

IMPROVEMENT OF PEARL MILLET (*Pennisetum glaucum* (L.) R. BR) PROLAMIN EXTRACTABILITY: CHROMATOGRAPHIC SEPARATION, CHARACTERIZATION AND FUNCTIONAL PROPERTIES

Rafika Bibi^{1,2}, Hind Mokrane^{1*}, Khaled Khaladi^{1,2}, Houria Amoura¹

Address(es): Prof. Hind Mokrane, PhD.

¹Laboratoire des produits bioactifs et de valorisation de la biomasse, Ecole Normale Supérieure, B.P. 92, 16050 Vieux-Kouba, Algiers, Algeria, phone number:00213555253835.

²Département des sciences de la matière, Université Alger 1 Benyoucef Benkhedda, Algiers, Algeria.

*Corresponding author: hind.mokrane@g.ens-kouba.dz or mokraneh2017@gmail.com

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ABSTRACT

Pearl millet is a gluten free cereal resistant to drought, diseases and pests. The major protein fraction in pearl millet is prolamin the alcohol soluble fraction called pennisetin. Researches on the factors affecting pennisetin extraction and on its functional properties are still very scant. In this paper, the effect of temperature, reducing agents (β -mercaptoethanol, dithiothreitol or sodium metabisulfite) and sodium hydroxide on pennisetin extraction was assessed. Samples were characterized by protein and amino acid (AA) analysis, SDS-Page and reversed phase high-performance liquid chromatography (RP-HPLC). Pennisetin was extracted with high protein purity (90.1%) from pearl millet grain flour (PMF) using 70% aqueous ethanol containing 1% sodium metabisulfite and 0.2% sodium hydroxide at 60°C. SDS-Page confirmed pennisetin extraction and showed three subunits corresponding to the 27-, 22- and 12 kDa-pennisetins. The percentage of essential amino acids in pennisetin (68.55%) was higher than that in PMF (41.64%). The hydrophobic character of pennisetin was confirmed by the presence of 45.49% of hydrophobic amino acids. Functional properties of pennisetin and PMF were investigated. Compared to PMF, pennisetin exhibited comparable oil and water binding capacities and higher foaming capacity and emulsifying activity index but with lower emulsion stability. Pennisetin functional properties were similar or lower relatively to common used proteins of wheat, sorghum, rice or peanut. Excluding water, oil binding and foam capacities, all the functional properties of pennisetin and PMF were significantly different ($p \leq 0.05$). The findings suggest increasing the use of pennisetin and PMF as nutritional and health promoting agent for vegans and celiac patients.

Keywords: Pearl millet, Effect of solvent, Pennisetin extraction, RP-HPLC, Functional properties

INTRODUCTION

Pearl millet [*Pennisetum glaucum* (L.) R. Br.] is an important cereal crop classified sixth in term of consumption after wheat, rice, maize, barley and sorghum. Mostly it is grown in the arid and semi-arid tropics of south Asia, sub-Saharan Africa, China, Russia and Latin America (Bora *et al.*, 2019; Ranasalva and Visvanathan, 2014; Sarita, 2016). Pearl millet is a drought-resistant crop with short growing season and good resistance to diseases and pests (Sarita, 2016). This gluten free cereal contains higher levels of essential amino acids (AA) than maize, rice, wheat and sorghum particularly methionine, cysteine and lysine (Akinola *et al.*, 2017; Kasaoka *et al.*, 1999; Mokrane, 2010). It is rich in dietary fibers, minerals, unsaturated acids and bioactive proteins (Bora *et al.*, 2019; Esfandi *et al.*, 2019; Gwamba *et al.*, 2019). The protein concentration of pearl millet cultivars measured so far by several researchers varied from 9 to 17% and could reach for some samples 21% (Kasaoka *et al.*, 1999; Marcellino *et al.*, 2002), while it varied from 6.8 to 7.4% in rice (Kasaoka *et al.*, 1999), 11 to 16% in sorghum (Amoura *et al.*, 2020; Mokrane *et al.*, 2009) and 9.8-17.9% in wheat (Hajas *et al.*, 2018). As in almost all cereals, the most abundant seed protein fraction in pearl millet is prolamin: a class of alcohol-soluble proteins. This protein called pennisetin exceeded 40% of the total seed protein fraction. Like other cereal prolamins such as gliadin of wheat, kafirin of sorghum and zein of maize, pennisetin is rich in proline and other hydrophobic amino acids (Sainani *et al.*, 1989; Schalk *et al.*, 2017). SDS-Page of pennisetin exhibited three distinct bands with molecular masses of 27, 22 and 12kD (Marcellino *et al.*, 2002; Ricks, 2007). Pennisetins were similar in composition and sequence to α -prolamins from maize and sorghum, but this amino acid similarity was not as high to be called α -, β -, γ - or δ -prolamins like sorghum kafirins or maize zeins (Adebisi *et al.*, 2017; Marcellino *et al.*, 2002; Ricks, 2007).

In the two last decades, several studies have aimed to improve cereal protein extractability. Chromatography and SDS-Page were used to investigate the factors affecting cereal prolamin extraction such as extraction time, pH, detergent

