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Microstructural changes to proso millet protein bodies upon cooking and digestion

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Abstract

Cooking results in a drastic decline in digestibility of proso millet proteins, panicins. Scanning electron and confocal microscopy were used to observe morphological changes in proso millet protein bodies upon cooking and digestion that could be associated with the loss in digestibility. Spherical protein bodies (1–2.5 mm) were observed in proso millet flour and extracted protein. Cooking did not result in any noticeable change in the size or shape of the protein bodies. However, upon digestion with pepsin the poor digestibility of cooked proso millet protein was clearly evident from the differences in microstructure of the protein bodies: large cavities were observed in the uncooked protein bodies while cooked protein bodies had only tiny holes. When proso millet was cooked in 8M urea and then digested, the protein bodies appeared similar to uncooked digested protein bodies. The morphological changes observed in proso millet protein upon cooking and digestion did not show any visible aggregates, but the inability of pepsin to digest cooked protein bodies was clearly evident under microscopy and is in agreement with the chemical analyses reported previously.

Keywords: Scanning electron microscopy, Confocal microscopy, Wet milling, Enzymatic hydrolysis

1. Introduction

Millets are a group of small seeded grains known for sustaining agriculture and ensuring food security in semi-arid regions (Amadou et al., 2013). The production and cultivation of millets is comparatively new to the western world and they are mostly cultivated to provide agricultural benefits rather than nutritional advantages (Lyon and Baltensperger, 1995). In the past few years there has been a rising interest in the nutritional quality of millets mainly due to the abundance of phytochemicals (phenolics and flavonoids) and their gluten free protein profile (Amadou et al., 2013).

Among different millet varieties (finger, foxtail, little, pearl etc.), proso millet (*Panicum miliaceum*) is the only millet variety grown on a commercial scale in the US. The majority of this crop is used as bird feed but recently there has been an increased interest in proso millet for human food due to the rapidly growing gluten free foods market (McDonald et al., 2003). Being gluten free with a protein content similar to wheat and higher than commonly consumed gluten free crops, proso millet is an ideal food choice for people with Celiac disease and individuals with gluten sensitivity. Thus, many researches are focused on ensuring nutritional adequacies of proso millet as human food or developing novel foods from proso millet (Taylor et al., 2014; Gulati et al., 2016; McSweeney et al., 2017).

Previously, we reported a unique property of proso millet protein that could be a matter of concern when promoting the crop as a gluten free food (Gulati et al., 2017). Specifically, we found that there was a significant decline in digestibility (more than 50%) of proso millet protein when it was heated above 55 °C. The effect observed was similar to the decrease in digestibility reported for sorghum proteins (Hamaker et al., 1986), but more dramatic and with a different mechanism of action. Rather than being driven by disulfide bond formation as in sorghum, the digestibility of proso millet proteins declines upon heating due to intramolecular hydrophobic protein aggregation (Gulati et al., 2017).

The storage proteins of cereals are present along with minerals and enzymes required during seed germination in subcellular spherical organelles called protein bodies. Protein bodies typically have diameters ranging from 0.5 to 2.5 mm. Cereal protein hydrolysis by enzymes

appears as protein body degradation initiated either at the periphery (from external enzymes) or internally which leaves behind large cavities (Ashton, 1976). Several researchers have reported the presence of spherical protein bodies (up to 2.5 mm in diameter) in proso millet and their association with starch granules (Jones et al.,1970; Zarnkow et al., 2007) but there has been no report on the morphological changes or appearance of these protein bodies when subjected to heating or enzymatic hydrolysis.

In the present study, microscopy was used to examine morphological changes that occur in proso millet protein bodies upon cooking both in water and urea. Based on our chemical findings we expected to observe 1) aggregates of protein bodies upon cooking as a result of hydrophobic association and 2) visual evidence of the inability of enzymes to hydrolyze cooked proso millet protein bodies. The objective of this research was to strengthen our understanding of temperature-induced changes in panicins that can help in preventing the loss in digestibility.

2. Materials and methods

2.1. Materials

Commercially available de-hulled proso millet grains were obtained from Clean Dirt Farms (Sterling, CO, USA) and milled using cyclone sample mill (UDY, Fort Collins, CO, USA) with a screen size of 1 mm. The flour was stored at 4 °C until analysis. Proso millet protein and starch were extracted from proso millet grains using a wet milling method (Xie and Seib, 2000) as modified by Gulati et al. (2017).

