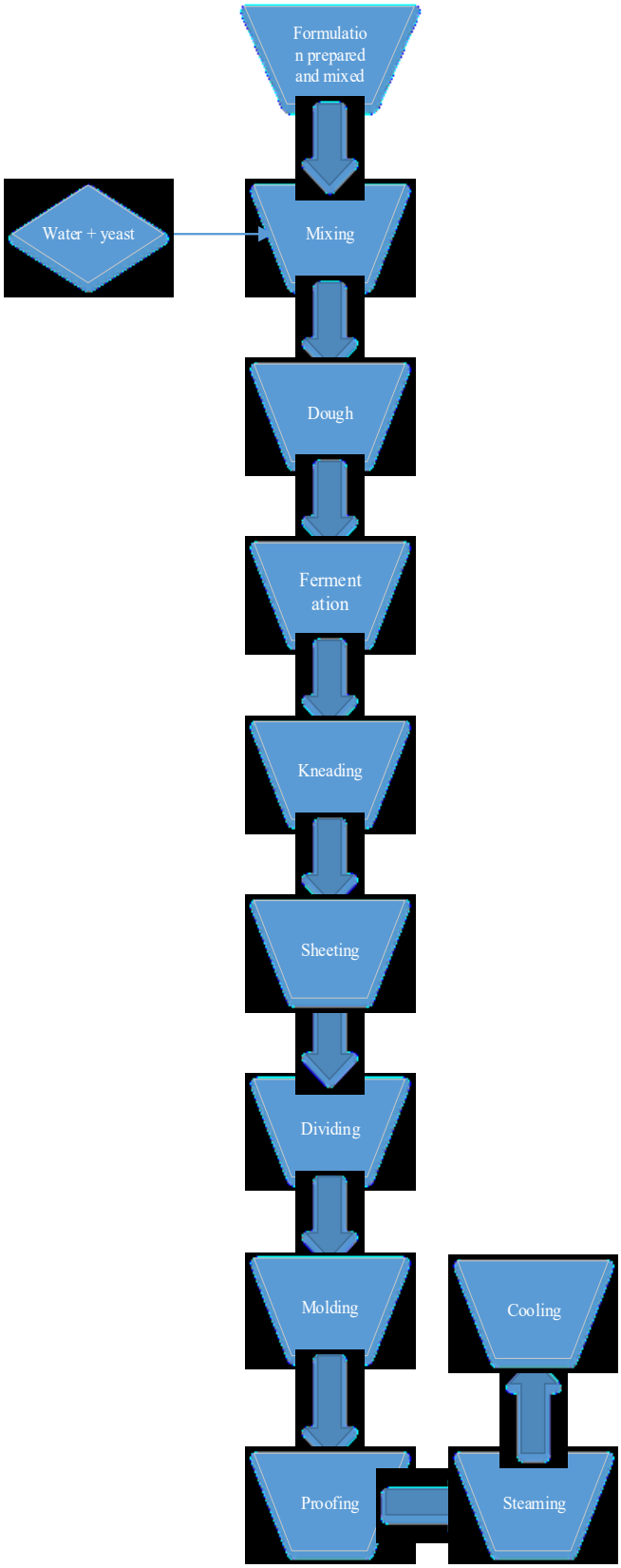

Figure 3.2 Laboratory scale production of HPCSB

3.2.4 Thermal characterisation

The gelatinisation (thermal) characteristics of the HPCSB formulations and control (wheat flour) and freshly cooked samples were determined using differential scanning calorimetry (DSC) (DSC; TA Q100, TA Instruments, Newcastle, DE). An aluminium pan contained HPCSB formulation samples (approx. 5.0 mg, dry wt basis) was prepared, and distilled water was added to obtain a starch-water ratio of 1:3 (w/w). The pan was sealed and stored for at least 2 hours at room temperature before analysis. The sample pans were then heated from 20 to 100 ℃ at a rate of 10 ℃ / min. The temperature at the onset of gelatinisation (T_o), peak temperature (T_p), conclusion temperature (T_c); enthalpy of gelatinisation and (ΔH) temperature range (ΔT) were determined.

3.2.5 Texture profile analysis

Double compression test of bread was performed using a Stable Micro Systems TA-XT2 texture analyser, equipped with a 35 mm diameter cylindrical flat probe and a 5 kg load cell. The curst of samples was removed, and bread crumb was cut into a 3*3*2 cm cuboid. The pre-test and post-test speed of the probe were set at 2mm/s and the test speed was 0.2mm/s. Hardness, gumminess, chewiness, springiness and cohesiveness were counted by the TA software.

3.2.6 Puncture test

Puncture test of bread was conducted using a texture analyser with a 5 kg load (TA XT plus, Stable Micro Systems). The force recorded was the one required to penetrate the bread crust by punching the bread surface at three different points. Experiments were carried out using a cylindrical probe, 2 mm in diameter. The test speed was 0.5 mm/s (Altamirano Fortoul, Hernando, & Rosell, 2013).

3.2.7 Tensile test

The purpose of carrying out pull-apart (tensile) analysis was for mimicking hands pulling apart bread. The bread was griped at two sides by metal fixator, and the position for griping the sample is 3.5cm from the centre, the analysis consisted of recording the force required to pull the sample apart. The experiments were conducted for three replicates. Since this analysis was barely conducted by previous studies, it has not been

fully standardised yet. The major problem with the experimental method is that the fracture point of the sample is not always in the middle but the grip part, which enlarges the error range. The results obtained from the tests were recorded and evaluated by the Texture Expert 1.22 software.

3.2.8 Scanning electron microscopy (SEM) for high protein Chinese steamed bread and dough

After the fresh bread samples and fermented dough were sent for freeze drying. Freeze dried bread samples were cut into sizes of about 5mm thickness and then coated with gold particles for 110s (SCD 050, Blazers, Liechtenstein). The morphology of the samples was evaluated by the SEM (FEI Quanta 200 FEI Electron Optics, Eindhoven, The Netherlands).

3.2.7 Gastro-small intestinal starch digestion *in vitro* of HPCSB

Oral processing simulating (Tamura, Okazaki, Kumagai, & Ogawa, 2017), as well as A two-stage gastro-intestinal in vitro digestion model simulation human gastric and small intestine, was used in this study (Dartois, 2010). The prepared steamed bread samples and an appropriate amount of distilled water were added into beakers to reach a 4% concentration of total starch content.

The ratio of saliva to sample was 1:1, samples were weighed and torn apart into small pieces and the volume each piece for around $0.5m^3$, then they were transferred to a mortar, soaking for 15 seconds and pounced for 15 times simultaneously.

Different formulations of steamed bread with saliva were adjusted their starch content by adding RO water to 170 g (formulation is shown below, **Table 3**) and were transferred to the mesh net in a 500ml jacketed glass reactions and agitated by magnetic stirrers continuously at 300 rpm. The bread samples were placed into a commercial mesh bag. A circulatory water bath was connected to the reactor jacket to maintain its temperature at 37 ± 1℃. The solution pH was adjusted to 1.20 ± 0.1 by adding simulated gastric fluid (SGF, containing pepsin) and was maintained during the simulated gastric part by adding different concentrations HCl. 0.5ml aliquots were collected and the enzyme was inactivated using absolute ethanol to analyse the glucose content after 0 (G0),

15(G15) and 30 min (G30) of simulated gastric digestion; After 30min of simulated gastric digestion, the pH was varied to 6.80 ± 0.1 by the addition of NaOH for pepsin inactivation.

Simulated intestinal fluid (SIF) containing pancreatin, amyloglucosidase and invertase was added into the juice to begin the second step of digestion, and the pH was adjusted to 6.80 ± 0.1. After 0 (I0), 5 (I5), 10 (I10), 15 (I15), 30 (I30), 60 (I60), 90 (I90), 120 min (I120) of simulated small intestinal digestion, 0.5 ml sample aliquots were collected, and the enzyme was inactivated by mixing with absolute ethanol. The solutions were centrifuged for 10 min at 1800×g and incubated at 37°C for 10 min with invertase and amyloglucosidase. Before analysing for glucose samples were pre-treated with D-glucose assay (GOPOD Format K-GLUK 07/11, Megazyme International Ireland Ltd., Wicklow, Ireland).

The hydrolysis index (HI) of each sample was calculated by dividing the area under its digesto-gram by the area under the digesto-gram of fresh white bread (Isabel Goñi *et al*., 1997). They suggested that starch digestion at 90 min in samples can also be used to estimate GI (GI_{H90}). Hence, according to (Yong, Chan, Garcia, & Sopade, 2011), the average estimated GI (eGI) for each sample was fined as below:

$$eGI = \frac{(39.21 + 0.803H_{90)} + (39.51 + 0.573HI)}{2}$$

3.2.8.1 Microstructure during in vitro digestion

Scanning electron microscopy (SEM) was used to examine the structural changes of HPCSB throughout the *in vitro* digestion process. After adding saliva for oral processing for 1 min, HPCSB cubes (~0.5-1mm^3) were digested in vitro using the procedure described in Section 3.3.4. A rotary shaker was used at a speed of 60rpm to shake the reactor during in vitro digestion. Digested HPCSB cubes were taken out right after oral processing (O), at 30min(G30) after gastric digestion, and at 5 (I5), 30 (I30) and 120 (I120) min of simulated small intestinal digestion (Tamura *et al*., 2017). The cubes were immediately put into a test tube and immersed in liquid nitrogen and freeze dried. The freeze-dried bread cubes were then sent for SEM (see section 3.2.7).

3.2.8 Statistic analysis

The reported data are averages of at least three measurements. All measurements were performed in triplicates. Minitab version 17.3.1 Statistical Software (Minitab Inc., State College, PA) was used for statistical analysis. The data was subjected to analysis of variance (ANOVA) and Tukey's test to 5% significance level was used for comparison. Standard deviation (SD) was shown in figures as error bars and was also shown in the tables. All graphical presentations were generated by Microsoft Excel.

Chapter 4. Results and discussion

4.1 Physico-chemical characterisation of HPCSB

Physicochemical properties of HPCSB i.e. appearance, colour, proximate composition, total starch content and specific volume was determined in comparison to control.

4.1.1 Colour characteristics of HPCSB

Colour and appearance are important parameters when it comes to product development. Ma *et al*. (2014) also stated in a survey that citizens were more focused on colour than other properties of steamed bread. This is because colour can provide an intuitive feeling to consumers, thus the variation of colour is also treated as a significant physical indicator for steamed bread quality (Luo, Liang, Xu, Kou, *et al*., 2017).

The physical appearance of the bread made from four different HPCSB formulations and the control sample are presented in **Figure 4.1.** The colour of HPCSB varied in accordance with different protein substitution compared to control. **Table 4.1. (A)** denotes the crust colour of HPCSB and control while **Table 4.1. (B)** denotes the crumb colour of HPCSB and control.

As observed in **Table 4.1 A.** Control bread was significantly lighter ($p<0.05$) as compared to other formulations. RC showed the highest L^* value and the lowest white index (WI). The higher the rennet casein in the bread, the darker the bread became. The L^* values of crust and crumb of HPCSB were reduced from 81.55 ± 0.76 (control) to 79.01 ± 0.50 (RC II) and white index from 74.58 ± 0.39 (control) to 70.80 ± 0.49 (RC II). The colour characteristic such as L^* value and white index of crumb also reduced (from 77.24 ± 0.77 for control to 73.54 ± 0.58 for RC II and from71.29 ± 0.72 (control) to 67.72 ± 0.51 (RC II). There was no significant difference between crust colour of RC I and RC II. whereas the a^* values and b^* values were increased compared to the control, respectively. Dose-dependent lower L^* values and white index (WI) in RCI and RCII indicated that rennet casein incorporation darkened the appearance of CSB. The a^* values for the control as well as samples containing rennet casein at different levels stayed closed to a light green colour (slightly negative).

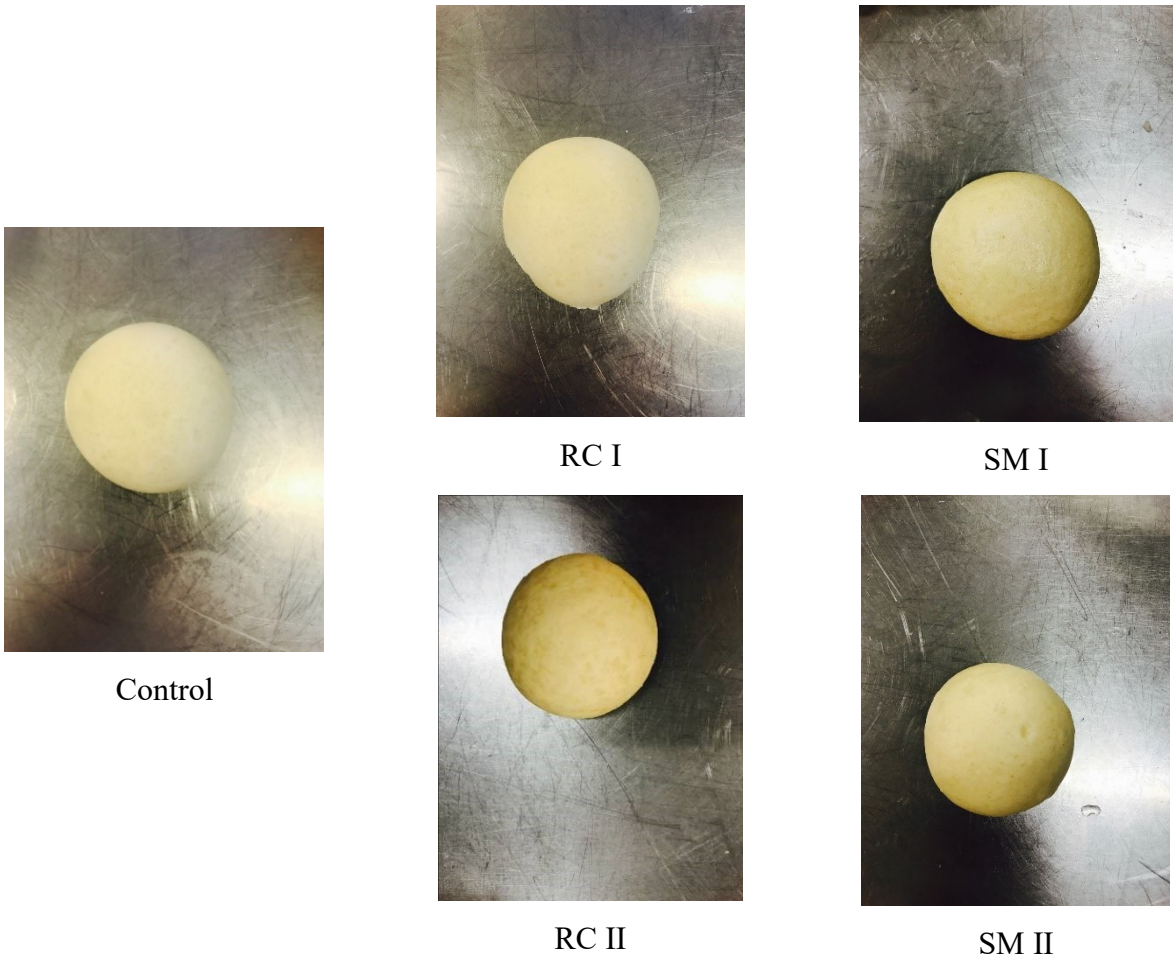

Figure 4.1 Physical appearance of HPCSB (RC I, RC II, SM I and SM II). Control: pure wheat Chinese steamed bread; RC I: rennet casein%:14%(w/w); RC II: rennet casein%: 33% (w/w); SM I: SPI%:7%(w/w), MPC%:5% (w/w); SM I: SPI%:7% (w/w), MPC%:8% (w/w).