type, reducing agent type and sample-to-solvent ratio (De Brier *et al.*, 2015; Gessendorfer *et al.*, 2010; Hamaker *et al.*, 1995; Nałecz *et al.*, 2017; Park and Bean, 2003; Redant *et al.*, 2017). To date only few studies have been conducted on the factor affecting pearl millet proteins extractability. Chandna and Matta (1990) characterized the seed protein extract of eight pearl millet lines by SDS-Page and two dimensional electrophoresis. Later, Marcellino *et al.* (2002) reported the use of 55% aqueous isopropanol containing β -ME to extract pennisetins and characterized them by SDS-Page, bidimensional gel electrophoresis, MALDI-TOF/MS and RP-HPLC. In a similar study, Ricks (2007) adapted a method used for zein extraction to isolate pennisetin in a single extraction step and separated it using SDS-Page. Then, Mokrane (2010) characterized the protein fraction of eleven pearl millet cultivars by RP-HPLC, SE-HPLC and SDS-Page with emphasis to their prolamins without examining the impact of solvent, reducing agent and temperature. Functional properties of pearl millet grain flours (PMF) or other millet varieties (Foxmail, Little, Barnyard, Koda, Proso and Finger) have been studied in their native form or as affected by processing (Akharume *et al.*, 2020; Akinola *et al.*, 2017; Ali *et al.*, 2012; Hassan *et al.*, 2007; Kamara *et al.*, 2009; Kamara *et al.*, 2010), however few studies aimed to investigate the functional properties of extracted pearl millet proteins particularly the prolamin fraction: pennisetin (Sainani *et al.*, 1989; Taylor *et al.*, 2016).

Due to the above mentioned reasons, the objective of this study was to investigate factors affecting pearl millet pennisetin extraction using RP-HPLC. Pennisetin was characterized by protein analysis, SDS-Page and AA analysis. Water and oil binding capacities, gelling, foaming and emulsifying properties of extracted pennisetin and PMF were investigated.

MATERIAL AND METHODS

Sample preparation

Pearl millet samples from local cultivars of Bechna Beldia were harvested in 2014 from In-Salah situated in Tidikelt Region in Southern Algerian Sahara. This cultivars is growing under harsh conditions characterized by low rainfall.

Pearl millet grains were sorted and screened to remove undesirable material such as dust, broken grains and debris. The grains were then ground to flour in a commercial coffee grinder for 30s. The flour was further sieved over a 500 µm sieve and then defatted with n-hexane (1:10 w/v) for 24h at room temperature with continuous stirring. PMF was recovered using an air Buchner funnel under a hood overnight. All chemicals and reagents were of analytical grade and from Sigma-Aldrich.

Protein Analysis

Protein analysis of extracted samples was performed by micro-Kjeldahl method of the Association of Official Analytical Chemists (AOAC) (AOAC, 1990) using 6.25 as conversion factor. Moisture content was determined according to American Association of Cereal Chemists (AACC) methods 44-15A (AACC, 2000). The amount of extracted protein was deduced on dry matter basis (dmb) from the initial protein content and the remaining protein content after extraction.

Pennisetin extraction

Pennisetin was extracted from 500 mg of PMF (1h) using a modified method of Espinosa-Ramirez et al. (2016) previously described for sorghum kafirin extraction. The basic solvent consisted of 70% aqueous ethanol containing a reducing agent. The effect of three reducing agents at different concentrations was investigated: dithiothreitol (DDT) or Sodium metabisulfite (MBS) (0.25, 0.5 or 1% (w/v)) and β-ME (0.25, 0.5 or 1% (v/v)).

Four temperatures 30, 40, 50 and 60°C were tested. The effect of adding Sodium hydroxide (NaOH) was assessed at different concentrations varying from 0 to 0.4% (w/v). All the obtained suspensions were centrifuged at 3000×g for 5 min and the supernatants were saved for further analysis by RP-HPLC and SDS-Page. For RP-HPLC analysis, samples were filtered (0.45 µm, nylon, Millipore) before storage in the freezer.

For protein and AA analysis and functional properties determination, pennisetin was precipitated and dried from the supernatant with the highest protein content as described in the following. First, ethanol concentration in the supernatant was brought below 20% (v/v) by adding distilled water. pH was then adjusted to 4.5 with hydrochloric acid HCl (1 M). The suspension was centrifuged at 3000×g for 10 min and the obtained residue was rinsed 3 times with distilled water and dried in the oven at 40°C overnight. All the extractions were repeated at least three times.

SDS-Page

Pennisetin extracted with or without reducing agent was dispersed in a Tris(hydroxymethyl) aminomethane-hydrochloric acid (Tris-HCl) sample buffer at pH 6.8 containing 125 mM Tris, 30% (w/v) glycerol, 4.0% (w/v) sodium dodecyl sulfate (SDS) and 0.002% (w/v) bromophenol blue. The samples were boiled for 5 min and centrifuged at 11,000×g for 3 min. Electrophoresis was carried out in 20% (w/v) polyacrylamide mini-gel using a PhastSystem unit (GE Healthcare, Uppsala, Sweden), then the gel was silver-stained using the GE Healthcare development Technique file no. 210. The molecular markers (MM) (GE Healthcare) used were α-lactalbumin (14.4 kDa), trypsin inhibitor (20.1 kDa), carbonic anhydrase (30 kDa), ovalbumin (43 kDa), bovine serum albumin (BSA) (67 kDa) and phosphorylase (94 kDa).

Amino acid composition

AA composition of PMF and pennisetin were determined using a high performance anion exchange chromatography with an Integrated Pulsed Amperometric Detection (IPAD) equipped with a gold electrode on an analytical AminoPac PA 10 analytical column (2×250 mm) preceded by an AminoPac PA 10 guard column (2×50 mm) (AAA-Direct Amino Acid Analyzer, Dionex Corporation, Sunnyvale, CA, USA). The operating conditions were previously described by Rombouts et al. (2009) and Mokrane et al. (2010).

Reversed Phase High Performance Liquid Chromatography (RP-HPLC)

Aliquots (100 µl) of the pennisetin were loaded on a Supelco C18 column (5µm, 4.6×250mm, 300Å, Machery-Nagel, Düren, Germany) preceded by a Supelco C18 guard column (2×50 mm) (Machery-Nagel, Düren, Germany). The HPLC system (Shimadzu, Tokyo, Japan) was equipped with LC10 ATVP pump and a SPD 10AVP UV-VIS detector. A Rheodyne 7725 sample injector (Coati, CA, USA) was fitted with a 20µl sample loop. The column was equilibrated at sample loading conditions 25% solvent B (Acetonitrile (ACN) containing 0.1% (v/v)

Trifluoroacetic acid (TFA) and solvent A (demineralized water containing 0.1% (v/v) TFA). Pennisetin fractions were separated at 50°C with a flow rate of 0.5 mL/min using the following gradient from 0 to 40 min, 25% B, from 40 to 46 min, 72% B and from 46 to 70 min, 80% B. The separation was monitored by recording extinction at 214 nm during 70 min. For each sample RP-HPLC analysis were repeated at least three times.

