Food Hydrocolloids for Health 3 (2023) 100111

Contents lists available at ScienceDirect

Food Hydrocolloids for Health

journal homepage: www.elsevier.com/locate/fhfh

Preparation and characterisation of plant and dairy-based high protein Chinese steamed breads (mantou): Microstructural characteristics and gastro-small intestinal starch digestion *in vitro*

Shiyuan Mao[a,b], Lovedeep Kaur[a,b], Tai-Hua Mu[c], Jaspreet Singh[a,b,*]

[a] School of Food and Advanced Technology, Massey University, Palmerston North 4442, New Zealand
[b] Riddet Institute, Massey University, Palmerston North 4442, New Zealand
[c] Institute of Food Science and Technology, Chinese Academy of Agricultural Sciences, Beijing, China

ARTICLE INFO

Keywords:
Chinese steamed bread
High protein breads
Starch gelatinisation
Microstructure
In vitro digestion

ABSTRACT

The effects of dairy and plant protein addition on microstructural characteristics and *in vitro* gastro-small intestinal starch digestion characteristics of Chinese steamed breads (CSBs) were studied. Breads containing rennet casein (RC) and a mixture of soy protein isolate and milk protein concentrate (SM) at two different levels (RC I, RC II; SM I, SM II) were prepared. Microstructural characteristics of the undigested and digested control (100% wheat flour) bread and high protein steam bread (HPCSB) versions were compared through scanning electron microscopy. The compact microstructure of HPCSBs displayed a network of proteins wrapped around starch granules and had fewer air cells compared to the control. The addition of both proteins influenced the microstructure of HPCSBs, which in turn affected their textural and starch digestion properties. The *in vitro* starch digestion of control CSB and HPCSBs confirmed that the addition of proteins is capable of lowering the starch hydrolysis (%). The highest starch hydrolysis was observed for the control wheat bread, followed by SM1 > RC I > SM II and RC II at the end of the small-intestinal digestion. The estimated glycaemic indices (eGI) for all HPCSBs were statistically lower than the control CSB. In comparison to control CSB, the microstructure of HPCSBs appeared more irregular, less porous, and compact during gastric and small intestinal digestion.

1. Introduction

Chinese steamed bread (CSB) is a traditional food product made from wheat flour dough and is cooked by steaming (Huang, 2014). It is a staple food in several parts of China and is now gaining popularity around the world (Hui & Evranuz, 2012; Zhu, 2014). Chinese steamed bread has a high glycemic index (GI) due to its high digestible starch content and low resistant starch and dietary fibre contents (Zhu, 2019). There are various strategies, such as incorporating diverse functional ingredients to develop low GI versions of the CSB. This study focuses on understanding the impact of the addition of plant and dairy proteins on the *in vitro*starch digestibility and estimated glycaemic index (eGI) of CSB.

Milk proteins such as casein are widely used in the manufacture of bakery products (Gallagher et al., 2003). Rennet casein is particularly rich in lysine, which can be used as a nutritional supplement in cereal products (Huppertz et al., 2018a). Similarly, milk protein concentrate (MPC) can be added to many food bakery formulations since it plays a major role in providing structural and functional properties (Augustin et al., 2011; Havea, 2006; Rollema & Muir, 2009). Milk proteins also can improve the absorption of vitamins A, B12 and folate in the intestinal tract (O'Regan et al., 2009). Soybeans are another rich source of lysine (higher than wheat flour), tryptophan and threonine (Nishinari et al., 2018). Soy proteins have been reported to be useful for cardiovascular health, while milk proteins are broadly utilised to support bone health and build muscle mass. Plant and dairy-based proteins, either alone or in combination can be used to improve the nutritional quality of popular processed foods (Dhinda et al., 2012). It has been reported that the presence of protein in food systems affects starch digestibility as the presence of globulins, glutenins and albumin help in creating a protein network surrounding the starch granules (Ezeogu et al., 2008; Hamaker & Bugusu, 2003). Also, the presence of protein during heat processing has been shown to reduce starch digestibility as conformational transitions in proteins occur that facilitate the formation of disulphide-linked polymer chains resulting in a lower glycaemic response (Ezeogu et al., 2008).

In summary, the addition of casein, MPC and soy protein might help not only reduce the GI but also have structural and health benefits when added to CSB. Therefore, the main objectives of this study were to in-

* Corresponding author at: School of Food and Advanced Technology, Massey University, Palmerston North 4442, New Zealand.
E-mail address: j.x.singh@massey.ac.nz (J. Singh).

https://doi.org/10.1016/j.fhfh.2022.100111
Received 24 March 2022; Received in revised form 12 December 2022; Accepted 12 December 2022

vestigate the effects of dairy proteins (rennet casein and MPC) and plant protein (soy protein isolate, SPI) on microstructural characteristics and *in vitro* gastro-small intestinal digestion of high protein Chinese steamed breads (HPCSBs). Microstructural properties of the digests obtained during gastro-small intestinal digestion were also studied. Investigating the microstructural characteristics of starch-based foods such as bread is crucial for understanding how the food structure breaks down during digestion. It also helps understand the starch and protein interactions during processing and how they impact the digestion behaviour of starch.

The physico-chemical and textural properties of the HPCSBs are discussed in a companion paper (Mao et al., 2022).

2. Materials and methods

2.1. Materials

Wheat flour, gluten (FLOURF25, Davis Trading), sugar, baking soda, yeast (2018, Edmonds) and SPI were bought from a local market. RC and MPC were purchased from Fonterra Ltd. (Palmerston North, New Zealand).

Amylo-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).

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

2.2. Methods

2.2.1. Preparation of HPCSBs

The formulations for control and HPCBs prepared in this study are shown in Table 1. The gluten level was calculated based on the amount of gluten in substituted wheat flour. Before mixing the dough, yeast and baking soda were dissolved in water (at 30 °C). For HPCBs, different dry protein powders were mixed with wheat flour beforehand. To begin dough mixing, sugar and yeast solutions were poured into the dry ingredients. Mixing was then conducted at speed 2 in a mixer (KMM021, Kenwood, China) as depicted in Table 2. After fermentation at 35 °C for 30 min, the dough was kneaded, and then sheeted by hand 5-10 times. The dough was then divided into eight pieces, hand-shaped and proofed for 15 min followed by steaming for 20 min in a steamer (ST6650, Sunbeam, China). After steaming, the breads were cooled to room temperature (27 °C) for 20 min and then sealed in a plastic bag until further analysis.