Table 4.1 Colour parameters of HPCSB made from different formulations. A) Crust colour of HPCSB made from different formulations. B) Crumb colour of HPCSB made from different formulations.

Table 4.1 (A). Crust colour of HPCSB-control

Crust	*L**	*a**	*b**	White Index
Control	81.55±0.76^{a}	-1.86±0.06^{d}	17.36±0.62^{c}	74.58±0.39^{a}
RC I	79.08±0.33^{b}	-1.77±0.08^{c}	17.71±0.49^{c}	72.53±0.41^{b}
RC II	79.01±0.50^{b}	-1.42±0.10^{b}	20.24±0.57^{a}	70.80±0.49^{d}
SM I	78.20±0.55^{c}	-1.41±0.08^{b}	18.55±0.66^{b}	71.33±0.37^{c}
SM II	78.89±0.78^{b}	-1.33±0.06^{a}	20.08±0.49^{a}	70.82±0.40^{d}

Table 4.1 (B). Crumb colour of HPCSB-control

Crumb	*L**	*a**	*b**	White Index
Control	77.24±0.77^{a}	-1.68±0.05^{c}	17.41±0.35^{c}	71.29±0.72^{a}
RC I	74.80±0.82^{b}	-1.63±0.12^{bc}	18.09±0.27^{b}	68.93±0.67^{b}
RC II	73.54±0.58^{c}	-1.58±0.08^{b}	18.41±0.32^{b}	67.72±0.51^{c}
SM I	72.80±0.72^{d}	-1.11±0.12^{a}	18.13±0.61^{b}	67.28±0.50^{c}
SM II	73.41±0.90^{cd}	-1.05±0.10^{a}	18.88±0.45^{a}	67.36±0.73^{c}

a-d, means in each column with the same superscripted letter are not significantly different (p<0.05). Results are demonstrated as average (n=3) ± SD

An increase in *b** values was also observed. Bread with a higher level of rennet casein was consequently more green and yellow than the control. These changes were attributed to the presence of the colour of rennet casein which is creamy yellow (Morr, 1984). Su (2005) reported that for CSB the effect of Maillard reaction on the colour of CSB was negligible as the steaming temperature is approximate 100℃.

For SPI & MPC (SM) samples, with the increase in protein level, the *L** values (78.20) and white index (71.33) of the crust decreased compared to the control. A similar trend was observed for crumb colour characteristics. However, there was no significant difference in white index values, *L**values and *b** values among SM samples, which may be attributed to the fact that SPI levels in both SM samples were not much different. Du *et al*. (2016) reported similar results for extruded soy protein incorporated into steamed bread where the white index decreased and *a** showed a positive trend.

4.1.2 Proximate composition and specific volume

Proximate composition including total starch content and specific volume of HPCSB and control and control are presented in **Table 4.2**.

In comparison to control, moisture content for all the formulations was reportedly higher. RC II (42.90 ± 0.54%) and SM II (42.79 ± 0.8%) showed the highest moisture content whereas control (39.13±0.46%) was the lowest. This might be attributed to the hydration properties of proteins which increase the hydration properties of the dough during processing. Another possibility might be that, during steaming, the protein might have absorbed more moisture than the control sample (Kenny, Wehrle, Stanton, & Arendt, 2000).

As expected, the protein content of HPCSB increased significantly from 8%±0.03 (control) to 13 ± 0.11% (RC I) and 19 ± 0.13% (RC II) and to 13 ±0.14% for SM I and 15±0.11% for SM II. With the increase of protein content, subsequently the starch content decreased followed by control (67.59 ± 1.58%), RC I (58.73 ± 2.03%), RC II (48.85 ± 4.98%) and SM I (54.82 ± 0.10%), SM II (52.25 ± 0.67%).

Table 4.2 The composition of protein, moisture and total starch content of steamed bread made with different HPCSB formulations in comparison to control, specific volume of HPCSB is also included for comparison

	Moisture (%)	Protein (%)	Total starch (%)	Specific volume (ml/g)
Control	39.13±0.46^{c}	8±0.03^{c}	67.59±1.58^{a}	2.22±0.06^{a}
RC I	41.73±0.25^{ab}	13±0.11^{c}	58.73±2.03^{b}	1.59±1.59^{c}
RC II	42.90±0.54^{a}	19±0.13^{a}	48.85±4.98^{c}	1.39±1.39^{d}
SM I	41.05±0.17^{b}	13±0.14^{c}	54.82±0.10^{bc}	1.96±1.96^{b}
SM II	42.79±0.82^{a}	15±0.11^{b}	52.25±0.67^{bc}	1.89±1.89^{b}

a-d, means in each column with the same superscripted letter are not significantly different ($p<0.05$). Results are demonstrated as average (n=3) ± SD

On the other hand, in terms of specific volume, with the increase of protein content, the specific volume decreased. The control bread had the highest specific volume (2.22),

while RC II (1.39) obtained the lowest specific volume in RC samples. These results are in agreement with other studies (Patel, Patel, & Singh, 2016), who reported that the specific volume decreased progressively with the addition of rennet casein. Similarly, SM II (1.89) also had the lowest specific volume in SM samples. This might be attributed to the addition of proteins that make the structure of steamed bread denser, as also confirmed through measurements of texture analysis. Another explanation might be the addition of proteins might have caused disruption in the well-defined protein-starch complex of the dough due to a reduction in the wheat structure forming proteins and starch (Dhinda *et al*., 2012). Similar results were also observed by Kenny *et al*. (2000) and Dhinda *et al*. (2012) who reported that the addition of soy protein isolate and milk protein decreased the volume and increased the crumb firmness of bread.

4.2 Pasting properties- Rapid Visco Analyser (RVA)

4.2.1 RVA characteristics of HPCSB Formulation

RVA parameters such as pasting temperature, peak, final, breakdown, setback, holding strength viscosity and peak time of different HPCSB formulation were determined and are summarized in **Figure 4.2** and **Table 4.3**. As shown in **Figure 4.2**, significant differences were observed among all the formulations when compared control (p<0.05). The incorporation with RC and SM decreased the pasting curve after gelatinisation as shown in **Fig 1**. As the protein percentage increased, the peak, final, breakdown, setback, and holding strength viscosities decreased, whereas the pasting temperature increased with increasing protein content. In all cases, the extent of change in pasting properties effect was higher with a higher protein content of the HPCSB ingredients. Similar trends were reported by Gökşen and Ekiz (2016).

4.2.1.1 Pasting temperature

Pasting temperature is the point where wheat starch granules start to absorb water and swell when suspending in water (Ledezma, 2018).

Generally, the addition of proteins increases the pasting temperature. Compared to the control, RC I had a higher pasting temperature of 68.80 ± 0.43 ℃ while RC II had the

highest pasting temperature of 86.97 ± 0.89 °C (**Table 4.3**). This finding corroborates with previous studies which reported that the addition of sodium caseinate increased the pasting temperature of normal rice starch (Noisuwan, Bronlund, Wilkinson, & Hemar, 2008). A possible explanation for this might be casein is capable of forming micelles due to its self-association properties, and it might absorb onto the surface of starch granules and strengthen the granules, hence restricting starch swelling thus affecting the pasting temperature (Noisuwan, Hemar, Bronlund, Wilkinson, & Williams, 2007).

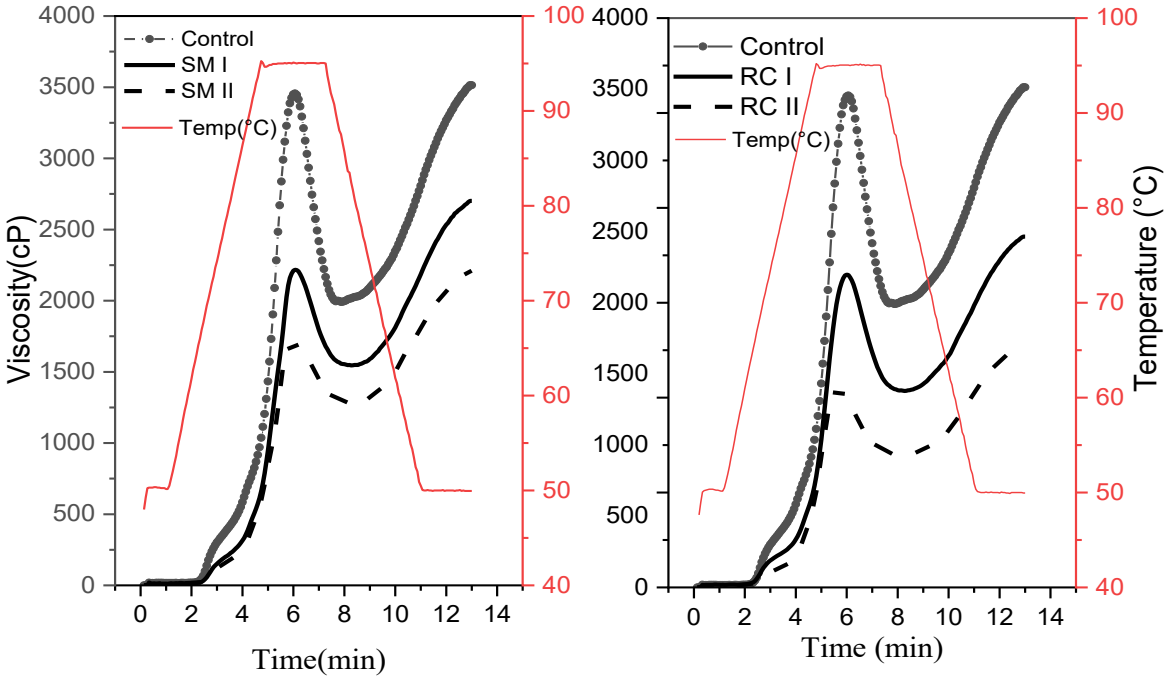

Figure 4.2 Pasting curves for HPCSB ingredients.

As shown in **Table 4.3,** the addition of SM (SPI + MPC) also led to an increase in pasting temperature, with a more prominent effect at higher protein levels, ranging from 68.52 ± 0.10 °C to 70.97 ± 0.08 °C (SM I) (p>0.05) and 83.20 ± 8.40 °C (SM II) (p<0.05). These findings are consistent with those of (Sai Manohar, Urmila Devi, Bhattacharya, & Venkateswara Rao, 2011), who suggested that the addition of a mixture of SPI and skim milk protein increases the pasting temperature of coarse wheat grit. The possibility for the increased pasting temperature of SM may be attributed to an increase in the total solid contents due to the addition of soy proteins. (Lim & Narsimhan, 2006).

Kumar, Brennan, Zheng, and Brennan (2018) and Noisuwan *et al*. (2008) also reported that the whey protein concentrate is more resistant to swelling in an oat starch and rice starch system. Also, the addition of SM might lead to a minimizing in the availability of water for starch leading to an increased pasting temperature (Kaur, Singh, & Singh, 2005). These results also support the DSC finding (**Table 4.5**)

However, Ribotta, Ribotta, Colombo, Leon, and Anon (2007) indicated that the addition of soy protein increased the pasting temperature of wheat starch. The possible reason for this might be the ratio (50%) of soy protein to wheat starch was much higher than the current study (7%), leading to the self-aggregation of soy globulins in soy protein leading to increased pasting temperature..

4.2.1.2 Peak viscosity

Peak viscosity is associated with the point where all the starch granules are swollen during heating. It is indicative of the water binding viscosity of the mixture and it corresponds to the degree of swelling of starch granules (Kaur, Singh, McCarthy, & Singh, 2007). It also attributes to the diastatic activity and polymer leaching. In this study, it also relates to the amount of amylopectin, within the total starch and protein-starch interaction (Kumar *et al*., 2018). The peak viscosity for the control wheat flour was 3452 ± 120.86 cP, which is in line with previous study (Caramanico *et al*., 2017; Ktenioudaki, O'Shea, & Gallagher, 2013). As shown in **Table 4.3**, the higher the protein content, the lower the peak viscosity. This possibly indicates that starch contributes majorly to network formation at a low level of protein components and therefore no phase transition was detected (Kumar *et al*., 2018).

For all protein formulations, the peak viscosity reduced significantly, which might be attributed to the lowering of total starch content (Bravo Núñez & Gómez, 2019). Proteins can also aggregate and act as a filler and bind to water to form a gel, leading to the availability of less water for starch gelatinisation leading to a lower viscosity whereas pure starch can form a more viscous gel (Noisuwan *et al*., 2008). Consequently, low peak viscosity demonstrates a decreased swelling and rapturing of starch granules (Ktenioudaki *et al*., 2013). According to Ribotta *et al*. (2007), protein molecules can influence the starch gelatinisation process in many ways owing to their different

properties of retaining water and their capabilities of interacting with starch surface granules and starch molecules.

In terms of RC formulations (RC I and RC II), as expected, peak viscosity decreased dramatically from 3452 ± 120.86 cP (control) to 2193 ± 9.54 cP (RC I) and 1419 ± 27.62 cP (RC II). It is hypothesised that caseins, especially β-casein aggregates around starch (due to their micelle forming capacity) have led to a decrease in starch granule swelling, which did not allow amylose to leach out, and thus the resulted in a decreased peak viscosity. (Kenny *et al*., 2000; L. Kumar, Brennan, Mason, Zheng, & Brennan, 2017). Similarly, as for SM based formulations (SM I and SM II), the peak viscosity also dropped dramatically to 2215 ± 72.63 cP (SM I) and 1774 ± 123.72 cP (SM II), compared to control wheat flour sample. This finding is in agreement with Sai Manohar *et al*. (2011), who reported that the combination of SPI and skim milk protein have decreased peak and final viscosity, setback and breakdown viscosity of wheat porridge. Milk protein concentrate (MPC) contains whey protein and casein. The addition of whey protein has been reported to decreases peak viscosity, which may be attributed to the interaction of leached out amylose and amylopectin with whey protein to form non-covalent bonding and also lowering of starch content of the formulation. Furthermore, whey protein has poor water holding capacity than wheat protein (Sarabhai & Prabhasankar, 2015), which might be another explanation for its lower peak viscosity.