Functional properties of pennisetin and pearl millet whole grain flour

Gelling properties

The least gelling concentration (LGC) determination of PMF and pennisetin at different concentrations 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20% (w/v) was carried out according to the method of Sathe et al. (1982). Samples were heated for 1 h in boiling water followed by cooling in ice and further cooling for 2 h at 4°C. LGC was defined by the concentration of sample that did not fall down or slip when the test tube was inverted.

Water binding capacity and oil binding capacity

Water binding capacity (WBC) and oil binding capacity (OBC) were determined using the method of Beuchat (1977). One gram of PMF or pennisetin was weighed into a pre-weighed centrifuge tube and mixed with 10 mL of distilled water for WBC or 10 mL of corn oil (Sigma-Aldrich) for OBC. Samples were vortexed for one min and allowed to stand for 30 min at 25 ± 2°C before being centrifuged at 4000×g for 25 min. Excess water was removed by inverting the tubes over absorbent paper and samples were dried before being weighted. WBC was expressed as grams of water per gram of dry sample and OBC was expressed as grams of oil per gram of dry sample.

Foaming properties

Foaming capacity (FC) and foam stability (FS) of PMF and pennisetin were determined according to the method of Coffman and Garcia (1977). FC was measured in term of percentage of volume increase after whipping reported to original volume of the liquid. Foam stability (FS) was expressed as percentage of foam volume remaining after 1, 5, 10, 20, 30, 40, 50 and 60 min related to initial foam volume at room temperature 25 ± 2°C.

Emulsifying properties

The emulsifying properties of PMF and pennisetin were expressed by the emulsifying activity index (EAI) and emulsion stability (ES) as previously defined by Pearce and Kinsella (1978) using the turbidimetric method. EAI (m²/g) was measured after 0, 10 and 30 min and calculated as follows:

$$EAI \left(\frac{m^2}{g} \right) = \frac{2 \times 2.303 \times A \times D}{\Phi \times L \times C} \quad (1)$$

Where A is the absorbance at 500 nm, D is the dilution factor (D=100), Φ is the oil volumetric fraction (Φ = 0.25), L is the curve path length (L =0.01 m), C is the protein concentration of the sample (g/m³).

ES (%) is defined by the percentage of EAI remaining after 0, 10 and 30 min. PMF and pennisetin functional properties were analyzed at least in duplicate.

Data treatment

All statistical analyses were performed using the Statistical Analysis System R 4.0.2. The extraction procedure and the functional properties were carried out at least three times. The analysis of variance of AA composition, reducing agent, NaOH concentration, temperature, WBC, OBC, foaming and emulsifying properties of pennisetin and PMF were performed with a Tukey multiple comparison procedure on a 5% significance level (R Core Team, 2020).

RESULTS AND DISCUSSION

Protein contents

High protein content was obtained in PMF analyzed in this study up to 18.22 ± 0.80 % on dmb with low humidity of 9.0 ± 0.29%. These results are probably deeply related to the drought growing conditions in the city of In-Salah situated in South Algeria. This high protein pearl millet cultivars content is locally known to support harsh growing conditions such as low rainfall and hyper arid climate. In the past ten years (2009-2019) the minimal and maximal average temperatures were 6 to 45°C, respectively and the minimal and maximal average rainfall were 0.20 and 3mm, respectively. According to Ozturk and Aydin (2004) climate and environment have a remarkable effect of the cereal grain yield, ash and protein content. The highest protein content was obtained in the most water stressed accessions. These conclusions were in agreement with the pearl millet cultivars analyzed in the present study.

The level of protein in the pennisetin extract was 90.1 ± 0.20 % on dmb. To the best of our knowledge, this is the highest level of protein obtained in pennisetin extract to date probably because this extraction procedure initially applied for sorghum kafirin by **Espinosa-Ramirez et al. (2016)**, was applied on pearl millet proteins for the first time.

Amino acid composition

Table 1 shows the AA composition of PMF and extracted pennisetin expressed in g of amino acid by 100g of crude protein (%). All AAs in PMF and pennisetin were significantly different ($p \leq 0.05$) in individual AA concentration. The percentage of essential AA in the pennisetin extract (68.55 %) was higher than that in the PMF flour (41.64%).

Pennisetin was rich in Lysine (21.44%), while in the PMF the percentage of Lysine was 11 times lower, this may improve the nutritional quality of the extracted pennisetin. In a previous study, **Chandna and Matta (1990)** have also found high level of Lysine in the prolamin fraction of pearl millet exceeded only by its level in the albumin fraction. They assumed that in stead of screening high lysine pearl millet lines, it would be better to screen high prolamin pearl millet lines.

As shown in table 1, pennisetin is also rich in hydrophobic AA: Valine (22.44%) and Proline (10.24%). Pennisetin was made up of high percentages (45.49%) of hydrophobic AA (Alanine, Isoleucine, Leucine, Methionine, Phenylalanine, Proline and Valine), the remaining AA were basic (34.51%), non polar (12.80 %) and acid (7.20%). Such results show and confirm the hydrophobic properties of pennisetin.