Table 1
Formulations for control and high protein Chinese steamed breads.

	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	219	187.9	220	212
RC	0	31	62.1	-	-
SPI	0	-	-	17.6	18
MPC	0	-	-	12.5	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

RC I and RC II, samples containing rennet casein at 14% & 33% (dry wt. of added protein (excluding gluten)/dry wt. of wheat flour), respectively. SMI and SMII, samples containing soy protein isolate and milk protein concentrate at 7%, 5% & 7%, 8% (dry wt. of added protein (excluding gluten)/dry wt. of wheat flour), respectively.

* water addition and kneading time were adjusted based on the performance of the dough during preliminary experiments.

Table 2
Production parameters for control and high protein Chinese steamed breads.

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

RC I and RC II, samples containing rennet casein at 14% & 33% (dry wt. of added protein (excluding gluten)/dry wt. of wheat flour), respectively. SMI and SMII, samples containing soy protein isolate and milk protein concentrate at 7%, 5% & 7%, 8% (dry wt. of added protein (excluding gluten)/dry wt. of wheat flour), respectively.

* mixing and kneading times were adjusted based on the performance of the dough during preliminary experiments.

2.2.2. Moisture, protein, and starch contents

The moisture content of the bread samples was measured according to the standard AOAC method (AOAC, 1990). Fresh bread samples were used for the analysis. The oven temperature was 108 °C and the duration was 4 h.

The protein content of the freshly prepared CSBs was determined by the Kjeldahl method (AOAC, 2006).

The total starch content in the bread samples was determined by using the method developed by Bordoloi et al. (2012) and Goñi et al. (1996). HPCBs and control samples were freeze-dried, ground, passed through a 0.5 mm mesh sieve and kept in a desiccator until analysed (Tamura et al., 2016). A total starch kit (K-TSTA, Megazyme International Ireland Ltd., Ireland) was used to determine the starch content.

2.2.3. Microstructure analysis for undigested samples

Freeze-dried dough and bread samples were fractured into pieces of about 5 mm thickness and then coated with gold for 110 s (SCD 050, Blazers, Liechtenstein). The morphology of the samples was evaluated by a scanning electron microscope (SEM) (FEI Quanta 200 FEI Electron Optics, Eindhoven, The Netherlands).

2.2.4. Gastro-small intestinal starch digestion in vitro

A three-stage oral-gastro-small intestinal model was used to study *in vitro* digestion characteristics of the bread samples as described by Tamura et al. (2017) and Goebel et al. (2019). The prepared steamed bread sample and an appropriate amount of distilled water were added to a beaker to achieve a 4% concentration of starch.

The ratio of simulated salivary fluid (SSF) to the sample used was 1:1 (w/w). Samples were weighed and torn apart into small pieces and the volume for each piece was around 0.5 cm^3. Then they were transferred into a mortar, soaked in SSF for 15 s and pounced 15 times simultaneously.

Different formulations of steamed breads containing SSF were transferred into a mesh bag in a 500 mL jacketed glass reactor and agitated by magnetic stirrers continuously at 300 rpm. A circulatory water bath was connected to the digestion reactor jacket to maintain its temperature at 37 ± 1 °C. 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 1M HCl. Aliquots (0.5 mL) were collected, and the digestive enzyme was inactivated using absolute ethanol after 0 (G0), 15 (G15) and 30 min (G30) of simulated gastric digestion. After 30 min of simulated gastric digestion, the pH was varied to 6.80 ± 0.1 by the addition of 1M NaOH.

Simulated intestinal fluid (SIF) containing pancreatin, amyloglucosidase and invertase was added to the reactor 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 digestive enzymes were inactivated by mixing with 2 mL absolute ethanol.

The solutions were added with amyloglucosidase and invertase and centrifuged at 1800 × *g* for 10 min and then incubated for 10 min at 37°C. The glucose concentration for the solutions was measured using a D-glucose assay kit (GOPOD Format K-GLUK 07/11, Megazyme International Ireland Ltd., Wicklow, Ireland). Starch hydrolysis (%) was calculated using the following equation:

$$\%SH = S_h/S_i = 0.9 \times G_p/S_i$$

Where %SH is starch hydrolysis percentage, S_h is the amount of hydrolysed starch, S_i is the initial amount of starch, and 0.9 is a conversion factor. G_p is the amount of produced glucose.

To calculate starch hydrolysis kinetics, a first-order equation model was applied (Goñi et al., 1997). Parameter estimation was carried out using Origin9® 2017.

The hydrolysis index (HI) of each sample was calculated by dividing the area under its digesto-gram by the area under the digestogram of fresh white bread (Goñi et al., 1997). The average *eGI* for each sample could be defined as the following equation (Liu et al., 2018):

$$eGI = 8.198 + 0.862 * HI$$

Where, HI is the hydrolysis index, 8.198, and 0.862 are the parameters of the modified first-order kinetic model.

2.2.5. *Microstructure analysis during* in vitro *digestion*

SEM was also used to examine the microstructural changes in HPCBs throughout the *in vitro* digestion process. After adding saliva for oral processing for 1 min, HPCSB cubes (~0.7-1 cm^3) were digested *in vitro* as described in Section 2.2.4. Samples were added straight into a reactor and a water bath was used to maintain its temperature at 37 ± 1°C and shake at a speed of 60 rpm during *in vitro* digestion. The pH was adjusted to 1.2 after adding SGF to initiate gastric digestion. After 30 min of simulated gastric digestion, the pH was adjusted to 6.8 and SIF was added to the reactor to initiate the small intestinal digestion. Digested HPCSB cubes were taken out immediately after oral processing (O), 30 min (G30) after gastric digestion, and 5 (I5), 30 (I30) and 120 (I120) min of simulated small intestinal digestion (Tamura et al., 2017; Goebel et al., 2019). The cubes were immediately put into a test tube and immersed in liquid nitrogen for freeze-drying. The freeze-dried bread cubes were then examined for microstructure as described in Section 2.2.3.