On the other hand, the peak viscosity of SM I (2215 ± 72.63 cP) was higher than RC I (2193 ± 9.54 cP) although the protein content for these two samples is the same (13%). The possibility for this might be that the portion of SPI (7%, w/w) is higher than MPC (5%, w/w) in SM I. According to Ribotta *et al*. (2007), proteins contain various hydrophilic groups (-NH_2, -SH -COOH, -OH) which can form crosslinks with starch, thus resulting in an increased paste viscosity. However, for SM II, the peak viscosity decreased when compared to SM I and control sample. The decrease in peak viscosity of the sample containing additional proteins may be attribute to the decrease in starch content of the composition of the samples. The SM I samples have displayed higher viscosity than SM II samples due to its higher starch content in the formulations (Singh et al, 2006).

Table 4.3 Pasting characteristics of HPCSB formulations compared to control

Sample	Peak viscosity (cP)	Holding strength (cP)	Break down (cP)	Final viscosity (cP)	Setback viscosity (cP)	Peak Time (min)	Pasting Temp (°C)
Control	3452±120.86^{a}	1991.33±65.01^{a}	1460.67±82.81^{a}	3514.33±61.34^{a}	1523.00±31.19^{a}	6.07±0.07^{a}	68.52±0.10^{b}
RC I	2193±9.54^{b}	1381.33±10.50^{b}	811.67±11.06^{b}	2464.67±25.17^{c}	1083.33±20.98^{b}	6.00±0.07^{a}	68.80±0.43^{b}
RC II	1419±27.62^{d}	933.33±31.09^{c}	485.67±16.07^{d}	1704.67±53.46^{d}	771.33±24.11^{b}	5.62±0.14^{a}	86.97±0.89^{a}
SM I	2215±72.63^{b}	1546.33±39.46^{b}	668.67±41.86^{c}	2701.33±75.80^{b}	1155.00±39.34^{ab}	6.07±0.00^{a}	70.97±0.08^{b}
SM II	1774±123.72^{c}	1354.67±82.34^{b}	419.33±44.81^{d}	2307.67±152.58^{c}	953.00±70.55^{b}	6.02±0.08^{a}	83.20±8.40^{a}

a-d, mean values in the each column with the same superscripted letter are not significantly different (p<0.05). Results are demonstrated as average (n=3) ± SD Control: 100% wheat flour; RC I: Replaced with rennet casein (14% w/w); RC II: Replaced with rennet casein (33% w/w); SM I: Replaced with SPI (7% w/w) + MPC (5% w/w); SM II: Replaced with SPI (7% w/w) + MPC (8% w/w).

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4.2.1.3 Final viscosity

The final viscosity is the parameter most commonly used to determine the ability of the starch in starch-based products to gel after cooking and cooling as well as to undergo the retrogradation of starch molecules (Sasaki, Yasui, Matsuki, & Satake, 2002).

As shown in **Table 4.3**, compared to control, final viscosity decreased with increasing levels of the protein content from 3514.33 ± 61.34cP (wheat flour) to 1704.67 ± 53.46cP (RC II) and 2307.67 ± 152.58cP (SM II). Similar results were observed from other studies (Sarabhai & Prabhasankar, 2015; Yang, Irudayaraj, Otgonchimeg, & Walsh, 2004), which showed that the increasing content of whey protein decreased the final viscosity of starch and dairy ingredient-based food systems.

They further reported that for a gel containing whey protein and starch, the whey protein fraction incapacitated and diluted the gel network and acted as an inactive barrier during the process of amylose reordering by hindering H bonding between starch molecules. The drop in final viscosity of HPCSB formulations could also be ascribed to the reduced recrystallisation of amylose molecules during cooling as the starch content decreased along with the increase of protein content. During cooling process when the macromolecular complex was formed, a disturbance such as secondary forces, physical interference and steric hindrance might occur (Ktenioudaki *et al*., 2013; Yang *et al*., 2004). Moreover, the increased portion of lactose and lipid in the whey protein may also delay starch swelling and gelatinization. (Kim & Walker, 1992)

4.2.1.4 Holding strength and breakdown viscosity

Holding strength and breakdown viscosity are two fundamental factors which related to the ability to withstand heating and shear stress during the holding period at a high temperature which results in the breakdown of starch granules with amylose leaching and realignment (Wang, Li, Copeland, Niu, & Wang, 2015). As shown in **Table 4.3**, the holding strength of HPCSB formulations decreased with the increase of protein content compared to the control sample. Control showed the highest holding strength (1991.33 ± 65.01 cP) whereas RC II exhibited the lowest (933.33 ± 31.09 cP). The obtained trend

was in order of 1991.33 ± 65.01 cP (control) > 1381.11 ± 10.50 cP (RC I) > 933.33 ± 31.09 cP (RC II); and control > 1546.33 ± 39.46 cP (SM I), 1354.67 ± 82.34 cP (SM II).

In terms of breakdown viscosity, the trend obtained for HPCSB formulations followed the same pattern as that of holding strength viscosity (**Table 4.3**). Breakdown viscosity obtained was in the order of the control (1460.87 ± 82.81 cP), RC I (811.67 ± 11.06 cP), RC II (485.67 ± 16.07 cP); and SM I (668.67 ± 41.86 cP) and SM II (419.33 ± 44.81 cP). Breakdown viscosity can be correlated with the degree of swelling of the starch granules (Kumar *et al*., 2018). The high degree of swelling causes the starch to rapidly reach the peak viscosity. When the forces within the molecules become weaker, which consequently causes them to be sensitive to high temperatures (Ktenioudaki *et al*., 2013), This in line with the findings of increased pasting temperature obtained in the current study (**Table 4.3**). Also, the breakdown viscosity can be associated with the rigidity of swollen starch granules (Rani & Bhattacharya, 1995). Therefore, breakdown viscosity can be related to the extent of disruption of starch components in the presence of proteins. Interestingly, SM II showed the lowest breakdown viscosity among the five samples. The reason for it could be SM II contains whey protein, which attributed to a plasticity response that prevented the starch granule from swelling maintaining gelation even during the cooling cycle (Carvalho, Onwulata, & Tomasula, 2007).

4.2.1.5 Setback viscosity

The setback viscosity depicts the amylose content and reordering of starch paste after gelatinisation and the immensity of peak viscosity (Carvalho *et al*., 2007). It may also be related to the hardness of bread texture (Lei, Tian, Sun, & Chun, 2008). The setback viscosity for different HPCSB formulations decreased along with the increment of protein content compared to control in the order of wheat flour > (1523 ± 31.19 cP) > RC I (1083 ± 20.98 cP) > RC II (771 ± 24.11 cP) and control > SM I (1155 ± 39.34 cP) > SM II (953 ± 70.55 cP). It might be attributed to a low rate of starch retrogradation and syneresis. Therefore, RC II obtained the most stable forming paste among all the samples because it contained the highest protein among all the samples.

Similar results for pasting, peak, final, setback and breakdown viscosities have also been observed previously. (Gökşen & Ekiz, 2016; Jia, Huang, Abdel-Samie, Huang, & Huang, 2011; Ktenioudaki *et al*., 2013; Lei *et al*., 2008).

4.2.1.6 Peak time

Peak time refers to the speed at which viscosity increase, indicating the final product quality (Ragaee & Abdel-Aal, 2006; Yadav, Yadav, Kumari, & Khatkar, 2014). The variations of peak time among five samples were statistically insignificant at $p>0.05$, which points to the fact that the dough formed among all the formulations including the control were cooked within the steaming time. In conclusion, the viscosity decreased markedly by substitution of starch with dairy and plant proteins. Also, adding proteins to wheat flour made the starch granules requires higher energy to swell and gelatinize, leading to an increase in the paste stability and minimum temperature control.

4.2.2 Pasting properties of freeze-dried HPCSB.

The pasting properties of freeze-dried HPCSB are shown in **Figure 4.3** and **Table 4.4** Compared to raw material formulations, the pasting profiles of freeze-dried HPCSB were somewhat smooth flat-curves without showing a sharp decrease in viscosities. As shown in **Table 4.4** the pasting property values were lower than that of raw formulations. Moreover, pasting temperatures of freeze-dried samples were not detected, indicating that the starch in freeze-dried bread samples had almost lost crystalline order after 15 min of steaming. RVA properties, all the properties such as peak viscosity, final viscosity, holding strength and setback viscosity were decreased in a dose-dependent manner. Compared to control, pasting parameters showed the following order: control > RC I > RC II; control > SM I > SM II (**Table 4.4**). RC II showed the lowest RVA properties which may be attributed to its lowest starch content as well as the highest protein content compared to other samples. Low peak and final viscosities indicate higher heat damage to starch granules due to the gelatinisation and plasticization of starch-protein structure (Shittu, Raji, & Sanni, 2007). The pasting temperature could not be detected except for control wheat flour (95°C).

Table 4.4 Pasting characteristics of freeze-dried HPCSB

Sample	Peak viscosity (cP)	Holding strength (cP)	Breakdown (cP)	Final viscosity (cP)	Setback viscosity (cP)	Peak Time (cP)	Pasting Temp (°C)
Control	624.67±20.79[a]	597.33±19.76[a]	27.33±4.04[a]	938.00±11.36[a]	340.67±9.07[a]	6.49±0.03[b]	95.28
RC I	240.67±1.15[b]	236.67±3.79[b]	4.00±2.65[b]	422.00±4.58[b]	185.33±2.08[bc]	6.27±0.24[b]	ND
RC II	114.67±13.32[c]	111.67±13.43[c]	3.00±3.00[b]	198.33±21.39[c]	86.67±8.62[d]	6.40±0.13[b]	ND
SM I	248.33±16.74[b]	241.00±15.57[b]	7.33±3.21[b]	447.00±21.70[b]	206.00±6.24[b]	6.89±0.08[a]	ND
SM II	184.67±18.58[d]	179.33±16.07[d]	5.33±3.21[b]	343.00±29.87[d]	163.67±13.80[c]	6.58±0.17[ab]	ND

[1] a-d, mean values in each column with the same superscripted letter are not significantly different ($p<0.05$). Results are demonstrated as average (n=3) ± SD

[2] ND: not detect

Becker, Hill, and Mitchell (2001) reported that the existence of amylose-lipid complexes inside the starch granules could hinder amylose leaching and form rigid structures that impede swelling, which in turn provided a decreased peak viscosity. They also indicated that the presence of added ingredients can noticeably vary the starches properties in water. Another reason for the decrease in the peak viscosity of freeze-dried samples might be the freeze-drying process. According to Krystyjan et al. (2017), damage of hydrogen bonds that stabilise the structure may result from the removal of water from the frozen starch through the freeze-drying process. Juszczak, Fortuna, Witczak, and Dymel (2004) also supported this reason as they suggested that freeze-dried native starch gel has a low viscosity of the system due to amylose retrogradation at cooling. Wheat starch is especially susceptible to this process since amylose dendrites formed during the retrogradation process are not easily hydrated. Therefore the structure of the continuous phase of the paste, formed by amylose, is more susceptible to shearing (Juszczak et al., 2004).

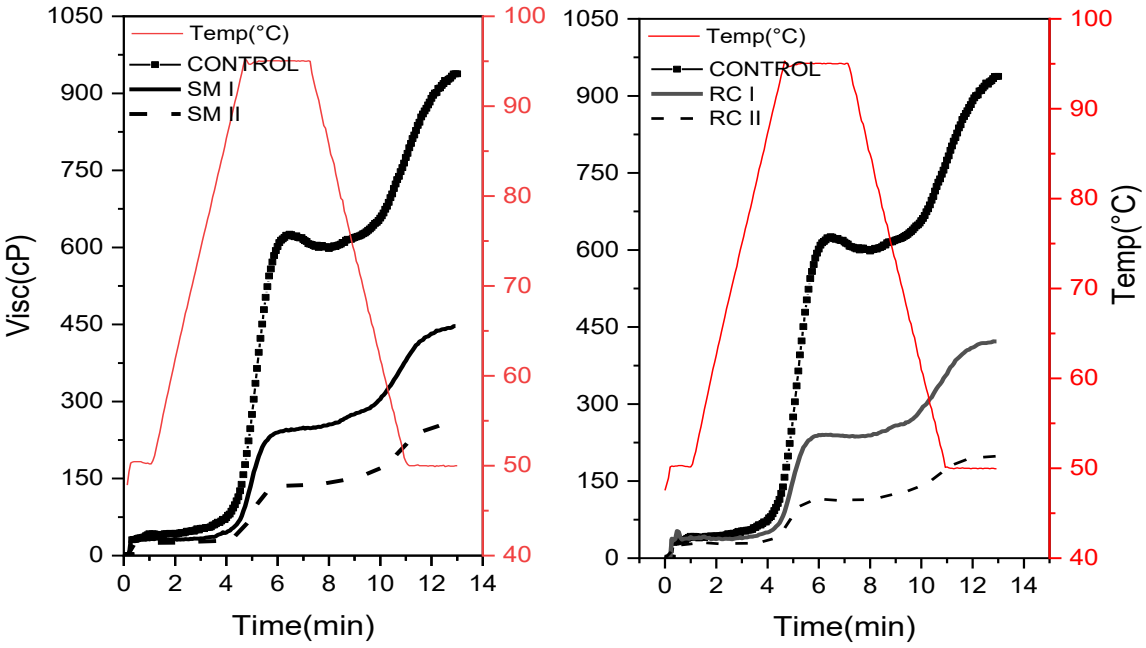

Figure 4.3. RVA profiles of freeze-dried HPCSB powder made from different formulation: (A) SM, (B) RC

However, Krupa, Rosell, and Sadowska (2010) demonstrated that the reason for the raw formulations and the freeze-dried sample showing a similar trend for viscosities is that the starch presented in the bread might not be fully gelatinised during the bread-baking process. The peak viscosity, final viscosity and setback viscosity were dramatically higher than found in the current study. Further, they reported the values for pasting temperature of freeze-dried bread could not be detected in the present study. These results could be corroborated through SEM images (**Figure 4.6**; **Figure 4.7**) and DSC values (**Table 4.5**) which show that for the present study, starch was fully gelatinised in freeze-dried bread samples. This might be attributed to the fact that the method of cooking for our study is less intense (i.e. steaming) and the cooking time varies.

4.3 Thermal properties-Differential scanning calorimetry (DSC)

DSC scans were performed to find out the effect of different protein addition on starch gelatinisation properties.