The flour and protein and starch fractions were analyzed for ash, fat, moisture, protein, and starch using approved methods (AACC International, 1999). Protein content was analyzed using a nitrogen analyzer (FP 528, Leco, St. Joseph, MI, USA) with a protein factor of 6.25. Total starch content was analyzed using total starch assay kit (K-TSTA, Megazyme, Bray, Ireland) following the KOH format.

2.2. Cooking

Four hundred milligrams of flour, 200 mg protein, or 2 g starch, were suspended in 5 mL of water or 8M urea in a centrifuge tube and heated at 100 °C for 20 min (time recorded after reaching boiling temperature) with intermittent mixing. After heating, the samples were cooled to room temperature and then either used directly for digestibility measurements or frozen at −80 °C for further analysis.

2.3. In vitro protein digestibility

Pepsin digestibility of cooked (water and urea) and uncooked proso millet flour and protein was measured using the residue method developed by Mertz et al. (1984) as described by Gulati et al. (2017). After digestion, the pellet was freeze dried (FreeZone 6, Labconco, Kansas City, MO) and used for microscopic analysis.

2.4. Scanning electron microscopy

A thin uniform layer of freeze-dried sample (cooked and uncooked millet flour, protein and starch) was fixed on an aluminum stub (26mm diameter, 6mm height) by tapping the sample tubes on adhesive conductive carbon tape (EMS, Hatfield, PA) and gently blowing off the extra sample using pressurized air. Samples fixed on the stub were kept overnight in a vacuum oven (Model 5831; NAPCO scientific, Tualatin, OR) at 20 KPa and 40 °C to remove any residual moisture. The dried samples were then sputter coated with chromium under an argon atmosphere using a Denton desk V TSC sputter apparatus (Denton Vacuum LLC, Moorestown, NJ) for 15 min (mean thickness of coating was 4–5 nm).

A field-emission scanning electron microscope (SEM) was used to study the morphological changes in millet proteins and starch upon cooking and digestion (Hitachi, S4700, Hitachi America Ltd., Tarrytown, NY) at an accelerating voltage of 5 kV and an emission current of 5 mA. Samples were studied under different magnifications ranging from 500x to 10,000x and images were captured using built-in software (HI-S027-0003, Version 3.8). The size of protein bodies was determined using image processing and analysis software (ImageJ, 1.51s, National Institute of Health, USA).

2.5. Confocal laser scanning microscopy

A thin uniform smear of millet flour, protein or starch sample in water was placed on a clean glass slide and covered with a cover glass and observed under Nikon A1 confocal laser scanning microscope (CLSM) mounted on a Nikon 90i upright fluorescence microscope (Nikon Instruments Inc., Melville, NY) at approximately 1200x magnification. The samples were subjected to an excitation wavelength of 405 nm and the protein auto-fluorescence was detected using a pseudo green colored filter at emission wavelength ranging between 425 and 475 nm. The transmitted light detector was used with a 561.4 nm laser. In order to confirm the observed auto-fluorescence was emitted by proteins in millets and not other substances, the protein and starch samples were stained with Fast Green FCF (Sigma-Aldrich, St. Lois, MO USA) at a concentration of 0.025 mg/mL in water for at least 15 min. The stained samples were excited at 561.6 nm and red fluorescence was detected at 570–620 nm. Images were processed using confocal acquisition software (NIS-Elements 4.4.0, Nikon Instruments Inc., Melville, NY).

