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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 μm) 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 8 M 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.

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

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have diameters ranging from 0.5 to 2.5 μm. 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 μm 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 8 M 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 (26 mm diameter, 6 mm 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 μA. 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 μg/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 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. 1a and b). The starch granules were polygonal in shape and were cohesively joined to one another resulting in compound starch

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.