4.3.1 Thermal properties of HPCSB formulations

Thermal properties of HPCSB formulation from various protein sources are presented in **Table 4.5.**

Table 4.5 Thermal properties of HPCSB formulations

	Onset (°C)	Peak (°C)	Conclusion (°C)	Enthalpy (ΔH)	R (°C)
Wheat Flour	57.06±0.41^{b}	62.95±0.30^{ab}	76.61±2.37^{a}	6.81±0.71^{a}	19.55±2.67^{a}
RC I	56.65±0.55^{b}	62.17±0.37^{b}	77.95±2.03^{a}	6.43±1.37^{a}	21.30±2.55^{a}
RC II	57.71±0.11^{ab}	62.42±0.82^{ab}	76.73±1.51^{a}	3.97±0.59^{a}	19.02±1.62^{a}
SM I	57.62±0.35^{ab}	62.21±0.45^{ab}	80.63±0.94^{a}	6.44±0.34^{a}	23.01±1.19^{a}
SM II	58.48±0.80^{a}	63.63±0.69^{a}	79.45±2.47^{a}	5.15±1.25^{a}	20.96±2.97^{a}

a-b, mean values in each column with the same superscripted letter are not significantly different (p<0.05). Results are demonstrated as average (n=3) ± SD

4.3.1.1 Transition Temperatures

Onset temperature (To), peak temperature (Tp) and conclusion temperature (Tc) of control wheat flour calculated from the endothermic peak are given in **Table 4.5**. These values are in accordance with the values for wheat flour reported in the literature (Wang, Opassathavorn, & Zhu, 2015) with minor differences. The minor difference might be due to the different botanical sources which vary in their amylose and amylopectin ratio and attributed to fact that dissimilarity in milling processes (Singh, Singh, Kaur, Sodhi, & Gill, 2003; Zhu, Sakulnak, & Wang, 2016). A single endothermic transition was observed in **Figure 4.5**, which correlates with the dissociation of the amylose and amylopectin as well as the disruption of crystalline and double helical structures in starches (Singh, McCarthy, Singh, Moughan, & Kaur, 2007).

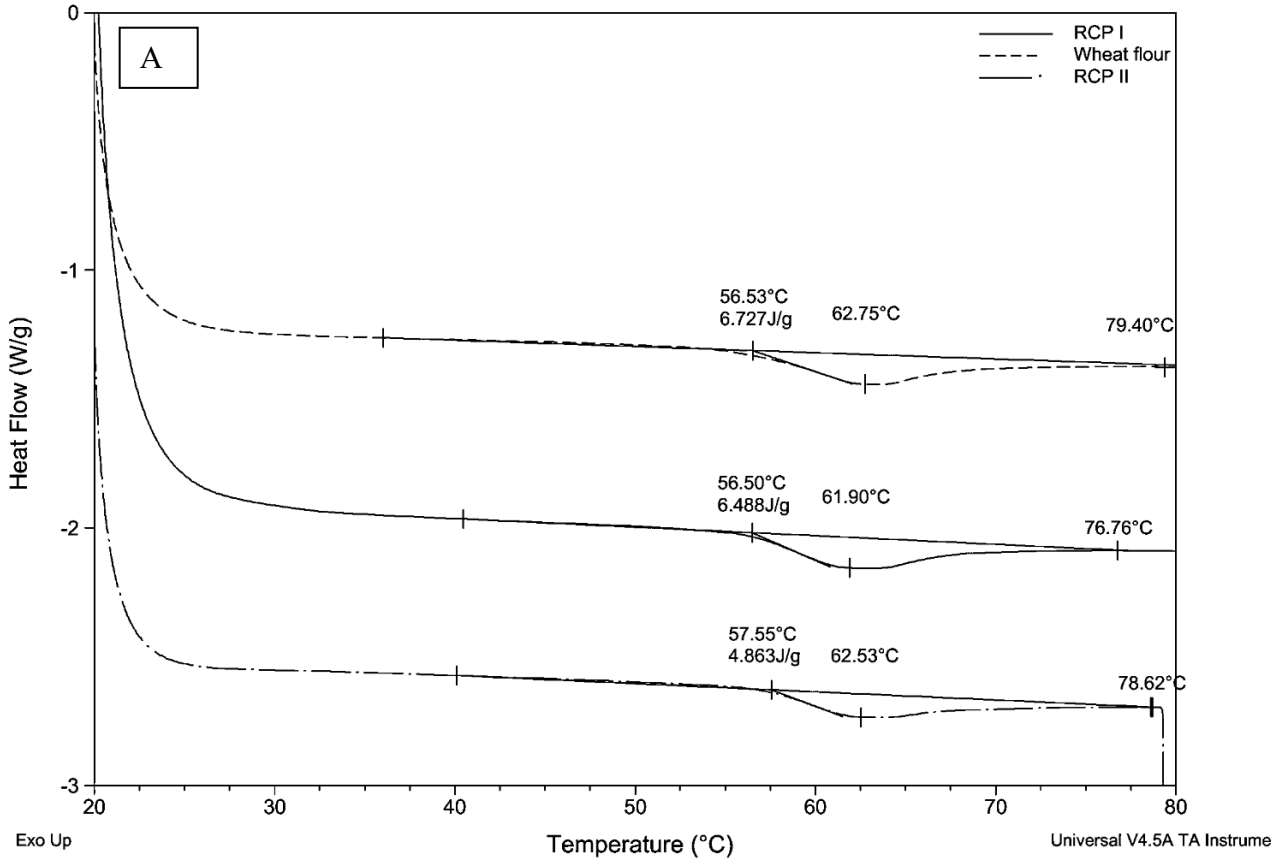

Figure 4.4 (A) Endothermic profile of RC formulations

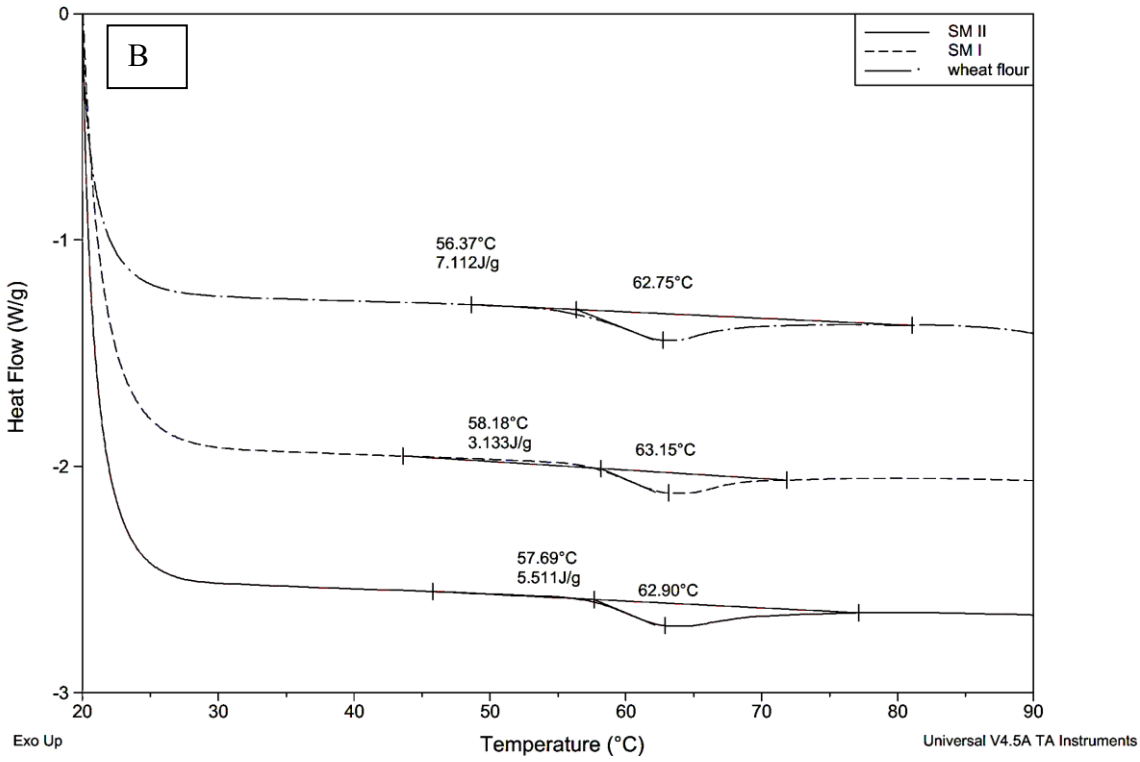

Figure 4.4 (B) Endothermic profile of SM formulations

Figure 4.4 Endothermic profile of HPCSB formulations. A, RC: rennet casein formulations; B, SM: SPI+MPC formulations

Among the five samples, not much differences were observed for the onset and peak transition temperatures whereas for samples containing added protein (except RC II) showed higher conclusion temperatures. Similar findings are reported in the literature (Bravo Núñez & Gómez, 2019; Noisuwan *et al*., 2008), who reported that minor changes were observed in transmission temperatures in the mixture of whey protein/milk protein and extruded maize flour (25%-50%, w/w), and the mixture of milk protein and rice starch (10%, w/w).

Yu, Liu, Li, and Jiang (2016) reported that all the DSC thermograms of mixture samples (soybean 11S globulin with nonwaxy maize starch) exhibited two peak temperatures in the range of (1) 60–80°C and (2) 80–110°C (II) when conducting the thermal analysis. However, this phenomenon did not appear in the current study (**Figure 4.5**). The potential reason for this might be the different protein types such as SPI which has a much tighter structure and higher crystallinity, which makes it more difficult to denature at given water ratio (Li, Wei, Fang, Zhang, & Zhang, 2014; P. Ribotta *et al*.,

2007; Ribotta *et al*., 2005; Yu, Jiang, & Kopparapu, 2015; Zhou, Liu, & Tang, 2018). The structure and molecule of SPI also resulted in differences in thermal characteristics. The thermograms obtained for our study presented an endotherm corresponding to the gelatinization of starch. The actual denaturation temperature for these proteins may be higher than the scanning temperature range (20-130 ℃) used in our study.

Onset and peak temperatures increased (p>0.05) with proteins addition. The increase in conclusion temperatures could be related to the interaction between the material leached out of the granules and protein and/or between surface granules and protein (Ribotta *et al*., 2007). This observation is similar to the study reported by (Li *et al*., 2014). They suggested that it might due to the migration of water from the starch to the protein which leads to lower water availability for starch gelatinisation.

Addition of SM influenced the gelatinisation transmission temperatures (To, Tp, Tc) to various extents. For example, the addition of SM at level II into wheat flour increased peak temperatures (Tp) to 63.63℃ whereas no change was detected at the level I of SM addition. The presence of SM at level II may have restricted the swelling of starch granule that caused a delay in gelatinisation to occur. Furthermore, SPI also absorbs more water compared to milk protein, thus resulting in less amount of water available for starch which may have delayed the gelatinisation resulting in higher transition temperature (Zayas, 1997).

Our results demonstrate that soy protein played a significant role in gelatinization of SM formulations (Yu *et al*., 2015). A plausible reason for this might be that when SM (SPI & MPC) addition was done at level II, more molecular interactions between starch and protein might have taken place expect to compete for water which may retard starch gelatinization thus, making starch difficult to gelatinize and increase the transition temperatures of starch. Similar results were reported for soybean protein isolates/maize starch mixtures (Colombo, León, & Ribotta, 2011; S. Li *et al*., 2014; Yu *et al*., 2015). The transition range R also differed among the five samples.

The difference among the thermal properties of samples containing RC and SM mix could be attributed to the high water-retention capacity and swelling characteristic of soy protein, which reduces the available water for starch and shifts the endotherm peaks to

higher temperatures (P. Ribotta *et al*., 2007; Zayas, 1997). Analysing the onset and peak temperatures in the endotherm, it has been observed that the SM samples shifted to higher temperatures with the increase in protein percentage when compared to control and rennet casein samples. However, the extent of increase was not statistically significant ($p<0.05$). These results suggest that the thermal properties of starch/ proteins mixture are influenced by the interaction among water, proteins and starch, and also from the molecular and structural properties of soy and milk protein gelatinisation temperature.

4.3.1.2 Enthalpy

The enthalpy (ΔH) is associated with the energy needed to dissociate amylopectin double helices. It reflects mainly the loss of crystallinity within the granule and the loss of the duplexes (double helix) order (Colussi *et al*., 2017). Enthalpy gives an overall measurement of crystallinity (both quantity and quality) and is an indicator of the loss of molecular order within the starch granule that occurs on gelatinisation (Singh, McCarthy, & Singh, 2006).

As shown in **Table 4.5,** the pure wheat flour had a ΔH for 6.81 J/g, which is consistent with previously published results (Wang *et al*., 2015). The pure wheat flour showed the highest enthalpy followed by SM I (6.43J/g), RC I (6.44J/g) the sample containing level II showed lower enthalpy values, which are SM II (5.15J/g) and RC II (3.97J/g). A similar thermal behaviour of rennet casein containing samples was reported by Noisuwan *et al*. (2008). The lower ΔH of HPCSB samples suggests a smaller proportion of starch compared to pure wheat flour, resulting in a lower percentage of organised structures (Colussi *et al*., 2017). Higher protein contents can retard starch from gelatinizing and decrease thermal enthalpy, due to the interaction between proteins and amorphous regions of starch granules, and also due to water competition between polymers, which makes it hard for the starch to gelatinize. (Bravo Núñez & Gómez, 2019; Kaur *et al*., 2005; Mohamed & Rayas Duarte, 2003).-In addition, starch gelatinization is a process of granule swelling and the expansion occurs in amorphous regions within the granule, affects the crystalline domains because of the stress applied to them. Consequently, a decrease in ΔH (i.e., the energy required to break the crystalline structure mainly) could be related to the interactions between proteins and amorphous regions of the granule (Colombo *et al*., 2011).

4.3.2 Thermal properties of fresh HPCSB

Thermal properties of fresh HPCSB are presented in **Table 4.6**.

Table 4.6 Thermal properties of fresh HPCSB

	Onset (°C)	Peak (°C)	Conclusion (°C)	Enthalpy (ΔH)
Control	41.51±0.99[a]	58.53±0.45[a]	ND	0.69±0.59[a]
RC I	43.05±0.53[a]	59.12±0.47[a]	ND	0.07±0.03[a]
RC II	39.08±2.27[a]	48.99±0.35[b]	ND	0.20±0.21[a]
SM I	40.54±1.40[a]	59.78±1.89[a]	ND	0.52±0.09[a]
SM II	39.10±1.31[a]	56.65±2.32[a]	ND	0.37±0.10[a]

[1] a-b, means in each column with the same superscripted letter are not significantly different ($p<0.05$). Results are demonstrated as average (n=3) ± SD

[2] ND: not detected

When steamed bread is reheated during DSC, an endothermic transition occurs which is attributed to the melting of recrystallized starch material recreated during retrogradation (Yu *et al*., 2016). The enthalpy of retrogradation is generally considered to correspond order-disorder transitions of crystallites, i.e. double helices present in extended order arrays and regions of lesser crystalline order. The retrogradation properties of starches are indirectly influenced by the structural arrangement of starch chains within the amorphous and crystalline regions of the un-gelatinized granule, which in turn, influence the extent of granule breakdown during gelatinization and the interactions that occur between the starch chains (Singh, Kaur, & McCarthy, 2007).