2.2.6. *Statistic analysis*

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

3. Results and discussion

3.1. *Microstructural characteristics*

3.1.1. *Microstructure of dough*

Representative SEM micrographs of the control and high-protein fermented doughs are shown in Fig. 1. The spherical- and lenticular-shaped small and large starch granules contained in all samples of dough were observed to be distributed throughout an extensible and continuous protein matrix. The starch granules were more organised and embedded in the matrix in the control sample and they were visible and not fully covered in the gluten matrix, which could be clearly distinguished in the images.

The structure was observed to be more compact in the high protein doughs than in the control sample, with particularly RC II and SM II showing greater levels of network disruption compared to RC I and SM I due to higher levels of protein incorporation. The changes observed in the network structure may have influenced the physicochemical properties of the high-protein doughs.

For high protein dough samples, starch granules were visible to a lesser extent than control dough. In conjunction, the gluten and other added proteins were incorporated into an inconsistent and interrupted protein matrix along with coating and adhering to starch granules (Fig. 1). This suggested that the extensibility and resistance of dough could be disrupted, along with the microstructure (Liu et al., 2016) that might be responsible for the observed reduction in the loaf volume for HPCBs (Mao et al., 2022). This coating of protein could restrict the increase in viscosity due to restrictions in the swelling of starch granules at high temperatures, and accordingly retard the starch gelatinisation (Liu et al., 2018). Furthermore, because of the starch-protein interactions, the retrogradation of gelatinised starch could be delayed. It can also stabilise the starch granules thereby preventing the linkage of amylopectin chains due to retrogradation at low-temperature storage (Liu et al., 2016, 2018; Sun et al., 2015). These observations are in line with the results obtained from rapid viscosity analysis and differential scanning calorimeter (Keeratipibul et al., 2010; Mao et al., 2022; Sun et al., 2015).

Insoluble rennet casein in RC-containing samples remained as separately dispersed entities due to its inability to be solubilized in a dough matrix as well as exclusion during the formation of a water/gluten network (Huppertz et al., 2018b; O'Kennedy, 2011). The addition of SM (MPC & SPI) also seemed to coat the starch granules very evenly, which made the dough microstructure appear denser than in RC dough. This may be attributed to the higher solubility of SM than RC. The dough containing SM also showed the presence of filamentous structures possibly provided by SPI (Du et al., 2016; Liu et al., 2016).

3.1.2. *Microstructure of HPCBs*

The microstructure of the control bread and HPCBs with different proportions of proteins are shown in Figs. 2 and 3. The control bread displayed a more uniform gluten matrix compared to the HPCBs. The absence of intact starch granules in the gluten matrix is attributed to gelatinization during steaming. On the other hand, HPCBs 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 in breads made with higher protein levels.

For HPCBs, the images appeared to display denatured protein wrapping around gelatinised starch granules. Clear differences were observed in the morphology of air cells between the control and HPCSB samples.

The air cells in the control breads appeared to be hollower and deeper compared to the HPCSB samples. The bread network depicted even and regular air cells which provided a good structural character to retain gas, as demonstrated by the highest bread loaf volume (Mao et al., 2022). The air cells for HPCBs appeared to decrease with increased levels of proteins, indicating that breads made with higher levels of protein addition were denser, more compact and tough in texture (Mao et al., 2022). Also, SM-containing bread samples appeared to be denser than RC. This might be due to the solubility of SPI and MPC, which can fill the open spaces in the bread matrix (Augustin et al., 2011; Morr, 1984; Nishinari et al., 2018).

3.2. *Gastro-small intestinal digestion* in vitro

After oral processing and during the simulated gastric digestion period (G0-G30), the starch hydrolysis percentage for all five samples was low (< 15%), which may be attributed to the absence of the starch hydrolysing enzymes in gastric juices (Dartois et al., 2010). The small extent of hydrolysis could have occurred due to the action of salivary α-amylases that may have infiltrated via the food bolus after mastication and can maintain their activity during the gastric stage