3. Results and discussion

3.1. Sample composition

The proximate composition of de-hulled proso millet flour and protein and starch fractions is shown in **Table 1**. Similar to other cereal grains, starch was the major component of millet flour while proteins constituted the second largest component. The protein fraction obtained by

Table 1. Compositional analysis of proso millet flour and protein and starch fractions.[a]

Sample	*Moisture*	*Protein*	*Starch*	*Fat*	*Ash*
Flour	8.12 ± 0.03	13.6 ± 0.0	71.9 ± 0.1	3.32 ± 0.06	1.17 ± 0.00
Protein	2.43 ± 0.01	82.5 ± 0.6	ND	11.9 ± 0.3	0.57 ± 0.00
Starch	1.22 ± 0.05	5.72 ± 0.21	89.9 ± 0.2	0.35 ± 0.07	0.37 ± 0.01

a. Mean ± SD (% wb); n = 3; ND, not detected.

wet milling of millet grains was composed of 80% protein and 11% fat while no starch was detected. On the other hand, the starch fraction contained about 90% starch, 6% protein and trace amounts of inorganic matter and lipids. The high protein content in the starch fraction was likely because of the similarity in size and density of some of the starch granules and protein bodies, which made their physical separation difficult.

3.2. Morphology of proso millet flour, protein, and starch

Starch granules and protein bodies were the main components visible when proso millet flour was observed under SEM (**Fig. 1**a and b). The starch granules were polygonal in shape and were cohesively joined to one another resulting in compound starch granules similar to those found in oats and rice (Thomas and Atwell, 1999). In proso millet flour, mainly two size of starch granules were observed: A-type (>9.9 mm) and B-type (<9.9 mm) (Yu et al., 2014). Spherical protein bodies were observed in crevices of compound starch granules observed mainly at higher magnifications.

The protein bodies isolated using wet milling appeared as nonuniform clusters (Fig. 1c and d). The clustering of the protein bodies may have been important, but was more likely a result created during extraction or sample preparation. Protein bodies with <2.5 mm diameter and 2.5–5 mm diameter were observed. Starch was not detected in protein samples by chemical analysis (Table 1), but granules that were probably starch, with a distinct hexagonal shape and large size when compared with protein bodies, were observed in SEM. These were likely present below the limit of detection of the chemical analysis.

In the starch sample both compound starch granules and individual starch granules, probably broken from their compound structure during milling, were observed (Fig. 1e and f). A third size of starch granule similar to that of the protein bodies (<2.5 mm) was also observed in these samples, but was not noticed in the flour samples. Some of the larger starch granules had depressions on their surface that have been reported as a characteristic of starches from the Panicoideae subfamily to which proso millet belongs (Fannon and Bemiller, 1992). These indentations have been claimed as sites where small starch granules and protein bodies associate with larger starch granules (Zarnkow