Based on the AA composition of the FAO/WHO (1991) reference protein, lysine and methionine were the most limiting essential amino acid in PMF while Threonine, Phenylalanine and Methionine were the most limiting AA in pennisetin. As compared to sorghum (**Mokrane et al., 2010**), wheat (**Abdel-Aal and Hucl, 2002**) and maize (**Harrigan et al., 2009**), PMF was richer in Lysine, Threonine, Isoleucine, Valine, Serine and Asparagine/aspartic acid and poorer in Proline. The high levels of proteins and essential AA in the extracted pennisetin suggest its potential use as a good source of bioactive peptides or as a peptide concentrate for food. The higher level of hydrophobic AA in pennisetin could enable its potential use as coating material for hydrophobic bioactive compounds.

Table 1 Amino acid composition of pearl millet grain flour and extracted pennisetin expressed in g of amino acid by 100g of crude protein (%)

	Pearl millet grain flour	Pennisetin	FAO/WHO ⁵
Essential amino acids (%)			
Lysine***	2.38 ± 0.05	21.44 ± 0.00	5.8
Threonine***	6.25 ± 0.01	0.00 ± 0.00	3.4
Phenylalanine***	5.13 ± 0.01	0.55 ± 0.56	6.3
Isoleucine***	4.13 ± 0.02	7.74 ± 0.29	2.8
Leucine***	14.80 ± 0.22	4.38 ± 1.47	6.6
Valine***	5.28 ± 0.02	22.44 ± 0.44	3.5
Methionine***	1.39 ± 0.17	0.07 ± 0.14	2.5
Tyrosine***	2.47 ± 0.11	11.92 ± 0.27	
Total Essential amino acids (%)	41.64	68.55	
Non essential amino acids (%)			
Arginine***	10.99 ± 0.11	7.20 ± 0.41	
Alanine***	7.33 ± 0.12	0.07 ± 0.63	
Glycine***	2.66 ± 0.02	0.68 ± 0.09	
Cystine**	0.44 ± 0.08	0.21 ± 0.05	
Proline***	6.12 ± 0.20	10.24 ± 0.53	
Serine***	4.21 ± 0.03	0.00 ± 0.00	
Glutamic acid/ Glutamine***	17.47 ± 0.24	0.65 ± 1.99	
Aspartic acid/ Asparagine***	7.24 ± 0.10	6.55 ± 0.54	
Histidine***	2.04 ± 0.04	5.86 ± 0.16	1.9
Total non essential amino acids (%)	58.16	31.45	
Amino acids percentage with various characteristics (%)			
Acidic amino acids ¹	24.71	7.20	
Basic amino acids ²	15.41	34.51	
Hydrophobic amino acids ³	43.72	45.49	
Non polar amino acids ⁴	15.98	12.80	

Legend: Values indicate the mean of three replicates ± standard deviation. ¹Acidic: Glutamic acid, Aspartic acid, ²Basic: Lysine, Arginine, Histidine, ³Hydrophobic: Alanine, Isoleucine, Leucine, Methionine, Phenylalanine, Proline, Valine, ⁴Non polaire: Glycine, Serine, Threonine, Tyrosine, Cysteine. ⁵FAO/WHO (1991). *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$.

SDS-Page

The pearl millet prolamins showed SDS-PAGE profiles comparable to those in previous reports (**Hadimani et al., 2001; Marcellino et al., 2002**). Figure 1, lanes 1 and 2 show the silver-stained SDS-PAGE profile of pennisetin extracted with or without reducing agent, respectively. Three main bands, corresponding to the 27-, 22- and 12 kDa-pennisetins subunits appeared on both lanes 1 and 2. As shown in Figure 1 lane 1, pennisetin subunits could be extracted without adding any reducing agent using 70% aqueous ethanol at 60°C. However, this unreduced pennisetin fraction included also high molecular weight (HMW) proteins probably corresponding to polymers, trimers and dimers of pennisetin subunits. The addition of reducing agent led to almost total disappearance of these HMW in lane 2, a faint band remained at 40 kDa which is probably a dimer of the 27 and 12 kDa -pennisetins. The 27, 22 and 12 kDa-pennisetin subunits appeared more accentuated in the reduced fraction (Figure 1, lane 2) than that in the unreduced fraction (Figure 1, lane 1). SDS-Page of pennisetin showed that adding reducing agent improved the pennisetin subunits extractability markedly; this may indicate the presence of SS cross-linked pennisetins.

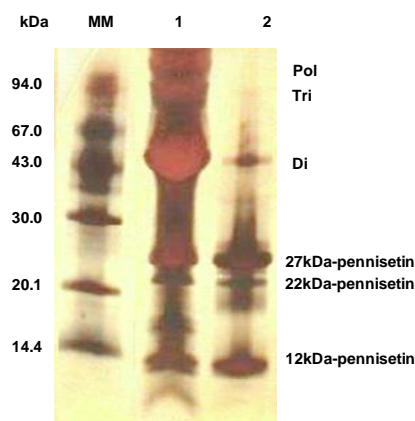


Figure 1 SDS-PAGE of pearl millet pennisetin: Lane 1: Pennisetin extracted without reducing agent; Lane 2: Pennisetin extracted with β-mercaptoethanol as reducing agent, lane MM: Molecular markers. The masses of the MM are indicated on the left side. The 27-, 22- and 12kDa-pennisetin monomers for pearl millet as well as the corresponding polymers (Pol), trimers (Tri) and dimers (Di) are indicated.

Reversed phase high performance liquid chromatography (RP-HPLC)

Protein extractability is highly affected by the solvent used, adding reducing agent, the experimental protocol and the conditions such as temperature and pH. In the following, to improve pennisetin extractability the effect of three reducing agents, temperature and NaOH was investigated by RP-HPLC.