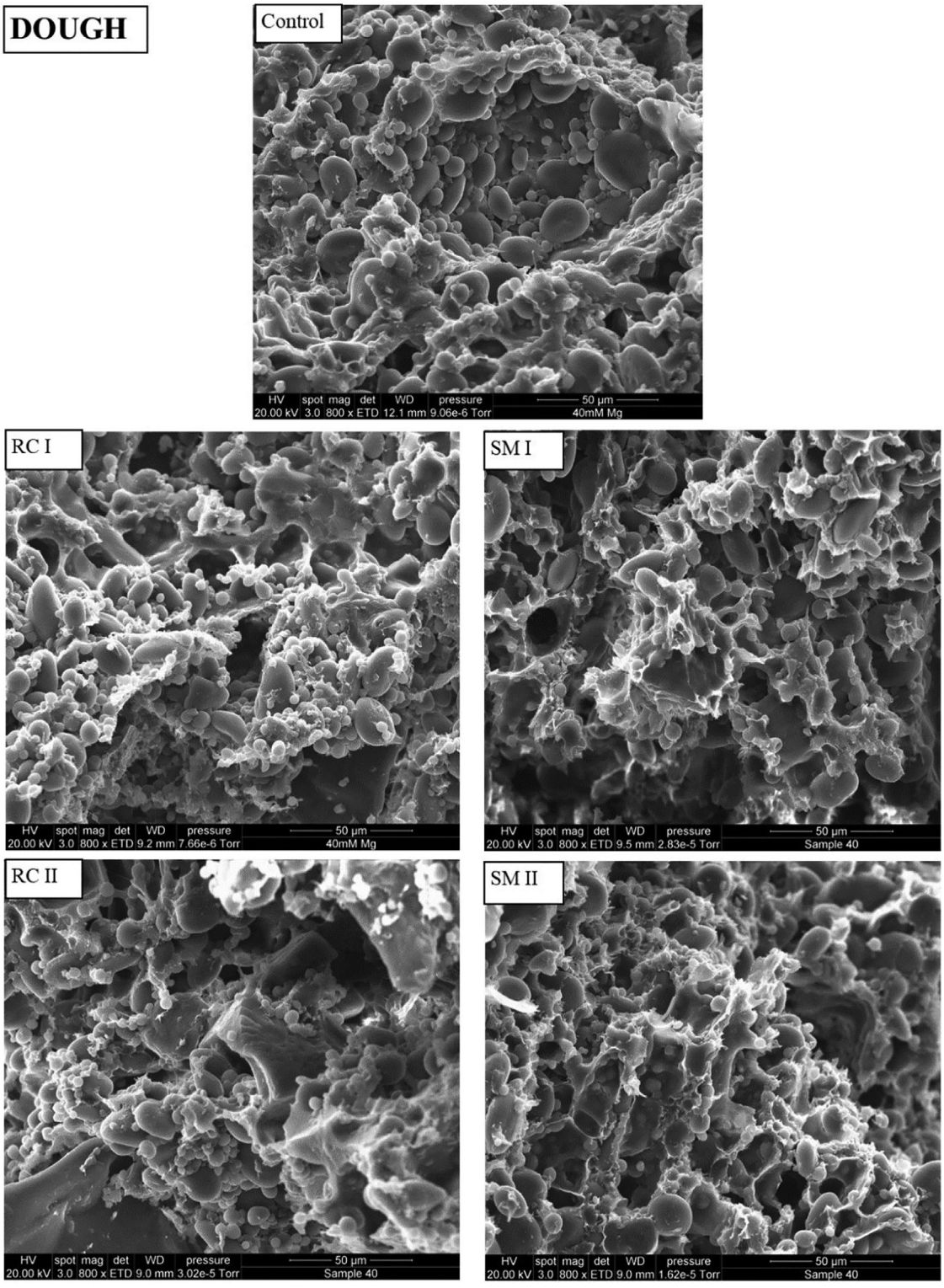

Fig. 1. Microstructure of doughs from control and HPCSB bread formulations (50μm).
RC I and RC II, doughs containing rennet casein at 14% & 33% (dry wt. of added protein (excluding gluten)/dry wt. of wheat flour), respectively; SMI and SMII, doughs containing soy protein isolate and milk protein concentrate at 7%, 5% & 7%, 8% (dry wt. of added protein (excluding gluten)/dry wt. of wheat flour), respectively.

(Bornhorst & Singh, 2013; Tamura et al., 2017). After, the addition of SIF, the rate of starch hydrolysis of all the bread samples progressively increased over the 120 min period of digestion.

During small intestinal digestion, the starch hydrolysis of the control sample increased to about 50% after 10 min, which was the highest among RC samples while no significant difference was observed between control and SM samples. After this, the rate of starch hydrolysis decreased until the end of the digestion (I120) and ended at 90%, which was the highest observed for all five samples.

Hydrolysis (%) of HPCSB was dose-dependently and delayed with the protein incorporation (Figs. 4a and b). The increase in protein concentration in the RC-containing samples decreased the hydroly-