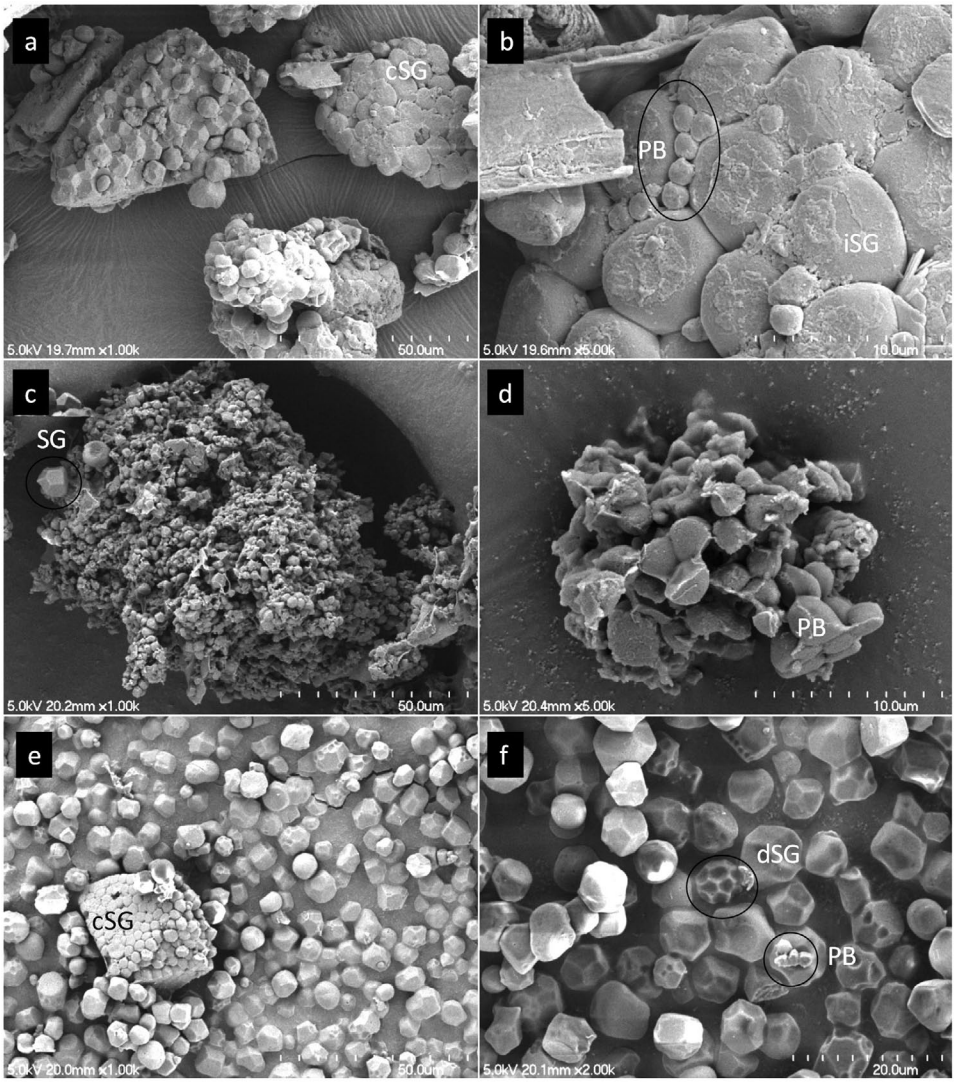

Fig. 1. Morphology of uncooked proso millet flour at 1000x (a) and 5000x (b) magnification, proso millet protein at 1000x (c) and 5000x (d) magnification, and proso millet starch at 1000x (e) and 2000x (f) magnification. PB, protein body; SG, starch granule; iSG, individual starch granule; cSG, compound starch granule; dSG, dented starch granule.

et al., 2007). The results obtained for morphology of starch granules and protein bodies of proso millet were similar to previous reports (Zarnkow et al., 2007; Serna-Saldivar and Rooney, 1995).

3.3. Morphological changes upon cooking

Upon cooking, proso millet flour and starch samples appeared as web-like networks of gelatinized starch when observed under SEM (**Fig. 2**a and e). At higher magnifications, the protein bodies could be seen intact and embedded in the starch network (Fig. 2b and f). In the protein