Fresh HPCSB samples showed dramatically lower onset, peak and conclusion temperatures (**Table 4.6**) than their raw flour-protein formulations (**Table 4.5**). This reduction is due to the fact that most starch granules were already gelatinised leading to the improper alignment of the amylopectin chains during re-association when samples cool down. This causes the formation of crystalline structures less stable and less organised compared with those of native starches. Consequently, less energy is demanded to melt the restructured crystals, and therefore, the enthalpy for retrogradation of starch is also lower than that of the raw formulations (Diamantino *et al*., 2019).

As expected, the control wheat bread has the highest To (51.51 ± 0.99 ℃) and Tp (58.53 ± 0.45 ℃)) while the RC II presented the lowest as and for To (39.08 ± 2.27 ℃) and Tp (48.99 ± 0.35 ℃) (p>0.05). Similar trends like raw formulations in thermal properties were also observed when protein content was increased.

4.4 Textural and microstructural characteristics HPCSB

Characterisations such as texture and microstructure were conducted in the current study.

4.4.1 Texture profile analysis (TPA)

The crumb structures of most bread are formed of holes of various sizes, shapes, and distributions. Each hole is embraced by a network of connects threads, coagulated gluten.

Table 4.7 demonstrates the texture parameters of HPCSB and control during double compression using TPA. The control sample showed the lowest hardness (15.55N), gumminess (10.97N) and chewiness (9.86N) whereas it possessed the highest springiness (0.90), cohesiveness (0.76) and resilience (0.38).

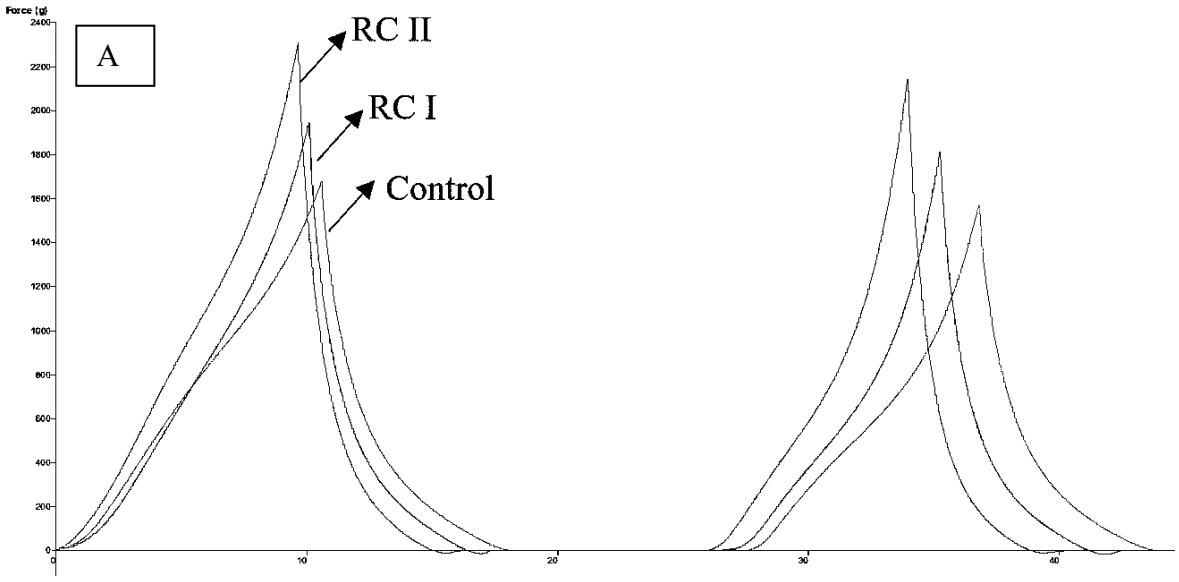

Figure. 4.5 (A) Texture profile analysis (TPA) curve of RC HPCSB

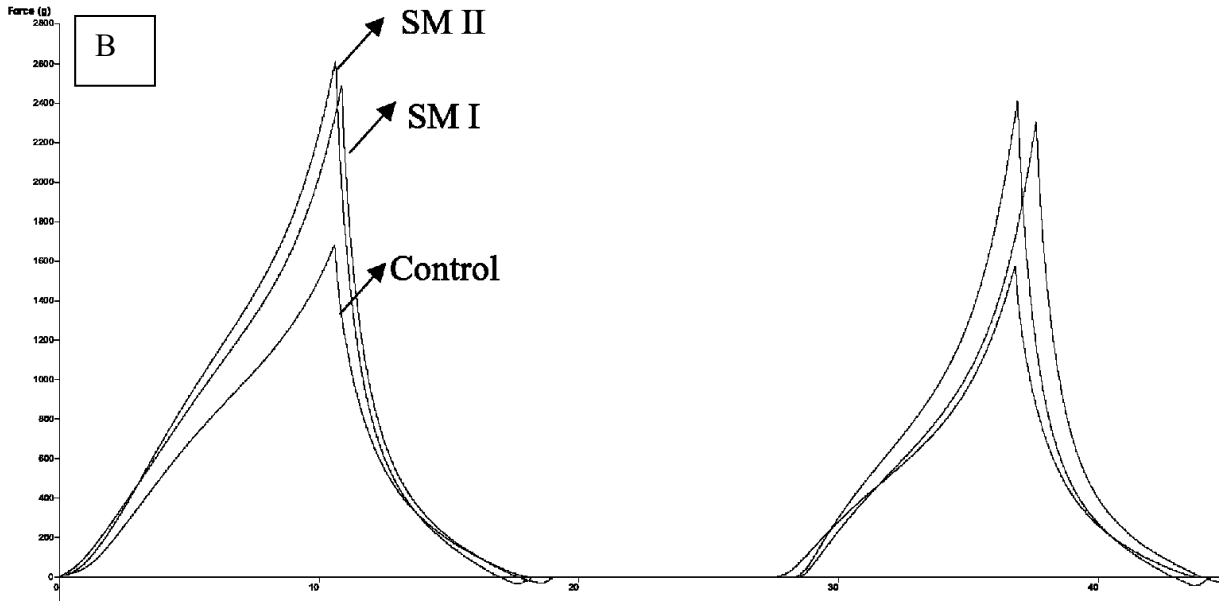

Figure. 4.5 (B) Texture profile analysis (TPA) curve of SM HPCSB

Figure. 4.5 Texture profile analysis (TPA) curve of HPCSB (A) Texture profile analysis (TPA) curve of RC HPCSB; (B) Texture profile analysis (TPA) curve of SM HPCSB.

The crumb deforms when it is compressed with fingers, and it springs back when the force is removed, this combination of a cellular crumb with the ability to recover after being a compressed is one of the distinguished properties of bread (Cauvain, 2012).

The addition of both RC and SM increased the hardness of bread, in the order of control (15.55N) < RC I (19.32N), RC II (22.25N); control < SM I (24.21N) < SM II (25.08N). A similar trend was observed for gumminess which is the energy required to disintegrate a sample to a state ready for swallowing (Stathopoulos & Kennedy, 2008), The chewiness also increased from 9.86N (control) to 14.29N (RC II), and to 14.32 N (SM II). These trends are in line with Zhou *et al*. (2018) who reported that the addition of whey and soy protein increased the hardness, gumminess and chewiness of the bread. Similarly, Ivanovski, Seetharaman, and Duizer (2012), Shin, Kim, and Kim (2013), Yang, Liu, Ashton, Gorczyca, and Kasapis (2013) reported that the addition of soy protein and whey protein increased the hardness, gumminess and chewiness of bread. Also, springiness, cohesiveness and resilience slightly decreased while adhesiveness increased slightly with the increase of protein content.

Table 4.7 Texture profile analysis (TPA) of HPCSB

Sample	Hardness (N)	Springiness (%)	Cohesiveness (%)	Gumminess	Chewiness	Resilience (%)	Adhesiveness (g· sec)
Control	15.55^{d}	0.90^{a}	0.76^{a}	10.97^{e}	9.86^{c}	0.38^{a}	0.00^{a}
RC I	19.32^{c}	0.89^{ab}	0.74^{b}	13.27^{d}	11.76^{b}	0.37^{a}	-0.15^{ab}
RC II	22.25^{b}	0.82^{c}	0.69^{d}	14.29^{c}	11.71^{b}	0.30^{b}	-0.09^{ab}
SM I	24.21^{a}	0.85^{abc}	0.72^{c}	16.16^{b}	13.79^{a}	0.31^{b}	-0.20^{ab}
SM II	25.08^{a}	0.85^{bc}	0.73^{bc}	16.92^{a}	14.32^{a}	0.31^{b}	-0.37^{b}

a-e, mean values in each column with the same superscripted letter are not significantly different ($p<0.05$). Results are demonstrated as average (n=3) ± SD

Dairy proteins especially casein contain strong water absorptive properties, which may lead to finer, denser crumb structures in the baked product (Stahel, 1983). A strong negative correlation was obtained in the current study between crumb hardness and loaf volume of HPCSB and control bead (-0.96). Thus, it may be attributed to the presence of gluten, which keeps the crumb softer in control bread as it slows the movement of water by forming an extensible protein network. Therefore, the absence of gluten in wheat flour will increase the movement of the water from bread crumb to the crust, resulting in a hardness increased crumb (Roach & Hoseney, 1995).

SM II obtained the highest texture parameters among the five samples, although its protein/ flour ratio was lower than RC II. Also, as mentioned the presence of casein in rennet casein and MPC also increased the hardness of CSB (O'Regan *et al*., 2009). Similar results were obtained from previous literature that the addition of casein and whey protein increased the hardness in bread (Erdogduarnoczky *et al*., 1996; Kenny, Wehrle, Auty, & Arendt, 2001; Kenny *et al*., 2000). The possible explanation for this might be due to dilution of the gluten matrix, interchange of disulphide bonds between gluten proteins and SPI, and increasing the dough viscosity as the absorption of water increased by SPI, this may lead to the low specific volume and increased density of soy protein (Du *et al*., 2016; Ribotta *et al*., 2005; Shin, Kim & Kim, 2013).

4.4.2 Tensile test

The effect of various protein incorporated at two levels on the tensile properties of CSB is shown in **Table 4.8.** Results showed that R value increased significantly with the increase in protein substitution from 2007 g (Control) to 2128 g (RC I) and 2431 g (RC II) and SM II obtained the highest resistance for 3086 g. Incorporation of both RC and SM significantly increased the extensibility and resistance to tear. The extensibility values for both RC and SM incorporated CSB (**Table 4.8**) increased to 2128.04 ±191.55 mm and 3218.96 ±98.87 mm for RC and SM, respectively at the level I and then decreased at level II (2431.45 ±91.18 mm for RC and 3086.67 ±119.40 mm for SM).

This decrease in extensibility might be partially attributed to the dilution of gluten due to increased water during dough making, leads to a sticky texture and difficult to tear thereby lowering its steaming performance (Shalini & Laxmi, 2007).

The highest R/E ratio was observed for control (-293), whereas it decreased significantly for both RC I (-172.21) and SM I (-255.27), both RC II and SM II showed highest R/E ratio and E values than other bread. The effect of SM affecting tensile properties of CSB was in agreement with (Tang & Liu, 2017) who reported that SPI products have a rubberier texture and hard to be torn.

Table 4.8 HPCSB properties of tensile analysis

	Resistance (g)	Extensibility (mm)	R/E
Control	2007.00±104.25^{c}	-6.85±1.13^{a}	-293.21±37.64^{c}
RC I	2128.04±191.55^{bc}	-12.36±0.29^{c}	-172.21±15.15^{a}
RC II	2431.45±91.18^{b}	-10.04±0.03^{b}	-242.25±10.83^{b}
SM I	3218.96±98.87^{a}	-12.61±0.02^{c}	-255.27±7.80^{bc}
SM II	3086.67±119.40^{a}	-11.36±0.57^{bc}	-271.71±9.77^{bc}

a-e, means in each column with the same superscripted letter are not significantly different (p<0.05). Results are demonstrated as average (n=3) ± SD

The tensile force which required to tear the sample was increased with the addition of protein but only up to level I for both RC and SM. This increase in SM samples was higher than those containing RC, this might be attributed to the properties of SPI, which

made the CSB rubberier and harder. Further, an increase in protein content to level II may have diluted the gluten content of HPCSB, which made HPCSB dough sticky and hard to sheet which lowered its processing performance.

Thus, control CSB was extensible and soft as demonstrated by its low resistance values, and also possessed less extension before rupture (extensibility). Control CSB showed extensibility of 6.85mm which increased to 12.36mm and 12.61mm when level I amount of RC and SM were added, respectively, whereas it was reduced again to 10.04 and 11.36 when the amount of RC and SM was increased to level II. Hence, the increase of R value of the bread enriched with protein might be due to the decrease of free water in dough or the rigid nature of protein hydration in HPCSB formulations (Morr, 1984; Nishinari *et al*., 2018; Zhou *et al*., 2018).

4.4.3 Penetration test

The penetration properties of HPCSB are shown in **Table 4.9**. No significant difference was found in crust hardness. The resistance of surface hardness ranged from 122.93g for control to a higher of 138.97g for RC II (**Table 4.9**). A similar trend was reported by Sołowiej *et al*. (2016) who indicated that the hardness of penetration values increased when testing a mixture of acid casein and modified waxy maize starch.

Table 4.9 Penetration analysis of HPCSB

	Resistance (g)	Extensibility (mm)
Control	122.93±2.80^{a}	11.94±0.82^{a}
RC I	134.21±15.39^{a}	8.91±0.55^{bc}
RC II	138.97±9.91^{a}	7.02±0.22^{c}
SM I	137.15±4.81^{a}	11.38±0.78^{a}
SM II	128.36±1.82^{a}	9.84±1.93^{ab}

a-c, mean values in each column with the same superscripted letter are not significantly different (p<0.05). Results are demonstrated as average (n=3) ± SD

The extensibility for RC I, RC II and SM II were observed to be lower than the control sample (**Table 4.9**) while there was no significant difference between SM samples (11.38, 9.84 for SM I and II respectively). This might be attributed to the addition of rennet casein, which leads to a weaker gluten network, resulting in less extensibility. On the other hand, the negative interaction between SPI and MPC might lessen the emended effect of protein towards starch, which maintained the extensibility of steamed bread (Augustin *et al*., 2011; Hui & Evranuz, 2012).

4.4.4 Microstructure characteristics-SEM of dough and HPCSB

Scanning electron microscopy (SEM) was used to visualise the cross-section of the HPCSB and to identify their structural features.