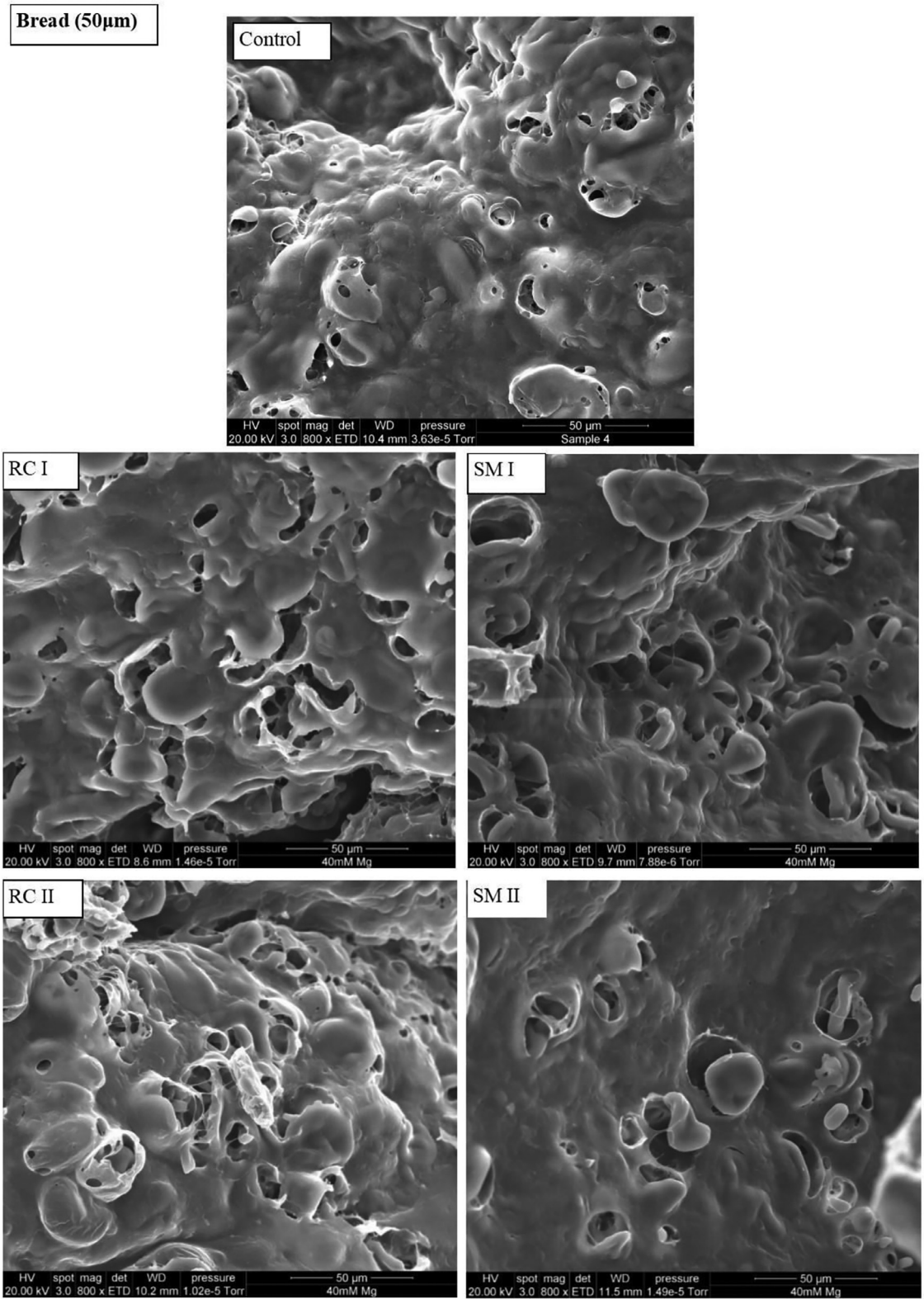

Fig. 2. Microstructure of breads for control and HPSCBs (50μm).
RC I and RC II, breads containing rennet casein at 14% & 33% (dry wt. of added protein (excluding gluten)/dry wt. of wheat flour), respectively; SMI and SMII, breads containing Soy protein isolate and milk protein concentrate at 7%, 5% & 7%, 8% (dry wt. of added protein (excluding gluten)/dry wt. of wheat flour), respectively.

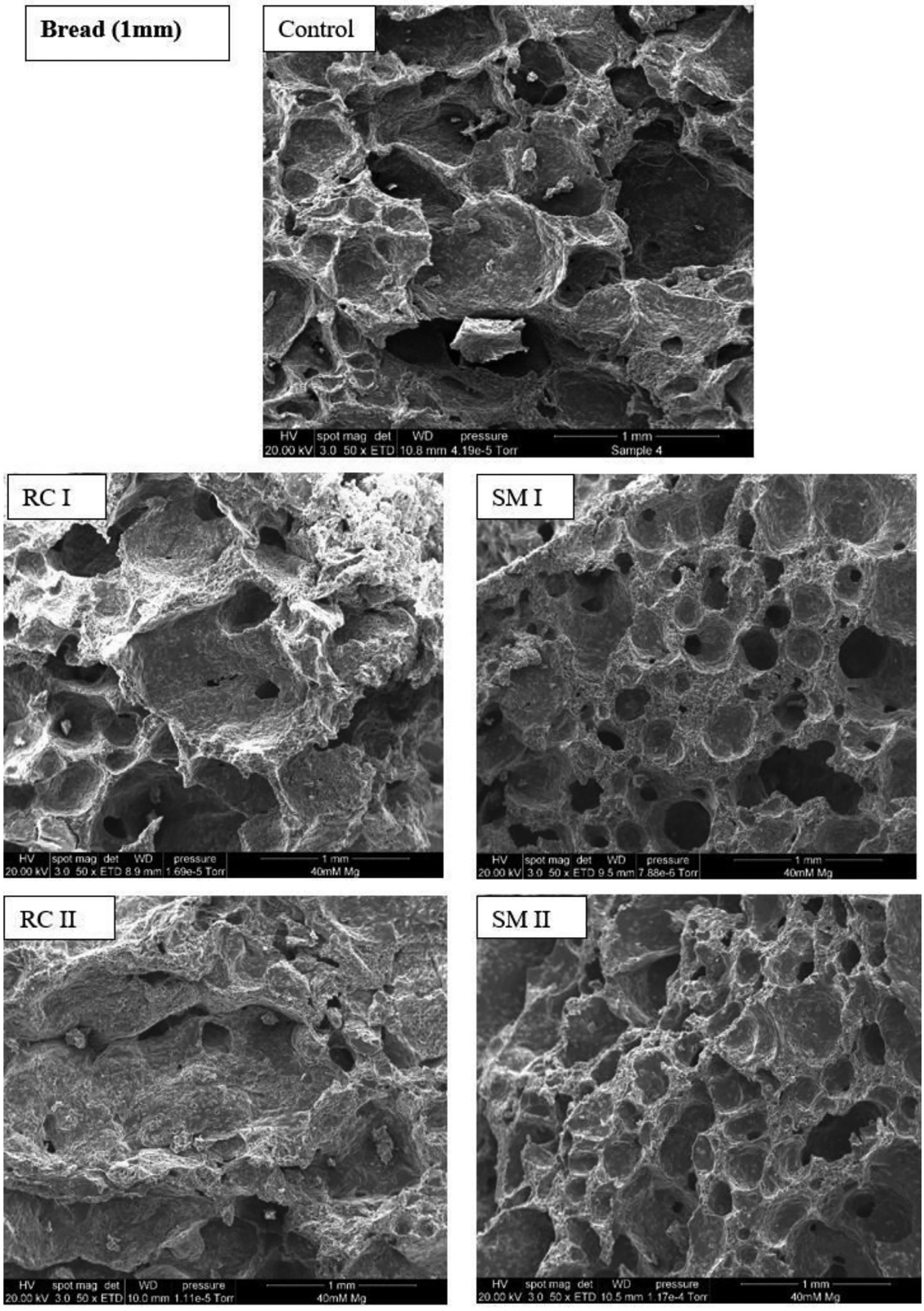

Fig. 3. Microstructure of breads for control and HPCSBs (1mm).
RC I and RC II, breads containing rennet casein at 14% & 33% (dry wt. of added protein (excluding gluten)/dry wt. of wheat flour), respectively; SMI and SMII, breads containing Soy protein isolate and milk protein concentrate at 7%, 5% & 7%, 8% (dry wt. of added protein (excluding gluten)/dry wt. of wheat flour), respectively.