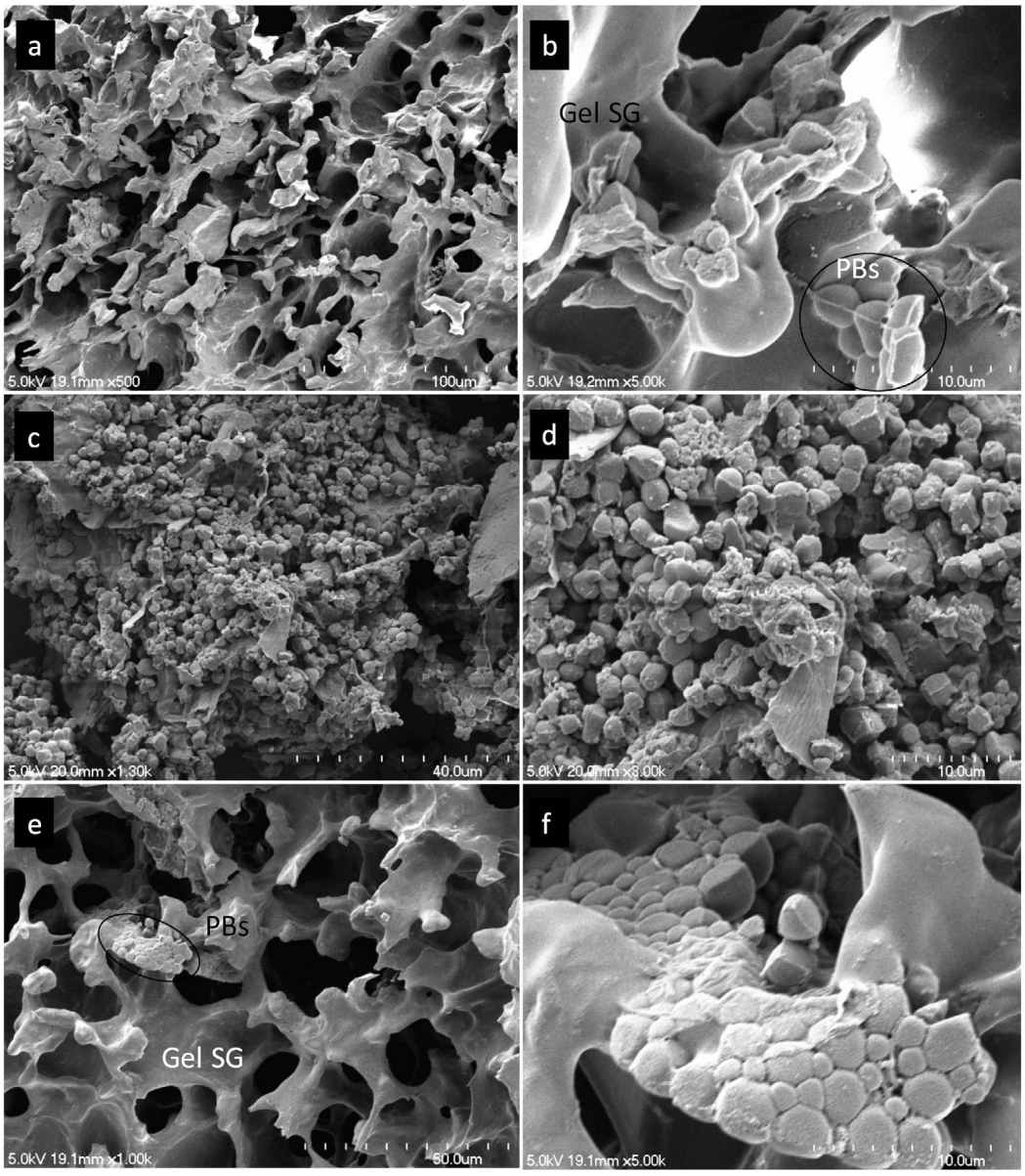

Fig. 2. Morphology of cooked proso millet flour at 500x (a) and 5000x (b) magnification, proso millet protein at 1300x (c) and 8000x (d) magnification, and proso millet starch at 1000x (e) and 5000x (f) magnification. PB, protein body; Gel SG, gelatinized starch granule.

samples the protein bodies did not show any visible change in structure upon cooking (Fig. 2c and d).

The loss in starch granular structure upon cooking due to gelatinization has been reported for other grains like maize and rice (Hu et al., 2011; Utrilla-Coello et al., 2013). Also, researchers have reported through microscopic and chemical analyses that protein bodies in cereals do not lose their structure upon cooking (Tanaka et al., 1978), which supports our observations. This is likely because the storage proteins of cereals are arranged along with other components in crystalloid subunits inside spherical protein bodies (Ashton, 1976). Thus, changes taking place inside the protein bodies could not be viewed by SEM as it is a tool for surface visualization rather than internal imaging.

The structures observed by SEM before and after cooking were supported by images from CLSM (**Fig. 3**). Cooked and uncooked samples of protein fractions were observed using both autofluorescence and fluorescence after protein staining. For autofluorescence, a green filter was used to detect proteins (Fig. 3a and e), while fluorescence