Effect of reducing agent

The effect of reducing agents on pennisetin extractability was the first factor investigated. Figure 2a shows the total peak area of RP-HPLC chromatogram of pennisetin using three reducing agents (MBS, β-ME and DTT) at various concentrations; all the values were highly significant at $p \leq 0.001$. Pennisetin could be extracted without reducing agent but at low level. Adding increasing amount of MBS allowed extracting higher level of pearl millet proteins. Controversially, adding increasing amount of both β-ME and DTT reduced the level of extracted pennisetin. Addition of 1% MBS allowed to extract the highest level of pearl millet protein. DTT extracted lower amounts of protein than MBS, with an optimal concentration of 0.25% DTT. β-ME extracted the least amount of proteins. Such findings show that MBS is more effective for pearl millet protein extraction than the other reducing agents. In addition, MBS is a food grade ingredient that could be used in food applications of pearl millet proteins without causing side effects (**Bean et al., 2006**). The amount of protein extracted with 1% MBS was then chosen for use in all the following experiments.

The effect of reducing agent on cereal protein extractability has been extensively investigated by several researchers, such as **Redant et al. (2017)** for rye protein and by **Bean et al. (2006), Hamaker et al. (1995)** and **Mokrane et al. (2009)** for sorghum protein and **Celus et al. (2006)** for barley protein. In a previous study, **Akharume et al. (2020)** extracted the prolamin fraction of two proso millet cultivars after albumin, globulin and glutelin extraction using 70% Isopropyl Alcohol at room temperature and without adding reducing agent, they obtained low protein recovery of 54-60% with a prolamin extract containing 64.3-77.3% of protein. However to the best of our knowledge, few studies have aimed to investigate the effect of reducing agent on pearl millet protein extraction. **Marcellino et al., (2002)** used 55% isopropanol in the presence of 2% β- ME without mentioning the protein recovery yield. **Hadimani et al. (2001)** reported the protein distribution of three pearl millet cultivars in the albumin, globulin,

glutelin and prolamin fractions, the later constituting more than 50% of total protein. To date the highest protein contents in pennisetin extracted did not exceed 60% (Mokrane, 2010). In the present study, using 1% of MBS allowed obtaining high protein concentrate (90.1%) with high purity.

Effect of NaOH concentration

Cereal prolamins extraction was reported to be markedly affected by alkaline pH (Hamaker et al., 1995; Park and Bean, 2003; Pontieri et al., 2019). To assess pennisetin extractability at various pH, increasing amount of NaOH ranging from 0 to 0.4% (w/v) were added to the previous extraction procedure using 70% aqueous ethanol at 60°C in the presence of 1% MBS as reducing agent. Figure 2b shows the effect of NaOH concentration on pennisetin extraction expressed by the relative areas of RP-HPLC chromatograms. A highly significant difference was obtained in NaOH concentration ($p \leq 0.001$). NaOH increased the relative peak area of solubilized pennisetin by up to 0.2% of NaOH. However, an increase in NaOH concentration of more than 0.2% decreased the amount of extracted proteins. Park and Bean (2003) also observed this decrease in sorghum kafirin extraction. Pearl millet prolamins were therefore best extracted at an alkaline medium without exceeding 0.2% NaOH.

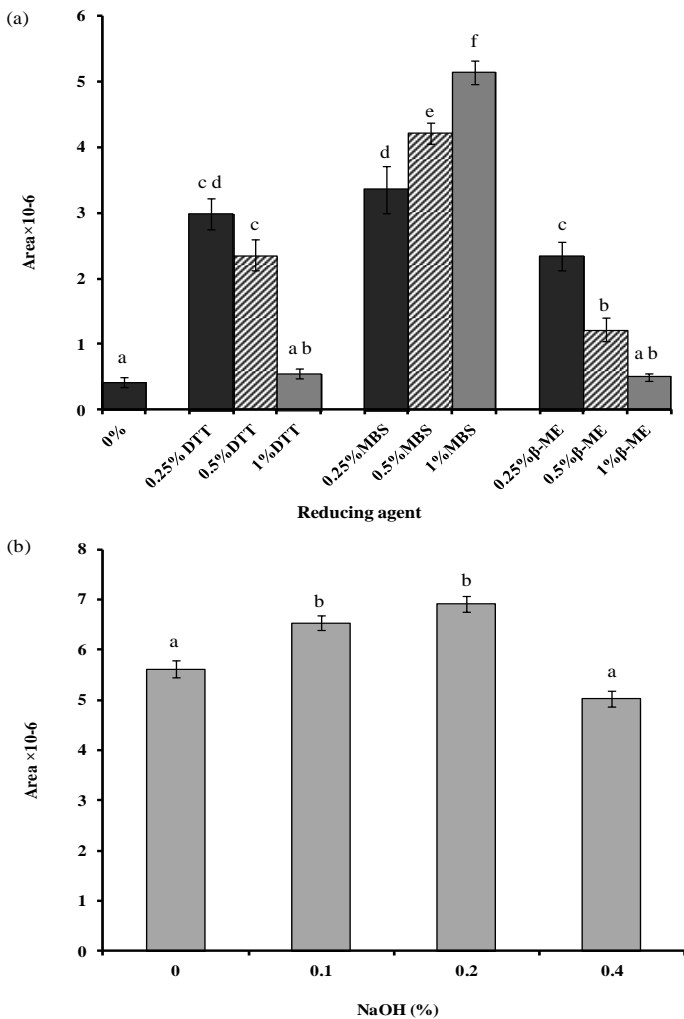


Figure 2 (a) Effect of reducing agent on extraction of pearl millet proteins. Samples were extracted with different concentrations and types of reducing agents in 70% aqueous ethanol. Samples were analyzed using RP-HPLC, and total peak area was calculated. (b) Effect of NaOH on extraction of pearl millet proteins. Samples were extracted with different concentrations of NaOH in 70% aqueous ethanol containing 1% MBS at 60°C. Samples were analyzed using RP-HPLC, and total peak area was calculated. Values indicate the mean of three replicates. Values with the same letter are not significantly different from each other ($p \leq 0.05$)

Effect of temperature

Pennisetin solubility is affected by heat. The effect of increasing extraction temperature was investigated in the following. The extraction procedure was performed with 70% aqueous ethanol and 0.2% NaOH at increasing temperature 30, 40, 50 and 60°C in the presence of 1% MBS as reducing agent. Figure 3a

shows the effect of temperature on the extractability of pearl millet proteins expressed by RP-HPLC area. RP-HPLC separations were compared both quantitatively by measuring peak area (Figure 3a) and qualitatively by visually comparing RP-HPLC patterns (Figure 3b).