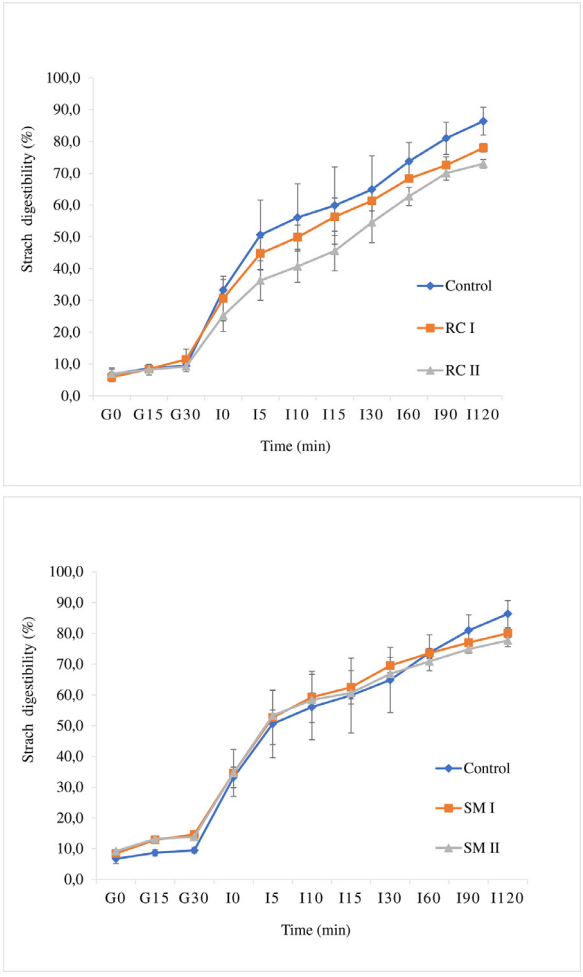

Fig. 4. a. Starch hydrolysis during *in vitro* gastro-small intestinal digestion of control, RC I and RC II for 30 min under simulated gastric digestion (G0-G30) followed by 120 min of simulated small intestinal digestion (I0-I120).
RC I and RC II, samples containing rennet casein at 14% & 33% (dry wt. of added protein (excluding gluten)/dry wt. of wheat flour), respectively; G0, 15 & 30 represent 0, 15 & 30 min in gastric conditions whereas I0, 5, 10, 15, 30, 60, 90 & 120 represent 0, 5, 10, 15, 30, 60, 90 &120 min in small-intestinal conditions following 30 min of gastric digestion.
b. Starch hydrolysis during *in vitro* gastro-small intestinal digestion of control, SM I and SM II for 30 min under simulated gastric (G0-G30) followed by 120 min of simulated small intestinal digestion (I0-I120).
SMI and SMII, samples containing Soy protein isolate and milk protein concentrate at 7%, 5% & 7%, 8% (dry wt. of added protein (excluding gluten)/dry wt. of wheat flour), respectively; G0, 15 & 30 represent 0, 15 & 30 min in gastric conditions whereas I0, 5, 10, 15, 30, 60, 90 & 120 represent 0, 5, 10, 15, 30, 60, 90 &120 min in small-intestinal conditions after 30 min of gastric digestion.

sis of HPCSB during the small intestinal digestion when compared to the control sample. On the other hand, the SM-containing samples showed no considerable decrease in starch hydrolysis compared to the control.

As expected, the highest starch hydrolysis index was noticed for the control (89.06), followed by RC I (80.25) > RC II (73.35) and whereas for SM samples, it was controlled > SM I (88.07) > SM II (85.54). However, only RC-containing samples showed starch hydrolysis indices that were significantly lower than the control ($p < 0.05$).

The rate and extent of starch hydrolysis in the small intestine are dependent upon several intrinsic and extrinsic factors. The addition of protein has been suggested to reduce the swelling of starch granules which results in a variation of the functional properties of starch, including its digestibility (Singh et al., 2013). Furthermore, the HPCSB preparation method could influence the water availability for starch gelatinisation (Hera et al., 2014). This influence could come into play became more noticeable at higher protein levels amount due to the increased competition for water molecules between the starch granules and protein (Li et al., 2015).

The digestion rates of RC-containing breads were markedly delayed when compared to those made from control and SM formulations. This can be explained by the fact that caseins and β-lactoglobulin are known as slow and fast proteins, respectively, attributed to the fact that casein micelles form a coagulum in the acidic environment of the stomach whereas β-lactoglobulin remains in the serum phase of the bolus that is rapidly evacuated from the stomach to the small intestine. Therefore, caseins are thought to remain in the gastric compartment longer than whey proteins (Barbé et al., 2014). Rennet casein has also been reported to be insoluble, therefore it may form a coating around the starch granules (O'Kennedy, 2011).

The observed differences in the starch hydrolysis of control and HPCSB samples could also be ascribed to other factors such as the microstructure of samples and the extent of starch gelatinisation. Other constituents present in samples, such as dietary fibre, cell wall materials, and polysaccharides may also interfere with starch digestion (Berg et al., 2012; Kaur et al., 2007; Singh et al., 2010).

As shown in Table 3, the rapidly digestible starch (RDS) of HPCSBs (particularly for RC II) was observed to decrease significantly ($p < 0.05$) with an increase in the amount of protein content, whereas the slowly digestible starch (SDS) increased significantly, and so did resistant starch (RS). Due to changes in the specific volume of the protein-containing breads (Mao et al., 2022), the physical structure became compact and dense, which may have reduced the contact between starch and enzymes, thus, the RDS content was lowered while SDS and RS contents were increased.

Breads containing higher levels of RC had the highest SDS, followed by RC I, whereas for SM-containing bread samples, there was no significant difference observed for SDS compared to the control sample. A possible explanation for this may be that the interaction between SPI and MPC receded the barrier behaviour, leading to starch granules being more easily accessible and susceptible to enzyme attack and eventual hydrolysis.

The values of the eGI ranged from 81.92 to 84.96, with the eGI of all HPCBs lower than the control bread, particularly for RC-containing breads. The eGI obtained from control wheat bread was consistent with the eGI values reported by Yang et al. (2006), although a small difference in eGI might have occurred due to the varied processing conditions and wheat flour to water ratio. In general, across the 5 samples, eGI decreased with an increase in protein levels, however, only the RC samples exhibited a significant difference from the control bread.