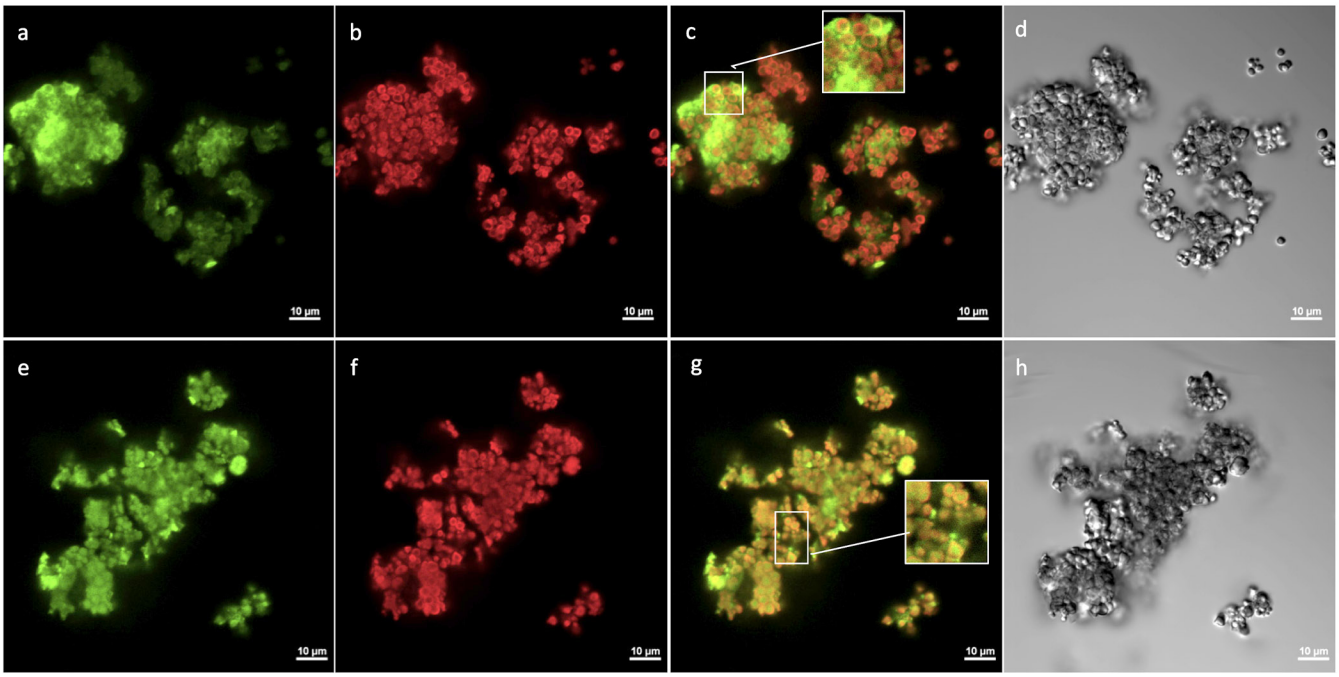

Fig. 3. Images of uncooked (a–d) and cooked (e–h) proso millet protein bodies under confocal microscopy at 1200x magnification; auto-fluorescence (a, e), red fluorescence after staining (b, f), merged auto- and stained fluorescence (c, g), and transmitted light image (d, h); scale bar: 10 mm.

of stained samples was observed in the far-red region (Fig. 3b and f). The auto-fluoresced and stained images merged well when fitted on top of one another (Fig. 3c and g), suggesting that the spherical bodies observed under both SEM and CLSM were indeed protein bodies. There were no differences observed in light images of uncooked and cooked protein bodies.

One striking difference observed in stained and unstained images of protein bodies (both cooked and uncooked) was that the dye stained protein bodies only on the periphery while autofluorescence (green) was mainly concentrated in the core (inserts in Fig. 3c and g). This could be due to the arrangement of amino acids in protein bodies. Fast green dye has a greater affinity towards basic amino acids (Tas et al., 1980) which may be exposed on the surface of the protein bodies while intrinsic fluorescence of proteins is mostly linked to aromatic amino acids (Eftink, 2000) which are hydrophobic and would likely be embedded in the core. Autofluorescence can also be linked to other aromatic compounds like phenolic acids and tannins, although it was unlikely that these compounds were responsible for the auto-fluorescence in the present experiment due to the low concentrations of phenolics and tannins in these samples (Gulati et al., 2017).

3.4. Microstructural change to proso millet flour and protein after digestion

A significant change in protein body morphology was observed when uncooked and cooked samples were digested with pepsin (**Fig. 4**). In uncooked proso millet protein samples (Fig. 4a and b), the protein bodies appeared shrunken with huge cavities or craters on their surface that appeared as a result of enzymatic hydrolysis. Similar peripheral enzymatic degradation of protein bodies has been reported previously for sorghum and yellow foxtail grass (Rost, 1972; Rom et al., 1992), but they were not as intense as those observed in our study. Similar structures have been observed when starches are digested by amylolytic enzymes, which suggests a common mode of hydrolysis by these enzymes on their substrates (Uthumporn et al., 2010).