The relative amount of total extracted pennisetin appeared to be extremely affected by increasing temperature and highly significant difference was obtained ($p \leq 0.001$). In this manner at 60°C the extractable amount of protein increased almost three times compared to that extracted at 30°C. This is probably due to the high protein folding in the pearl millet proteins. In previous works, FTIR showed that pennisetin the major protein fraction in pearl millet is arranged in secondary and tertiary structures which may be destroyed or highly reduced during heating (Bugs et al. 2004). Subsequently reducing agent along with heating might increase the solubility of pennisetin particularly at 60°C. Higher temperatures may cause pennisetin unfolding and may reduce its functional properties. Comparable results were obtained during sorghum prolamin extraction namely kafirin in several research (Amoura et al., 2020; Espinosa-Ramirez et al. 2016; Mokrane et al., 2009; Park and Bean, 2003).

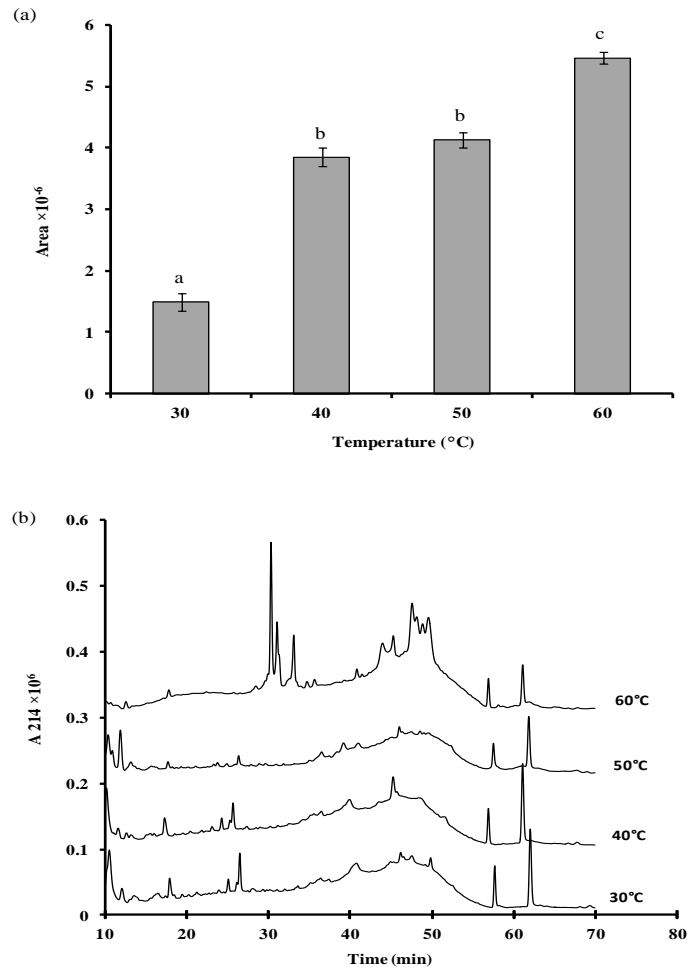


Figure 3 (a) Effect of temperature on extraction of pearl millet proteins. Samples were extracted at different temperature in 70% aqueous ethanol containing 1% MBS. Samples were analyzed using RP-HPLC, and total peak area was calculated. (b) Reverse phase-High performance liquid chromatography (RP-HPLC) profiles of proteins extracted from pearl millet with 70% aqueous ethanol containing 1% MBS at 30, 40, 50 and 60°C. Absorbance was recorded at 214 nm. Values indicate the mean of three replicates. Values with the same letter are not significantly different from each other ($p \leq 0.05$)

Water binding capacity and oil binding capacity

The measurement of WBC and OBC of pennisetin and PMF is required for their potential use as texture and flavor enhancers (Zayas, 1997). Figure 4 shows the WBC and OBC of pennisetin and PMF, the obtained values were not significantly different ($p \leq 0.05$). WBC and OBC of pennisetin were comparable but slightly lower than that of PMF. Pennisetin was likely denatured during extraction and precipitation at pH 4.5. In a similar study, Wu et al. (2009) obtained the same decrease in WBC and OBC of peanut proteins extracted by strong acid or alkali and alcohol solvent compared to peanut flour. As shown in Table 1 the amount of acidic AA and non polar AA was lower in pennisetin than those in PMF, this is probably due to their destruction during alkali extraction. WBC of PMF were in the same range of those obtained for pearl millet flour by

Ali et al. (2012) and Oshodi et al. (1999) and lower than that obtained by Akinola et al. (2017). WBC of both pennisetin and PMF were lower than those obtained for kafirin and sorghum flour (Amoura et al., 2020; Espinosa-Ramirez et al. 2016) and for quinoa flour (3.94 ± 0.06 g/g) and quinoa protein (1.3 ± 0.06 g/g) (Dakhili et al. 2016). Lower WBC is desirable for thinner gruels production (Simwaka et al., 2017). Pennisetin OBC was lower than that of kafirin, while OBC of PMF was higher (1.46 ± 0.05 g/g) than those obtained for PMF by Ali et al. (2012), defatted rice flour (1.10 ± 0.06 g/g) and defatted wheat flour (1.26 ± 0.15 g/g) by Joshi et al. (2015). OBC of PMF was almost two times higher than sorghum flour (Amoura et al., 2020) and in the same range of soybean flour (Ali et al., 2012; Joshi et al., 2015). Both pennisetin and PMF showed interesting WBC and OBC in the same range of other protein sources, which could allow their use as non gluten food additive in food products for better texture and flavour.