4.4.4.1 Microstructure of dough

Figure 4.6 shows the representative SEM micrographs of the control and HPCSB fermented dough. Generally, the spherical and lenticular shape of small and large starch granules contained in all samples of dough have been observed to be distributed throughout the gluten protein matrix. The starch granules were more organised and embedded in the gluten in the control samples. This observation is consistent with previously reported literature that the starch granules were almost embedded in the gluten network for wheat bread (Liu *et al*., 2018).

In our SEM study, the proteins influenced the dough structure when added at different levels. The protein appeared to be coating starch granules and lesser starch granules were observed. As for HPCSB dough, starch granules were embedded in both gluten and additional protein mixture network structure. The proteins were incorporated in the amorphous matrix along with embedded starch granules and a porous network was observed in the gluten structure in all dough samples.

For RC samples the protein seemed to wrap and adhere to starch granules tightly (arrows shown). This might restrict the increase in viscosity and hinders swelling of starch granules at high temperature, and accordingly retards the starch gelatinisation (X. Liu *et al*., 2018). Furthermore, because of the interaction between starch and protein, the ageing

of gelatinised starch could be delayed. Also, it can stabilise the starch granules thereby preventing the linkage of amylopectin chain during storage. (Liu, Mu, Sun, Zhang, & Chen, 2016; Liu *et al*., 2018; Sun, Zhang, Hu, Xing, & Zhuo, 2015; Xie, Dowell, & Sun, 2004). This observation is in line with the results obtained from RVA (**Table 4.3**) and DSC (**Table 4.5**).

The SEM showed that the presence of protein disrupted or interfered with the formation of gluten network (**Figure 4.6**), this observation was corroborated through RVA where the peak viscosity decreased for HPCSB sample (Keeratipibul *et al*., 2010; Liu *et al*., 2016; Sun *et al*., 2015). The discontinuity of the gluten matrix in HPCSB samples suggested that the extensibility and resistance of dough were interrupted, which could affect the HPCSB structure (Liu *et al*., 2016) and this was reflected in the TPA where an increase in hardness for samples was observed which might indicate a weaken damaged gluten network leading to a reduction in gas retention capability.

The SEM images of the control wheat dough exhibited a continuous gluten matrix wherein the starch granules were embedded. However, the starch granules were not fully covered in the gluten matrix and could be clearly distinguished in the images. While for HPCSB dough, the added protein behaved like a coating material, enwrapping starch granules, and due to the properties of the added proteins that are different from gluten. They disrupted the gluten matrix and formed an irregular network. Consequentially, this may decrease the capability of gas retention in the dough and thus result in a decrease in the bread volume (**Table 4.2**).

The discontinuous gluten matrix implied that the resistance and extensibility of dough were disturbed, which could further influence the textural properties of steamed bread (**Table 4.7**). (Li, Deng, Li, Liu, & Bian, 2015; Liu *et al*., 2016).

The addition of rennet casein made the dough structure denser, which is evident in the SEM image, where starch granule seemed to be embedded in the rennet casein-gluten protein matrix, due to the insolubility of rennet casein. Rennet casein lacks the capability of being solubilised in the dough matrix and remains as particles because as soon as gluten interacts with water, it forms a network, while insoluble rennet casein remains as separate entities dispersed in the system. (Huppertz, Fox, & Kelly, 2018b; Morr, 1984;

O'Kennedy, 2011). The addition of SPI and MPC also seemed to coat the starch granules very evenly and reduced it to a smaller pore size which makes the structure appear denser than RC dough. This may be attributed to the higher solubility of SM (MPC & SPI) than RC. The dough containing SM also showed the presence of filamentous structures possibly provided from SPI (Du *et al*., 2016; Liu *et al*., 2016; Zayas, 1997).

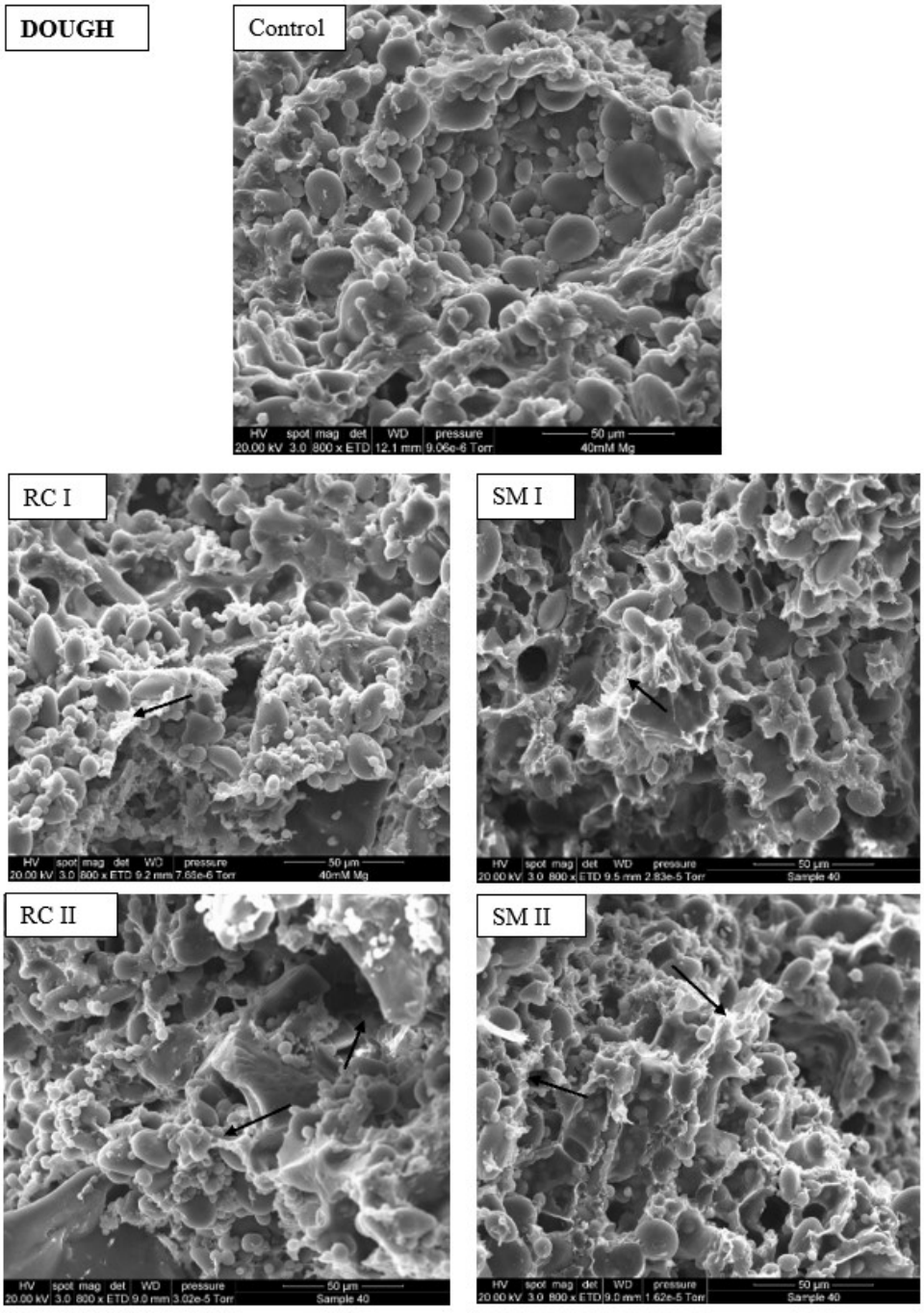

Figure 4.6 Microstructure of HPCSB dough

The structure of dough was observed to be more compact in the HPCSB dough with level II proteins indicating that higher level of protein incorporation led to a greater extent of network disruption compared to level I addition.

In conclusion, the dough made from control (pure wheat sample) had an extensible and continuous gluten network whereas the dough made from HPCSB formulations showed an inconsistent and interrupted protein matrix warping the starch granules. Further, the formation of an elastic network of cross-linked gluten molecules throughout steaming was also affected by the addition of proteins, causing the network to be disrupted easily and reduced the loaf volume of HPCSB (**Table 4.2**). In our study, the physicochemical properties of dough were influenced by the addition of different proteins (RC/SM), which might be due to the various functional and structural properties of proteins in RC and SM (Sun *et al*., 2015).

4.4.4.2 Microstructure of HPCSB

The microstructure of HPCSB with different proportions of protein in this study is shown in **Figure 4.7**. Control bread which is made of pure wheat flour, displayed a more uniform gluten matrix compared to the HPCSB. The absence of starch granules in the gluten matrix is attributed to gelatinization during steaming.

This observation is corroborated with previous results (Gao, Chen, Zhang, Bu, & Fan, 2016; Keeratipibul *et al*., 2010). Moreover, more air cells were observed in the control bread compared to HPCSB samples. HPCSB made of different proteins exhibited a discontinuous gluten network with a higher quantity of irregular cavities. Also, the gluten network structure of HPCSB appeared to be destroyed. This disruption was more evident for bread made with higher protein addition.

For HPCSB, the images appeared to display denatured protein wrapping around gelatinised starch granules. Clear differences were observed in the morphology of gas cells between control and HPCSB samples. Compared to RC, more discontinuous and more irregular protein structure was observed in the SM samples. A probable reason for this might be the high cooking temperature (100°C) and rapid heating during steaming, lead to protein-protein interaction between MPC and SPI in SM samples which might lead to the formation of aggregation which disrupt the continuous matrix (Li *et al*., 2015).

As shown in **Figure 4.8**, the air cells in control appeared to be hollower and deeper compared to HPCSB samples and the bread network depicted an even and regular air cells which provided a good structural character to retain the gas as demonstrated by the highest bread loaf volume (**Table 4.2**) and fine crumb air cell (**Figure 4.8**),The air cells for HPCSB appeared to decrease with increased addition of protein indicating that bread made with a higher level of protein addition was more tougher, compact and dense. Whereas for HPCSB samples, the air cells appeared irregular and shallow. Also, SM samples appeared to be more filled and denser. This might due to the solubility of SPI and MPC which able to fill the open space in the bread matrix (Augustin *et al*., 2011; Morr, 1984; Nishinari *et al*., 2018).

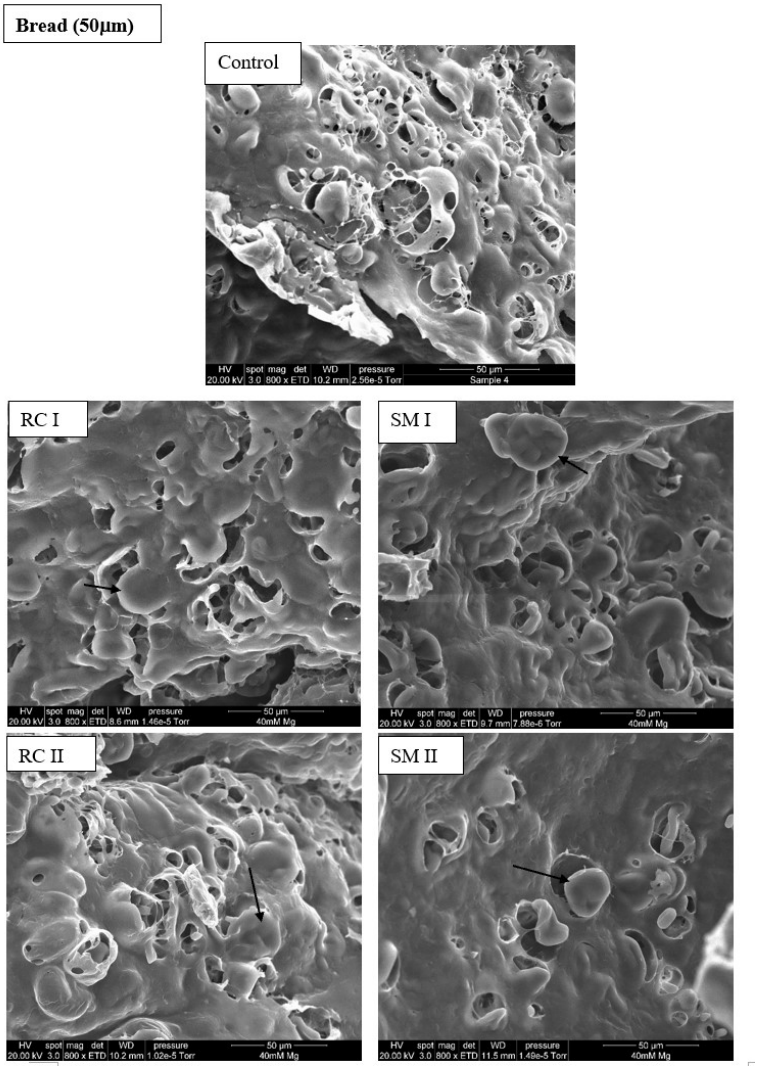

Figure 4.7 Microstructure of HPCSB (50µm)

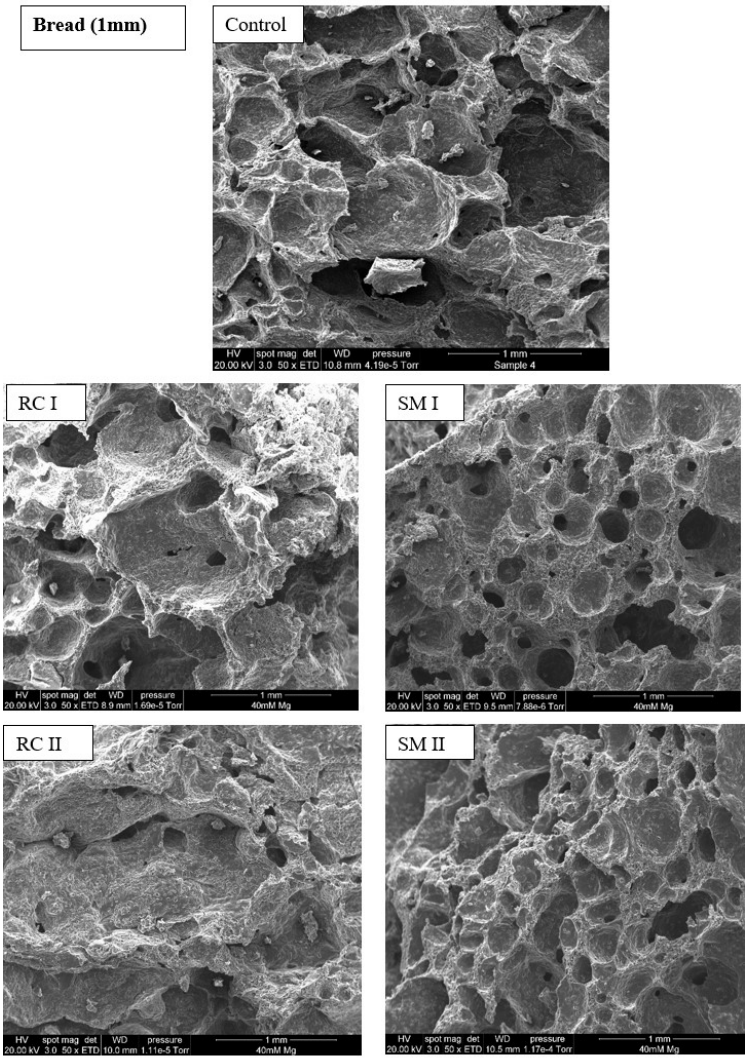

Figure 4.8 Microstructure of HPCSB(1mm)

4.5 Gastro-small intestinal digestion *in vitro* of HPCSB

After oral processing and during the simulated gastric digestion period (G0-G30), the starch hydrolysis percentage for all five samples was negligible (<5%), which may be attributed to the absence of the starch hydrolysing enzymes (Dartois, Singh, Kaur, & Singh, 2010). A very small percentage of starch hydrolysis may have occurred at low pH conditions for all five samples (Bordoloi *et al*., 2012). Another possibility of this small extent of hydrolysis might be due to the action of salivary α-amylases that may have infiltrated via the food bolus after mastication and is able to maintain its activity during the gastric stage (Bornhorst & Singh, 2013; Tamura *et al*., 2017). Generally, the simulated

intestine fluid (SIF) was added to the digestion reactor, after which the rate of starch hydrolysis of HPCSB progressively increased over the 120min period of digestion.