On the other hand, when the protein bodies were digested after cooking (Fig. 4c and d) they displayed only tiny holes on their surface indicating the inability of pepsin to digest cooked proso millet protein bodies. Based on the nitrogen content in these samples

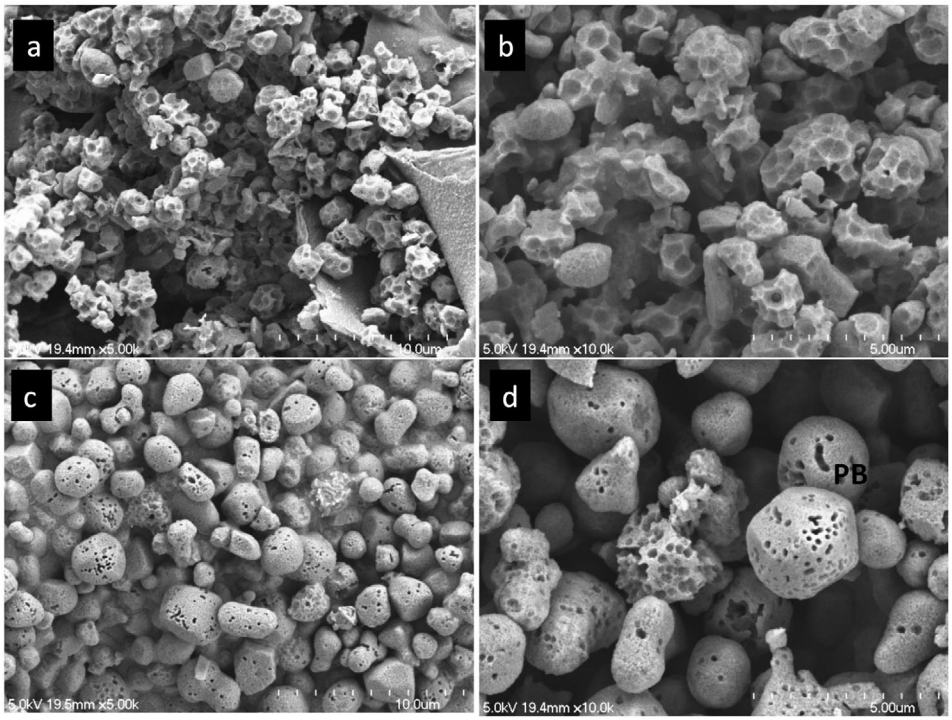

Fig. 4. Morphology of uncooked proso millet protein after digestion at 5000x (a) and 10000x (b) magnification, and cooked proso millet protein after digestion at 5000x (c) and 10000x (d) magnification. PB, protein body.

before and after digestion, protein digestibility of 79.7 ± 0.8% and 36.6 ± 1.5%was recorded for uncooked samples and cooked samples, respectively.

When these samples were observed using CLSM (images not shown), the results were not as prominent as SEM but there was diminished auto-fluorescence of proteins in uncooked proso millet flour and protein when compared to cooked samples following digestion. The reduced fluorescence is an indication that protein bodies do not have the same fluorescence properties as intact protein bodies. Also, since cooked samples were not digested they still maintained their auto-fluorescence.

When samples were cooked in 8M urea (**Fig. 5**), the protein bodies appeared to have digested the same way as raw protein bodies. We have reported that heating initiates denaturation of proso millet protein which exposes hydrophobic amino acids during a partial state of denaturation (Gulati et al., 2017). However, when the water