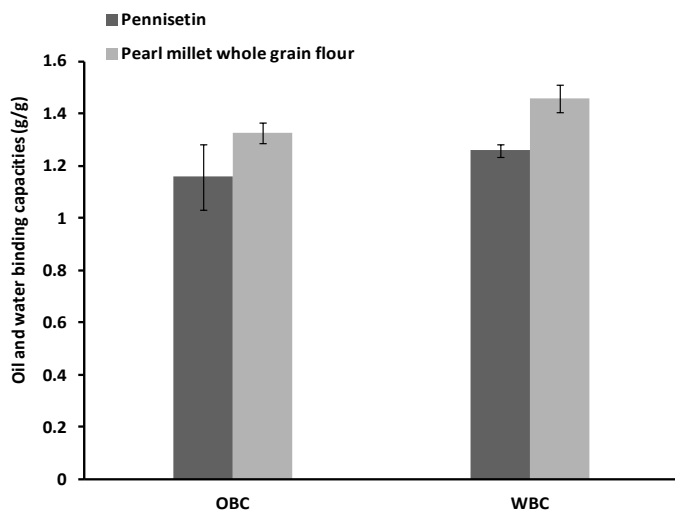


Figure 4 Water binding capacity (WBC) and oil binding capacity (OBC) of pennisetin and pearl millet whole grain flour. Values indicate the mean of three replicates.

Gelation properties

The gelation properties of pennisetin and PMF at increasing flour concentration are shown in Table 2. Pennisetin did not form gel while PMF began to form a gel at a concentration of 8% and gelled completely at 14%. LGC of PMF were significantly different at p<0.01. In previous studies, Ali et al. (2012) and Oshodi et al. (1999) obtained lower LGC of PMF 12% and 8%, respectively. However, PMF showed better gelation properties than sorghum flour (16%) (Amoura et al., 2020), rice flour (18%) (Joshi et al., 2015) and wheat flour (20%) (Dhankhar et al., 2019). Pennisetin could be used as co-gelling agent whereas PMF could be used as gluten free additive for better food sensorial perception and texture.

Table 2 Gelation properties of pearl millet whole grain flour and pennisetin

Concentration % (w/v)	2	4	6	8	10	12	14	16	18	20
Pearl millet whole grain flour**	0% ^a	0% ^a	0% ^a	<50% ^a	>50% ^{a,b}	>50% ^{a,b}	100% ^b	100% ^b	100% ^b	100% ^b
Pennisetin	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%

Legend: 0%, Not gelled; < 50%, gelled less than 50%; > 50%, gelled more than 50%; 100% gelled. Values with the same letter in one row are not significantly different from each other (p<0.05). ***p<0.001, **p<0.01, *p<0.05.

Table 3 Foaming properties of pennisetin and pearl millet whole grain flour

Time (min)	1	5	10	15	20	25	30	35	40	45	50	55	60
Pennisetin													
FC (%)	18.89 ^b	16.67 ^{a,b}	16.67 ^{a,b}	16.67 ^{a,b}	16.67 ^{a,b}	16.67 ^{a,b}	16.67 ^{a,b}	16.67 ^{a,b}	16.67 ^{a,b}	16.67 ^{a,b}	15.56 ^{a,b}	14.44 ^{a,b}	11.11 ^a
(%)	±1.11	±1.11	±1.11	±1.11	±1.11	±1.11	±1.11	±1.11	±1.11	±1.11	±2.22	±1.11	±2.22
FS*** (%)	100 ^c	100 ^c	88.19 ^{b,c}	88.19 ^{b,c}	88.19 ^{b,c}	88.19 ^{b,c}	88.19	88.19 ^{b,c}	88.19 ^{b,c}	88.19 ^{b,c}	81.94 ^b	76.39 ^b	58.33 ^a
(%)	±0.00	±0.98	±0.98	±0.98	±0.98	±0.98	±0.98	±0.98	±0.98	±0.98	±9.82	±1.96	±11.79
Pearl millet whole grain flour													
FC (%)	7.78 ^a	5.56 ^a	5.56 ^a	5.56 ^a	5.56 ^a	5.56 ^a	5.56 ^a	5.56 ^a	5.56 ^a	5.56 ^a	5.56 ^a	3.33 ^a	3.33 ^a
(%)	±1.57	±1.57	±1.57	±1.57	±1.57	±1.57	±1.57	±1.57	±1.57	±1.57	±1.57	±1.57	±1.57
FS** (%)	100 ^b	70.83 ^b	70.83 ^{a,b}	70.83 ^{a,b}	70.83 ^{a,b}	70.83 ^{a,b}	70.83 ^{a,b}	70.83 ^{a,b}	70.83 ^{a,b}	70.83 ^{a,b}	70.83 ^{a,b}	41.67 ^a	41.67 ^a
(%)	±0.00	±5.89	±5.89	±5.89	±5.89	±5.89	±5.89	±5.89	±5.89	±5.89	±5.89	±11.79	±11.79

Legend: FC: Foam capacity; FS: Foam stability; Standard deviation between brackets; Values indicate the mean of three replicates (Standard Deviation). Values with the same letter in one row are not significantly different from each other (p<0.05). ***p<0.001, **p<0.01, *p<0.05.

Foaming properties

Good foaming properties of food proteins are required for cakes, ice cream and whipped desserts. Foam enhances flavour dispersion, smoothness, lightness and palatability of food. Table 3 summarizes the foaming properties (FC and FS) of pennisetin and PMF. Among time, FC of pennisetin and PMF were not significantly different at p<0.05, while FS of pennisetin was highly significant (p<0.001) and FS of PMF was significantly different (p<0.05). Both pennisetin and PMF had the ability to form foam with FC of 18.89 ± 1.11 % and 7.78±1.57 %, respectively. FC of pennisetin was 2.43 times higher than PMF. FS of pennisetin and PMF decreased rapidly in the first 5 minutes to reach 88.19 and 70.83 %, respectively. Afterwards, FS remained stable for 50 min. FS of pennisetin and PMF decreased again rapidly to 58.33 and 41.67%. Similarly Ali et al. (2012) and Oshodi et al. (1999) found better PMF FC 24% and 11.3%, respectively with lower FS (16.69%) while Akinola et al. (2017) reported lower FC (3.36%) and FS (4.69%) respectively.