During small intestinal digestion, the starch hydrolysis of control sample increased till 10 min (I10) to about 40%, which was the highest among all the samples, after which the rate of starch hydrolysis decreased until the end of the digestion (I120) and ended at ~55%, which was the highest observed for all five samples.

The starch hydrolysis percentage increased significantly by the pancreatic amylase during this process, Hydrolysis (%) of HPCSB was dose-dependently delayed with protein incorporation (**Figure 4.9; Figure 4.10**). For RC sample, with the increase in protein concentration, the digestibility of HPCSB decreased dramatically during the first 5 min of small intestine digestion (I5), with ~27% and 21% for RC I and RC II, respectively, when compared to the control sample (~35%) (data not shown). On the other hand, as for SM samples, the apparent decrease in digestibility, was observed at I10 (37% for SM I and 36% for SM II) while a clear distinction between starch hydrolysis of SM I and SM II and control occurred at I15 (43% for control, 40.1% and 37.5% for SM I and II, respectively). The starch hydrolysis rate and extent of all the HPCSB samples during the rest of the digestion period decreased when compared to control, as observed from the slope of the curves (**Figure 4.10**).

As expected, the highest starch hydrolysis at the end of the small intestinal digestion (I120) was observed for the control wheat bread (52.5%), followed by RC I (48.7%) > RC II (44.6%) and control > SM I (49.8%) >SM II (47.9%).

RC and SM formulations significantly affected the final hydrolysis of the starch ($p<0.05$), where a 4% decrease was observed for RC I whereas 7% for RC II in starch digestibility compared to control sample. Similarly, a 3% decrease was observed for SM I whereas a 5% decrease was observed for SM II.

The rate and extent of starch hydrolysis in the small intestine are dependent upon several intrinsic and extrinsic factors (Singh, Kaur, & Singh, 2013). The addition of protein reduced the swelling of starch granules which results in a variation of the functional properties of starch, such as this digestibility.

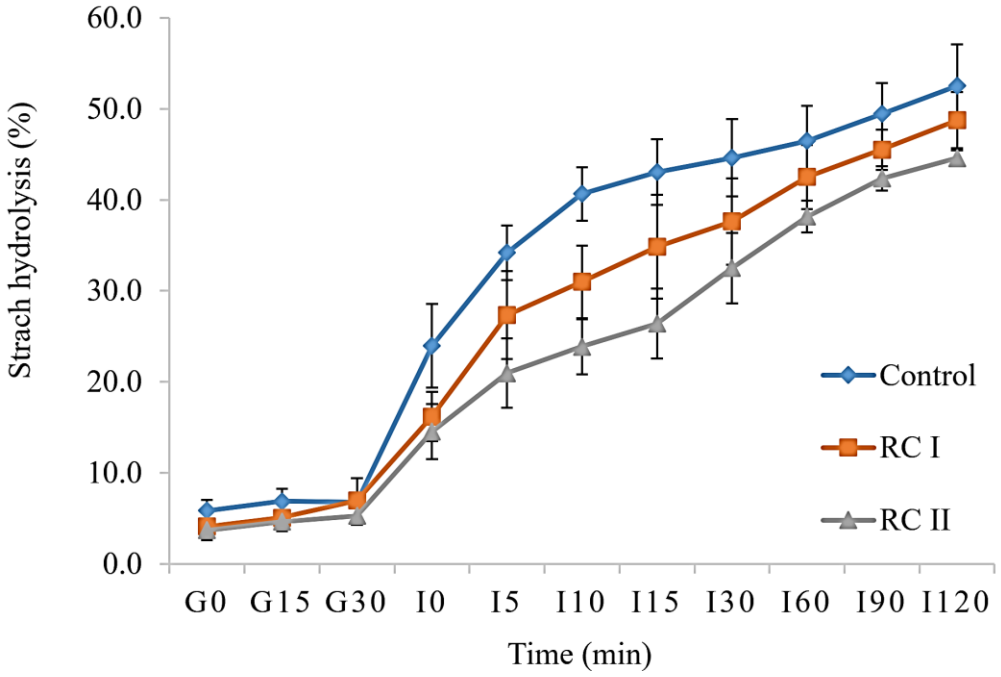

Figure 4.9 Starch hydrolysis during in vitro gastro-small intestinal digestion *in vitro* of control, RC I and RC II for 30 min at simulated gastric digestion (G0-G30) followed by 120min at simulated small intestinal digestion (I0-I120)

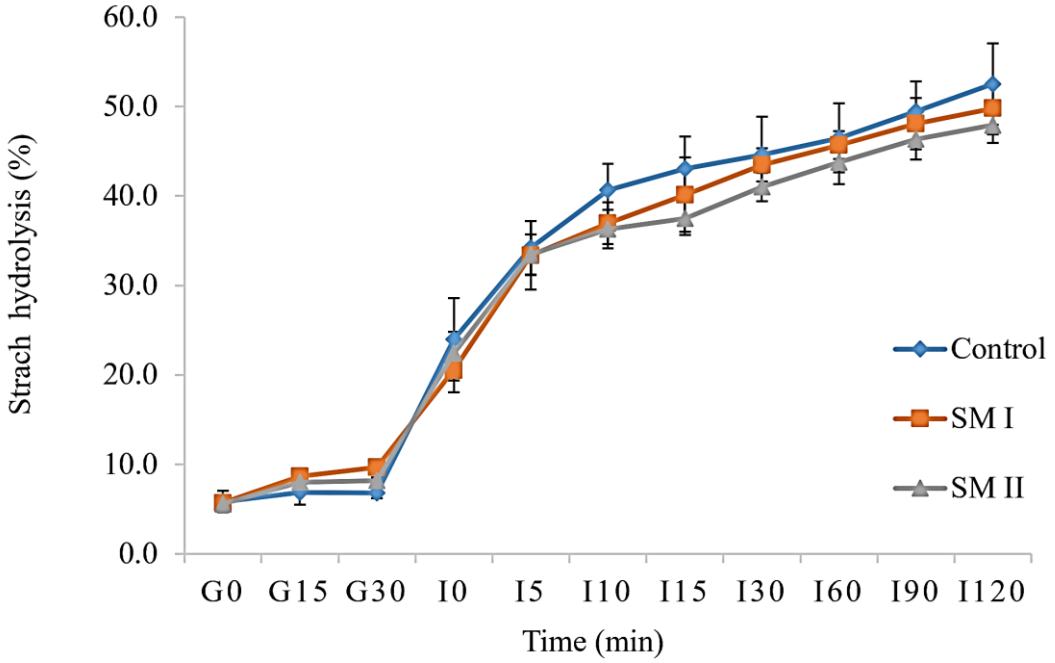

Figure 4.10 Starch hydrolysis during in vitro gastro-small intestinal digestion *in vitro* of control, SM I and SM II for 30 min at simulated gastric digestion (G0-G30) followed by 120min at simulated small intestinal digestion (I0-I120)

A possible explanation for these results may be that protein addition that limits the starch swelling during processing, results in lower starch hydrolysis (Eliasson, 2004). In our study, the protein network restricts the swelling of starch granules, hence indicating a structural explanation for the slow digestion of starch. Furthermore, the method of preparing the HPCSB could have an influence on the water availability for starch gelatinisation (Hera, Rosell, & Gomez, 2014). The influence became more noticeable at higher amounts of proteins due to the increased competition for water molecules between the starch granules and protein. (Li *et al*., 2015).

The digestion rate of RC bread was markedly delayed than those made from control and SM formulations. This can be explained by the fact that the RC II contained the lowest proportion of starch in its formulation, thus resulting in the lowest value of starch hydrolysis.

Although the SM starch hydrolysis curve was quite similar to control, the rate decreased after I15 compared to control, the reason for this might be the SM might not act as RC behaved. This may be because rennet casein is insoluble and forms a coating around starch (O'Kennedy, 2011).

Further, the noticed differences of starch hydrolysis among the control and HPCSB samples could also be ascribed to the interaction of many elements such as the susceptibility of starch towards hydrolysis, the microstructure of samples and the extent of starch gelatinisation. Other constituents present in samples may have interfered with starch digestion such as dietary fibre, cell wall materials, polysaccharides (Berg *et al*., 2012; Kaur *et al*., 2007; Singh *et al*., 2010). Nevertheless, the actual starch hydrolysis percentage of HPCSB may not be as estimated by invitro studies and needs further validation using in vivo studies/clinical trials (Bordoloi *et al*., 2012; Jenkins *et al*., 1987; Singh *et al*., 2010).

Table 4.10 Digestion properties and estimated glycaemic index (eGI) of HPCSB

	Control	RC I	RC II	SM I	SM II
RDS (%)	37.84±4.17^{a}	30.65±2.96^{bc}	26.19±2.24^{c}	33.85±0.93^{ab}	32.83±1.23^{abc}
SDS (%)	11.88±3.09^{b}	17.76±3.14^{ab}	22.08±3.12^{a}	14.64±2.33^{b}	11.66±0.23^{b}
RS (%)	47.49±4.57^{b}	51.25±3.08^{ab}	55.12±0.44^{a}	51.29±1.51^{ab}	52.07±2.00^{ab}
eGI	67±2^{a}	63±1^{b}	59±1^{c}	65±1^{ab}	64±1^{ab}

[1]RDS: rapid digestible starch; SDS: slow digestible starch; RS: resistant starch; eGI: estimated glycaemic index

[2]a-c, mean values in each column with the same superscripted letter are not significantly different (p<0.05). Results are demonstrated as average (n=3) ± SD

As shown in **Table 4.10,** with the increasing amount of protein content, the rapidly digestible starch (RDS) of HPCSB was observed to decrease significantly (p<0.05) whereas the slowly digestible starch (SDS) increased significantly (except SM II), and so did resistant starch (RS). SDS has been shown to slowly increase the levels of insulin and postprandial plasma glucose (Englyst & Hudson, 1996) while resistant starch refers to the sum of intact starch and retrograded starch, which passes into the large intestine.

The addition of protein reduced the percentage of RDS in HPCSB significantly (p<0.05). Due to changes in the specific volume of HPCSB, the physical structure became compact and dense, which may have reduced the contact between starch and enzyme, thus, the RDS content was lowered while SDS and RS content increased, our results are in agreement with the literature (Tahir, Ellis, and Butterworth (2010).

RDS ranged from 37.84% (control), followed by 30.65% and to 26.19% for RC I and RC II, respectively, and 33.85%, 32.83% for SM I and SM II, respectively. On the other hand, RC II obtained the highest SDS at 22.08%, followed by 17.76% for RC I compared to control sample (11.88%). This behaviour is consistent with the previous literature (Liu *et al*., 2016), whereas as for SM samples, there is no significant difference for SDS, a possible explanation for this may the interaction between SPI and MPC receded the barrier behaviour, leading to starch granules being more easily accessible and susceptible to the enzyme attack and eventual hydrolysis. RS contents of HPCSB were higher than the control bread. In our study, the percentage of RS ranged from 47.79% for control and up to 55.41% for HPCSB (**Table 4.10**).

Estimated glycaemic index (eGI) was calculated from the starch hydrolysis curve (Chen, Singh, & Archer, 2018). The values of the eGI ranged from 59.41 to 66.23, with eGI of all HPCSB lower than the control bread. The eGI obtained from control wheat bread was consistent with Lau, Soong, Zhou, and Henry (2015), who conducted an *in vivo* digestion and calculated GI, which was 68 ± 5 for their control wheat steamed bread sample. The small difference in eGI might be due to varied processing conditions such as mixing time, fermentation time, cooking time, and the ratio of wheat flour to water. In general, among the 5 samples, eGI decreased with an increase in protein addition. The highest eGI is 66.23 for control wheat bread, followed by 64.48, 59.14 for RC I and RC II, respectively; 65.46 and 64.43 for SMI and SM II, respectively.

The lower eGI for HPCSB than control bread may be attributed to the lower specific volume (**Table 4.2**), and porosity of steamed bread, resulting in decreased accessibility of amylases to starch, rendering the starch less susceptible to hydrolysis (Sui, Zhang, & Zhou, 2016). Also, digestibility decreased with the decreasing degree of gelatinisation (Liu *et al*., 2017), which is consistent with DSC results in our study (**Table 4.5**). Proteins reduce the starch granule surface accessibility and therefore influenced the enzyme susceptibility. More specifically, protein fractions, such as albumin, globulin and glutenin, were observed as glued into bread matrix surrounding starch granules acting as a barrier against starch digestion (**Figure 4.7**). This phenomenon has been confirmed by adding protease to corn flour or removing gluten from wheat flour, both of which resulted in a significant enhancement of *in vitro* starch digestibility. Many other studies have also reported the presence of a protein as a barrier towards starch digestion (Petitot, Abecassis, & Micard, 2009; Ren *et al*., 2016). Cooking or processing may sometimes reduce the starch digestibility as the conformational variations in proteins may occur that could promote the formation of disulphide liked polymers (Lau *et al*., 2015). The physical structure of bread could also be influenced by processing conditions such as mixing time and duration of fermentation, resulting in apparent changes in the glycaemic index (Lau *et al*., 2015).

4.5.1 Microstructural characteristics of HPCSB during and after gastro-small intestinal digestion *in vitro*

Figure 4.11 represents the scanning electron micrographs of HPCSB in freeze-dried samples taken after oral digestion *in vitro* (O), and *in vitro* simulated gastric digestion 30 min (G30), and *in vitro* simulated small intestinal digestion after 5 min (I5), 30min (I30), 120 min (I120).