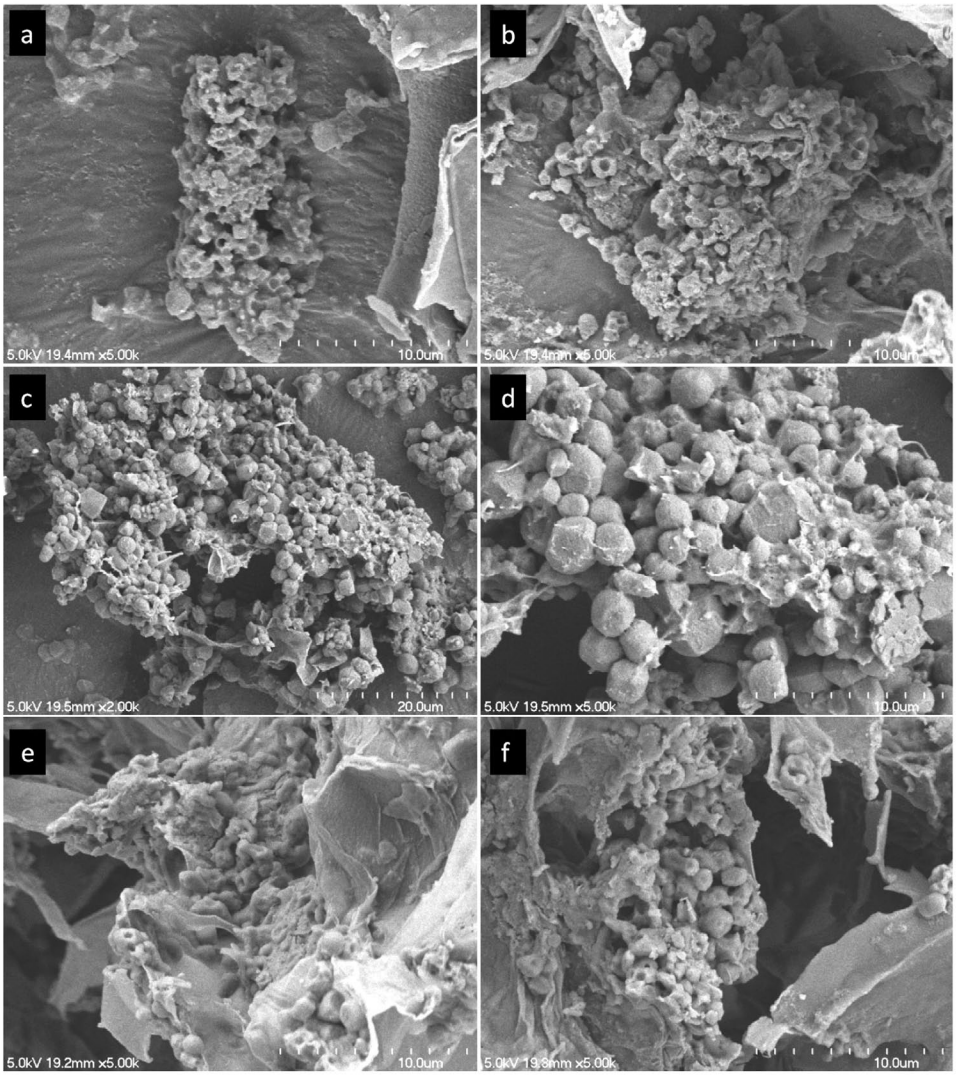

Fig. 5. Morphology of uncooked proso millet protein bodies in 8M urea after digestion at 2000x (a) and 5000x (b) magnification, cooked proso millet protein bodies in 8M urea before digestion at 2000x (c) and 5000x (d) magnification, and cooked proso millet protein bodies in 8M urea after digestion at 2000x (e) and 5000x (f) magnification.

is replaced by 8Murea it prevents the formation of hydrophobic interactions and results in high digestibility of protein even after cooking. The present microscopic images are a visualization of those previous findings.

4. Conclusion

Small, spherical protein bodies ranging from 1 to 2.5 mm in diameter were observed in proso millet flour and protein samples by both SEM and CLSM. Based on our chemical analyses we expected to observe aggregates of protein bodies upon cooking as a result of hydrophobic association and some visual evidence of the inability of enzymes to hydrolyze cooked proso millet protein bodies. When observed under SEM, protein bodies appeared as random clusters that were visually unchanged upon cooking. However, there was clear evidence that cooking reduced pepsin hydrolysis, which was observed as tiny holes on the surface of protein bodies after digestion compared with large craters appearing in the uncooked digested protein bodies. As expected, when samples were cooked in 8M urea and then digested the protein bodies had the same large cavities as observed for uncooked protein bodies after digestion. Thus, the visualization of proso millet protein bodies using microscopy provided conformational support for our chemical findings regarding the unique structure of millet storage proteins, panicins, and demands that future work should be focused on mitigating this effect.

Conflicts of interest — We (the authors) declare no competing interests.

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