The lower eGI for HPCSB compared to control bread may be attributed to the lower specific volume (Mao et al., 2022) and porosity of steamed bread, resulting in decreased accessibility of amylases to starch, rendering the starch less susceptible to hydrolysis (Sui et al., 2016). Also, hydrolysis has been reported to decrease with a decrease in the degree of starch gelatinisation (Liu et al., 2017), which is consistent with our DSC results (Mao et al., 2022).

Onset and peak temperatures increased ($p > 0.05$) with protein addition. The increase in conclusion temperatures could be related to the interactions between the material leached out of the starches granules and protein and/or between the granular surface and protein (Ribotta et al., 2007). It was also suggested that it might be due to the migration of water from the starch to the protein, leading to lower water availability for starch, which may have delayed the gelatinization resulting in a higher transition temperature (Li et al., 2014). The pure wheat flour sample showed the highest enthalpy followed by SM I (6.43J/g), RC I (6.44J/g), SM II (5.15J/g) and RC II (3.97J/g) samples. Higher protein content has been reported to retard starch gelatinisation and decrease the thermal enthalpy, also possibly due to the interactions among pro-

Table 3
Digestion properties and estimated glycaemic index (eGI) of high protein Chinese steamed breads.

	Control	RC I	RC II	SM I	SM II
RDS (%)	55.42±7.97[a]	49.91±0.24[ab]	45.32±4.22[b]	54.93±1.57[a]	53.01±0.59[ab]
SDS (%)	30.27±7.16a[b]	28.09±4.17[ab]	32.29±5.09[a]	20.81±6.20[b]	19.37±0.33[b]
RS (%)	13.67±3.58[c]	22.05±1.09[b]	27.05±1.12[a]	19.92±1.19[b]	22.29±1.59[b]
HI	89.06±8.20[a]	80.25±2.71[bc]	73.35±4.41[c]	88.07±2.95[ab]	85.54±1.37[ab]
eGI	84.96±1.07[a]	77.37±2.34[bc]	71.43±3.80[c]	84.11±2.54[ab]	81.93±1.19[ab]

RDS: rapidly digestible starch; SDS: slowly digestible starch; RS: resistant starch; eGI: estimated glycemic index
RC I and RC II, samples containing rennet casein at 14% & 33% (dry wt. of added protein (excluding gluten)/dry wt. of wheat flour), respectively. SMI and SMII, samples containing soy protein isolate and milk protein concentrate at 7%, 5% & 7%, 8% (dry wt. of added protein (excluding gluten)/dry wt. of wheat flour), respectively
[3] a-c mean values in each row with the same superscript letter are not significantly different ($p<0.05$). Data are reported as average ($n=3$) ± SD.

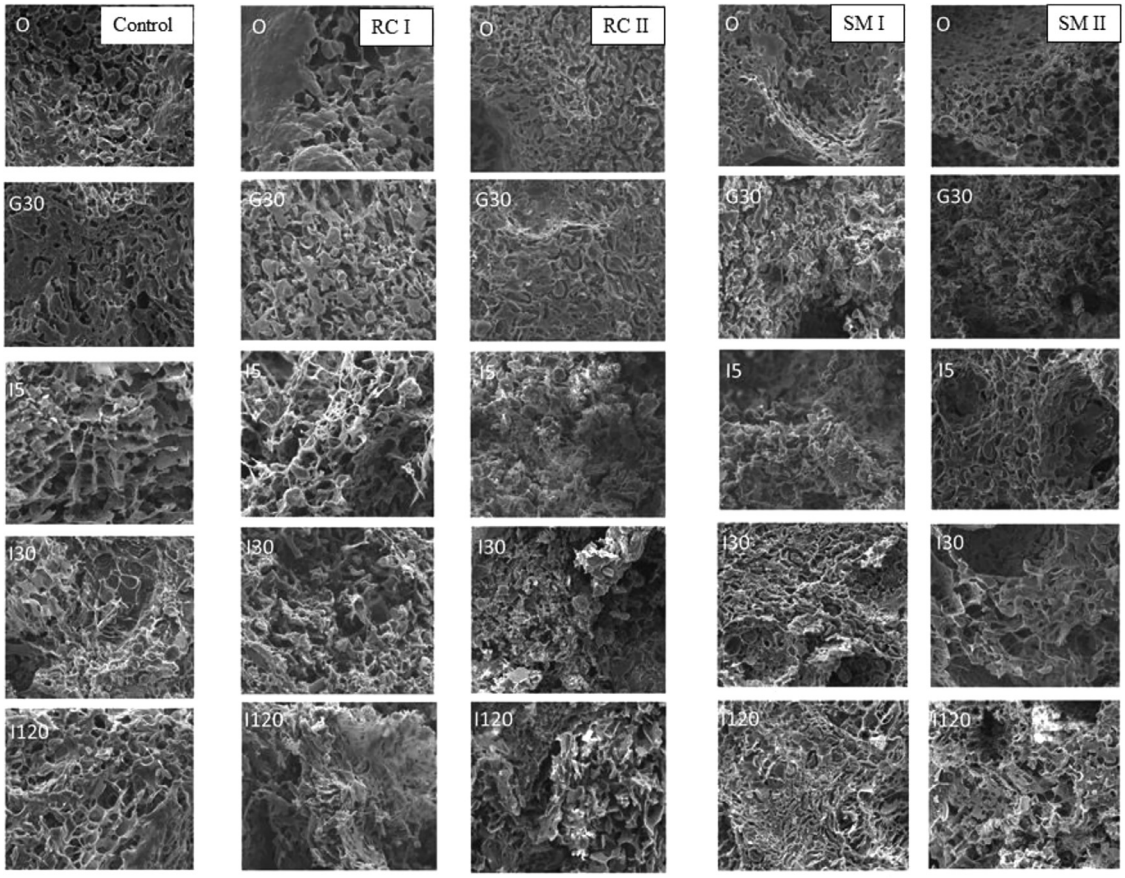

Fig. 5. Microstructure of HPCSB digests after O, G30, I5, I30, and I120 min of gastro-small intestinal digestion *in vitro* of HPCSB.
RC I and RC II, samples containing rennet casein at 14% & 33% (dry wt. of added protein (excluding gluten)/dry wt. of wheat flour), respectively; O, represents oral conditions; G30 represent 30 min of digestion in gastric conditions whereas I 5, 30 & 120 represent 5, 30 &120 min of digestion in small-intestinal conditions after 30 min of gastric digestion; SMI and SMII, samples containing soy protein isolate and milk protein concentrate at 7%, 5% & 7%, 8% (dry wt. of added protein (excluding gluten)/dry wt. of wheat flour), respectively.