4.5.1.1 Oral digestion

As shown in **Figure 4.11**, after oral digestion (O), the structure of control bread and HPCSB appeared to be more porous when compared to the undigested bread samples (**Figure 4.7**). This might be due to the action of α-amylase, which hydrolyses the gelatinised starch which is more susceptible to hydrolysis compared to semi gelatinised and ungelatinised starch (Ross, Brand, Thorburn, & Truswell, 1987). Some of the granules which might be un-gelatinised or semi-gelatinised were exposed more clearly as the gelatinised starchy mass is hydrolysed by the action of amylases during oral digestion. Control sample had a more open and porous structure compared to HPCSB, while RC had a more compact structure compared to SM. For HPCSB, during oral processing, the amount of exposed starch granules was reduced as the protein content was increased. The protein matrix was shown to engulf the starch granules.

This starchy mass was then hydrolysed by the α-amylase during simulated oral digestion, thus exposing the semi-gelatinised or ungelatinized starch granules. Moreover, since oral processing took place for a very short duration (10s), which might not be enough hydrolyse gelatinized starch resulting in ungelatinized and semi-gelatinised starch granules from the bread surface, but during gastric digestion these granules may have been washed or displaced by the digestion fluid, thus resulting in the bread structure not showing these granules (**Figure 4.11**, G30).

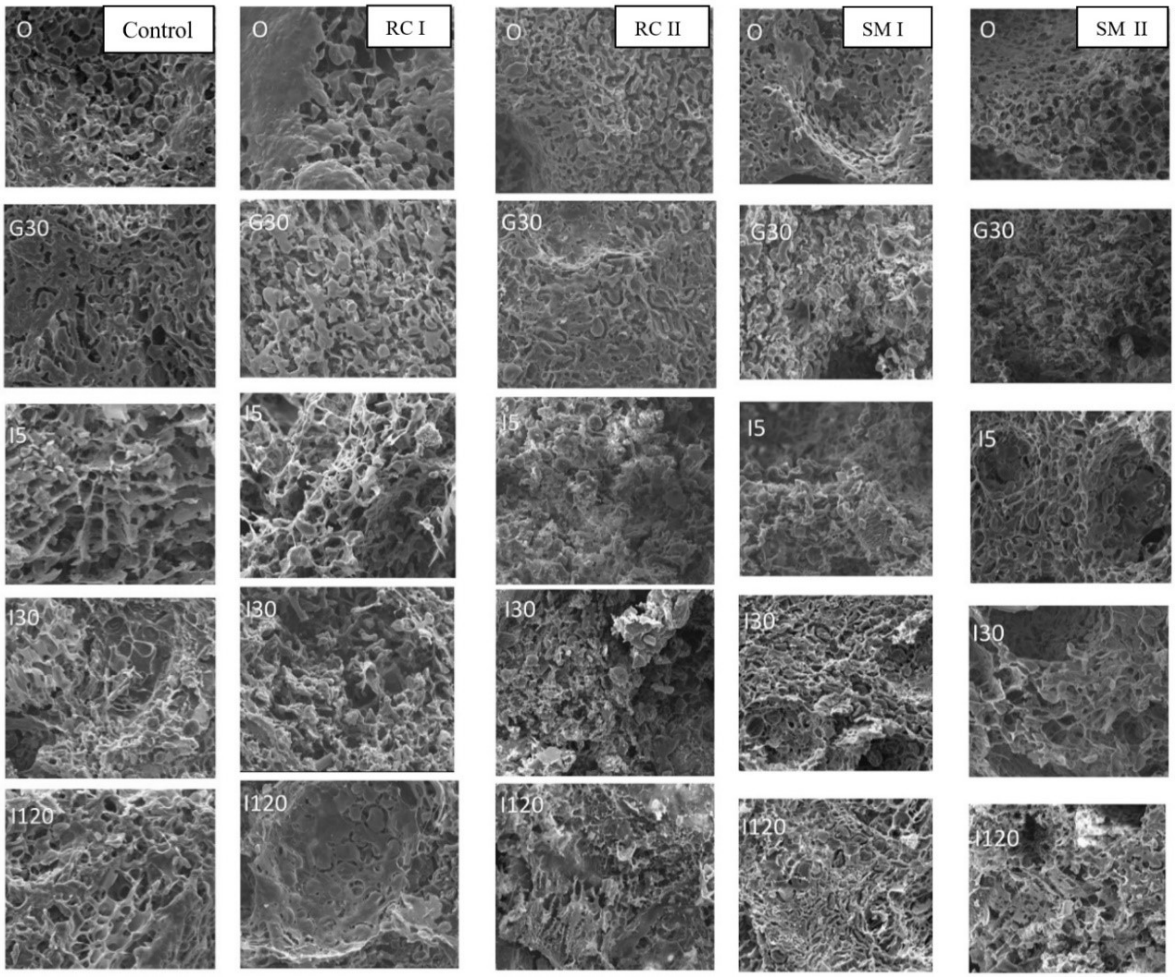

Figure 4.11 Scanning electron micrographs collected after O, G30, I5, I30, I120 min gastro-small intestinal digestion *in vitro* (digesta) of HPCSB.

During gastric digestion (G30), the structure also became more porous, due to protein digestion (SGF containing pepsin) mainly on the surface of the sample. The sample was shaken during gastric digestion phase (30min), therefore, there is a possibility that the ungelatinized and semi-gelatinised starch granules were washed out in the digestive juice due to the fact that the structure of ungelatinized and semi gelatinised starch is more loosely bound to the bread matrix compared to fully gelatinised starch (Singh et al., 2013).

The protein matrix of the control bread was almost intact until 30min of digestion (G30) whereas for HPCSB, little variation was noticed during gastric digestion, and the protein matrix of HPCSB presented a more irregular structure and compact where the

additional protein seemed to wrap the starch granules. Barely any difference was observed between SM samples and RC samples except that the structure appeared denser and compact with increase in the protein level.

4.5.1.3 Small intestine

During small intestine digestion, the structure was ill-defined for all five samples and the structures were appeared to be more damaged and more open spaces were observed. The extent of damage was less evident, and the structure appeared more compact for bread made with higher protein addition.

For control sample, the structure appeared to loosen up during the course of small intestinal digestion which might be due to the ungelatinized and semi-gelatinised starch granules being washed into the digestion fluid during gastric-phase whereas the remaining structure was further digested by the action of SIF (containing invertase and amyloglucosidase). It is important to mention that the starch hydrolysis enzymes can also act on ungelatinized starch although its catalysis efficiency is comparatively lower as compared to gelatinised starch (Slaughter, Ellis, & Butterworth, 2001).

At 30min, as shown in **Figure 4.11** (I30, control), the structure became more porous, ill-defined and damaged. Also, more starch granules were hydrolysed, and spherical voids were left behind. At the end of the digestion (I120), there were barely any starch granules that could be visualized as most of them were hydrolysed, leaving behind a protein network. The honeycomb-like structures shown at I120 for control sample in **Figure 4.11** may represent denatured cross-links of gluten network (**Figure 4.12**), and the voids or open spaces within the gluten network may have appeared during the course of simulated small intestine digestion due to the hydrolysis of starch.

As shown in microscopy of control dough (**Figure 4.6**), during dough making, the gluten was developed and formed a three-dimensional protein network, the cross-links which is attributed to the presence of disulphide bonds (disulphide-sulfhydryl exchange) (Mauritzen & Stewart, 1963; Shewry, Halford, & Tatham, 1992) and also to the the occurrence of tyrosine cross-links during dough formation and breadmaking processes (Tilley *et al*., 2001). Furthermore, as steaming is a milder process compared to baking, it may be less destructive on the structure of gluten. Nevertheless, it is unpleasant to

mention that the protein underwent minor modification during gastric digestion by the SGF containing pepsin. Cellulose and other cell wall fibrous materials are also present in the flour which might still be intact during gastric digestion.

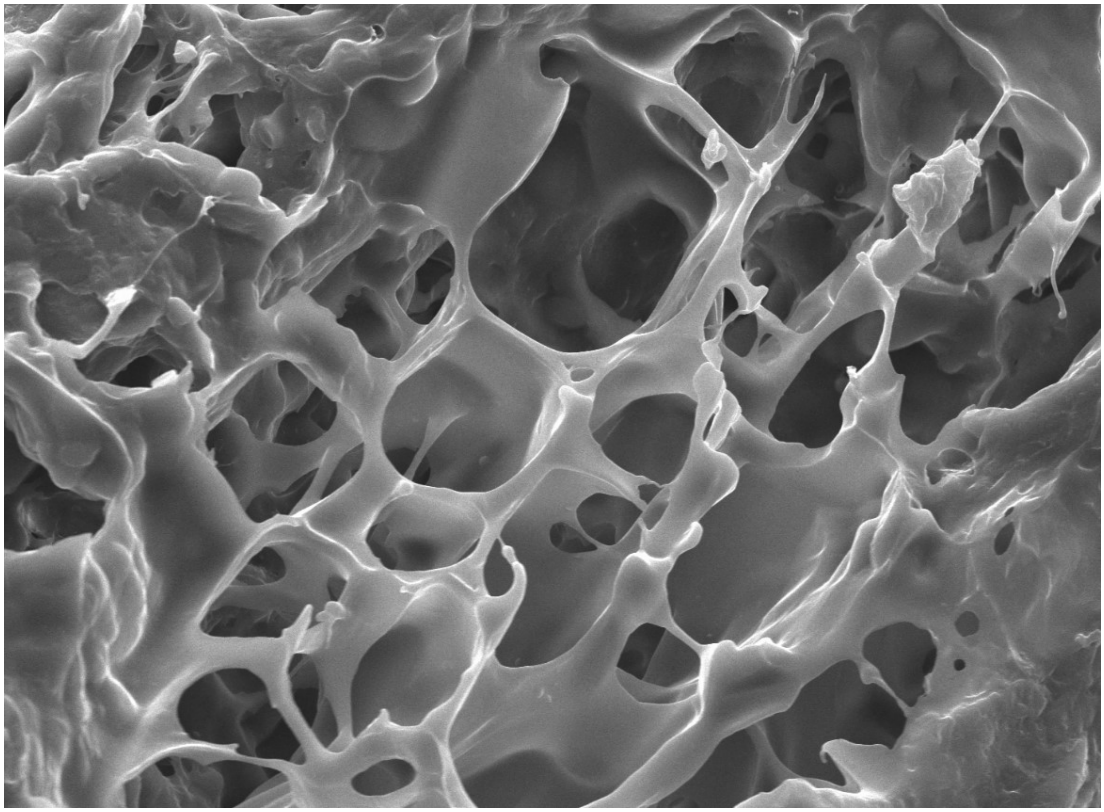

Figure 4.12 Scanning electron micrographs collected after I120 min gastro-small intestinal digestion *in vitro* (digesta) of control showing denatured cross-links of gluten network

As for HPCSB (RC & SM), the structure looked damaged which may have occurred upon the addition of SIF. During first 5 min of small intestinal digestion (Figure **4.11**, I5), more starch granules were observed for each HPCSB sample compared to control, possibly due to the fact that gelatinised starchy mass began to digest exposing ungelatinized along with semi-gelatinised starch granules. An increasing amount of starch granules appeared during the small intestinal digestion which were entangled in the protein matrix. This may be attributed to the less extent of starch hydrolysis in HPCSB, due to the presence of more protein (**Figure 4.9**; **Figure 4.10**).

A more porous structure was observed for SM samples with more starch granules present I 30, when compared to RC samples which also in line with the SM hydrolysis curve between I10-I30 (**Figure 4.9**; **Figure 4.10**).

As for RC, the protein skeleton structure was still present at 120min of the small intestine digestion (**Figure 4.11**, RC I & RC II, I120), and the in-depth starch granules also existed. Moreover, open voids were not observed for RC at I120 and appeared more compact and denser compared to SM and control. After the 120min of the digestion process, the RC protein skeleton structure was more continuous compared to SM. This might be the reason for RC being capable of protecting starch from enzymatic hydrolysis and may account for the lower starch hydrolysis of RC than SM (**Figure 4.9**; **Figure 4.10**).

In conclusion, for the control sample, the enzymes were able to penetrate into the structure, leading to a sharper increase in starch hydrolysis compared HPCSB (**Figure 4.9**; **Figure 4.10**), the structure was more compact for HPCSB.

Further experiment action needs to be conducted for the analysis of digestive juice for the presence of starch granules. Samples need to be taken every 2min, 5min, 10 min and 30min during gastric digestion for microscopy to see if the amount of starch granules decrease from the beginning to the end of gastric digestion. Also, if the starch granules are washed into the digestive juice, there is a possibility that the samples for glucose measurement may contain some ungelatinized starch, which may be further digested by the addition of invertases and converted into glucose. This might affect the calculated eGI values.

Chapter 5. Conclusions

The physico-chemical characteristics of HPCSB indicate that it is practical to include rennet casein and a mixture of SPI and MPC for producing HPCSBs.

The fortification of bread formulation with proteins especially RC can increase the protein content of CSB to more than double (up to 19% compared to control wheat CSB). Our studies indicated that with an increase in the level of protein (RC/ SM) supplementation, a decrease in specific volume and pasting behaviour occurred. An increase in moisture demand was also observed for HPCSB. The protein incorporation

had an effect on both crust and crumb colour. The colour became slightly darker as the level of proteins was increased. Crumb hardness, gumminess and chewiness increased whereas the cohesiveness springiness and resilience decreased with the increase in protein levels increased, indicating a firmer texture. Furthermore, the microscopy of both dough and bread indicated that the structure of HPCSB was more compact and denser compared to control. The protein matrix has been observed to act like an obstacle around starch thus preventing it from being hydrolysed by digestive enzymes. Therefore, the addition of proteins reduced the *in vitro* starch digestibility of HPCSB significantly than the control bread. The HPCSB sample with the highest protein content (RC II) showed a decrease in the eGI from 66.23 (control) to 59.41. The microscopy of digestion also confirmed that the addition of proteins, formed a layer around the starch that may have competed for water, leading to an insufficient amount of water being present for starch to gelatinise, thus leading to a decrease in starch hydrolysis during gastro-small intestinal digestion. The microstructure of HPCSB digesta during gastro-small intestinal *in vitro* digestion has also indicated that the protein matrix within HPCSB engulfed the starch granules by restricting the starch granule swelling, and thus decreasing their starch digestibility and eGI.

5.1 Further recommendation

The resulting product in HPCSBs provides an exceptional vehicle for nutritional improvement of the human diet for populations in Asia and elsewhere since the addition of different plant and dairy based proteins were tested in this study.

Further analysis that could be done includes optimisation of the water requirement during dough processing through the farinograph and the rheological characterisation of dough using a dynamic rheometer. Addition of salt could be done to solubilise the rennet casein prior to addition to wheat flour in order to improve the mouthfeel; Amino acids analysis of the digests can be conducted in order to further understand the nutritional attributes of HPCSB.

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