teins and amorphous regions of starch, and due to water competition between polymers, which makes it hard for the starch to gelatinise, consequently decreasing its hydrolysis (Bravo et al., 2019; Kaur et al., 2005; Mohamed et al., 2003). Protein coating reduces starch accessibility and therefore influences enzyme susceptibility. More specifically, protein fractions, such as albumin, globulin and glutenin, were observed as glued into the bread matrix, surrounding starch granules and act as a barrier against starch digestion (Ezeogu et al., 2008; Hamaker & Bugusu, 2003; Fig. 2). Many other studies have also reported the presence of protein to be a barrier to starch digestion (Petitot et al., 2009; Ren et al., 2016).

3.3. Microstructural characteristics of digesta

3.3.1. Oral digestion

As shown in Fig. 5, after oral digestion (O), the structure of the control bread and HPCBs appeared to be more porous when compared to the undigested bread samples (Fig. 2). This could be due to the hydrolysis of the gelatinized starchy mass, which is more easily accessible for hydrolysis by α-amylase as compared to semi-gelatinised and ungelatinised starch (Zhang & Hamaker, 2009). During oral processing, control samples showed a higher extent of porosity in their structure when compared with HPCBs.

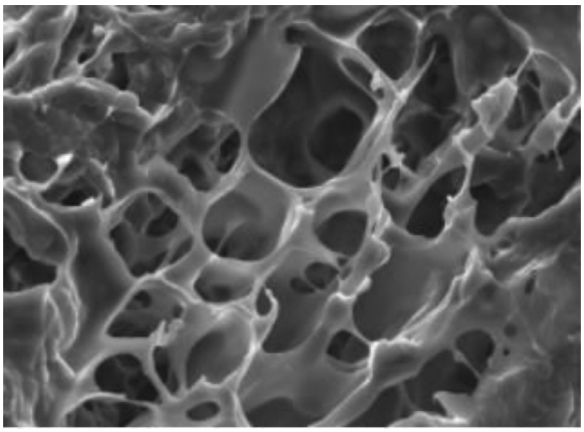

Fig. 6. Microstructure of the control sample after 30 min of gastric digestion followed by120 min of small intestinal digestion *in vitro*.

3.3.2. Gastric digestion

The matrices of all the samples remained intact during 30 min of gastric digestion (G30), with HPCB matrices presenting a more irregular and compact structure compared to control bread. The presence of additional proteins seemed to have wrapped around the starch granules.

3.3.3. Small intestinal digestion

The structure of the control sample appeared to loosen up during small intestinal digestion whereas the remaining structure during the gastric phase was further digested by the action of SIF (containing invertase and amyloglucosidase). At 30 min, as shown in Fig. 5 (I30, control), the structure became more porous and damaged. Spherical voids were left behind as more starch was hydrolysed. At the end of the digestion (I120), there was barely any gelatinized starch, either in the form of granules or gelatinised mass that could be visualised as most of it was hydrolysed, leaving behind a protein network. The honeycomb-like structures shown at I120 for the control sample in Fig. 5 may represent a cross-linked gluten network (Fig. 6), which formed during dough making (Fig. 1). In this process, gluten forms a three-dimensional protein network and cross-links due to the presence of disulphide bonds (Veraverbeke & Delcour, 2007).

For HPCBs, the structure was more intact than in control samples as observed during the first 5 min of small intestinal digestion (Fig. 5, I5). Starch granules entangled in the protein matrix were visible, particularly in the HPCBs with higher levels of protein addition. This may have happened due to more protein engulfing starch granules and possibly preventing them from being hydrolyzed (Fig. 4a and b).

A higher number of undigested starch granules were observed at the end of the small-intestinal digestion phase for HPCSB samples. Open voids were barely observed for RC-containing samples, which appeared more compact and denser compared to SM-containing samples and the control (Fig. 5, RC I & RC II, I120). Additionally, the RC protein skeleton structure was more continuous compared to SM-containing samples. This might be the reason for RC being capable of protecting starch from enzymatic hydrolysis and could account for the overall lower starch hydrolysis of RC than SM-containing samples (Fig. 4a and b).

In conclusion, for the control sample, the enzymes were able to penetrate the structure, leading to a sharper increase in starch hydrolysis compared to HPCBs (Fig. 4a and b). The bread structure was more compact for HPCBs, resulting in a tardy increment and lower final hydrolysis rates, particularly for RC-containing samples.

4. Conclusions

The microscopy of both dough and breads indicated that the microstructure of high protein doughs and breads was more compact and denser compared to the control. The protein matrix seemed to behave like an obstacle around the starch granules, protecting the starch from enzymatic hydrolysis. Therefore, the addition of protein particularly rennet casein at higher levels showed a reduction in the *in vitro* starch hydrolysis markedly. The sample with the highest protein content (RC II) showed a decrease in the eGI from 84.96 (control) to 81.92. The microstructure images of HPCSBs during gastro-small intestinal *in vitro* digestion showed that the protein matrix of HPCSBs engulfed the starch granules, leading to the restriction of starch granule swelling, in turn, decreasing the starch hydrolysis and eGI.

Ethical statement

No animals or humans experimentation was involved in this study.

Funding

This research was supported by funding from Riddet CoRE (NZ) and the School of Food and Advanced Technology, Massey University (NZ).

Declaration of Competing Interest

Authors declare no conflict of interest.

Data availability

Data will be made available on request.

Acknowledgments

The authors would like to thank Dr Matthew Savoian (Manawatu Microscopy and Imaging Centre, Massey University, Palmerston North, New Zealand) for assistance with scanning electron microscopy.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fhfh.2022.100111.

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