Compared to other cereals, FC of sorghum flour were higher 14% than that of PMF and kafirin formed negligible and unstable foam (Amoura et al., 2020). Foaming properties of pennisetin and PMF were lower than quinoa protein isolate (Dakhili et al. 2016), soybean protein isolate and soybean flour (Ali et al., 2012; Joshi et al., 2015). One possible reason could be the high ordered globular proteins in the native PMF. pH, salt contents and protein concentration might improve foaming properties of pennisetin and PMF. As suggested by Akinola et al. (2017) further studies should be undertaken to explain the low foaming properties of pearl millet flour. The effect of protein concentration, pH, salt concentration should be studied to improve the FC and FS of pennisetin and PMF.

Emulsifying properties

Good emulsifying properties are desired for oil emulsions stabilization by preventing droplets coalescence and increasing surface hydrophobicity. Figure 5 illustrates the emulsifying properties (EAI and ES) of pennisetin and PMF. EAI of pennisetin was highly significant (p<0.001), while EAI of PMF was only significantly different (p<0.05), among time. Pennisetin exhibited higher EAI than PMF (Figure 5a), this is likely due to pennisetin heating at 60°C during extraction. According to Pearce and Kinsella (1978), EAI of proteins is highly affected by temperature. In the mean time pennisetin had lower ES than PMF as shown in figure 5b, ES of both pennisetin and PMF were significantly different (p<0.05). This is probably due to the non protein content in PMF such as starch, which may contribute to improve the stability of emulsions. Pennisetin EAI obtained in this study were two times lower than those obtained for sorghum kafirin (Amoura et al., 2020) and kidney bean proteins (Makeri et al., 2017). While pennisetin EAI was 9.5 to 3.5 times higher than those obtained for quinoa protein (1.24-3.38 m²/g) (Dakhili et al., 2019) and comparable to sesame isolate (16.8 m²/g) (Makeri et al., 2017). Emulsifying data of pennisetin and PMF obtained in the present study might increase their potential use as emulsifying enhancers in salad creams, sausage and mayonnaise.

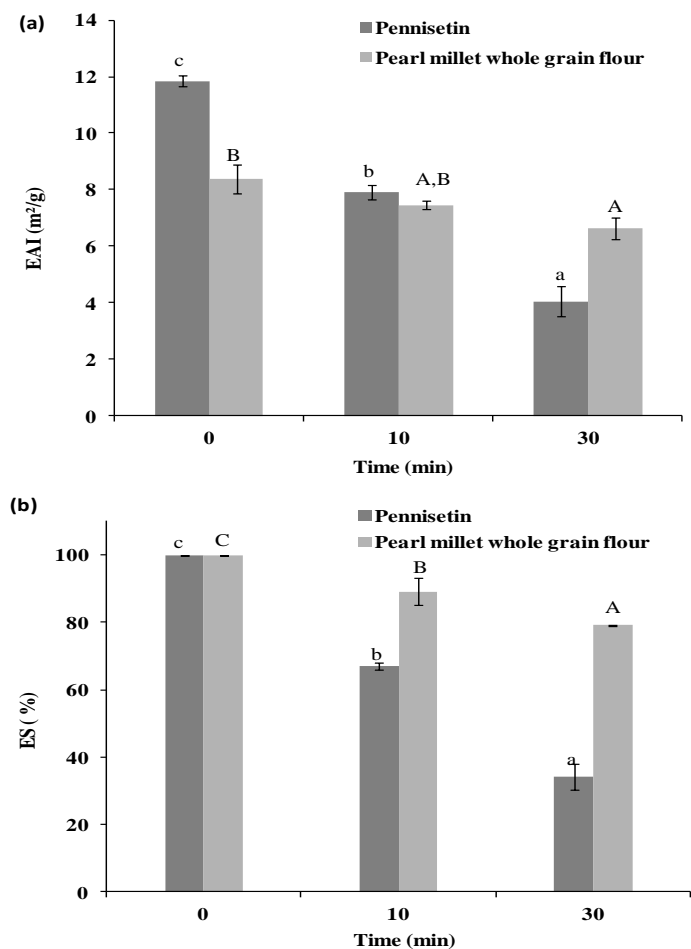


Figure 5 Emulsifying properties of pennisetin and pearl millet whole grain flour (a) Emulsifying activity index (EAI) and (b) Emulsion stability (ES). Values with the same letter (lowercase, uppercase) are not significantly different from each other ($p \leq 0.05$)

CONCLUSION

In the present study the effect of temperature, adding reducing agent and NaOH on the extraction of the alcohol soluble pearl millet proteins (pennisetin) was investigated. The best conditions were found to be the following 70% aqueous ethanol containing 0.2% NaOH and 1% MBS at 60°C. To the best of our knowledge, this solvent allowed extracting the highest percentage of pennisetin with protein content exceeding 90 % and higher level of essential AA (68.55%) than PMF (41.64%).

Compared to sorghum, soybean, wheat and rice flours, PMF exhibited higher or in the same range OBC and slightly lower WBC. While, WBC and OBC of pennisetin were lower than cereal protein isolates such as kafirin and quinoa protein isolate. Pennisetin had no ability to form a gel while the LGC of PMF was better than sorghum, wheat and rice flours. Foaming and emulsifying properties of pennisetin were better than PMF with better FS and lower ES.

According to their functional properties, PMF and pennisetin could be used as gluten free additive for better food sensorial perception and texture and as emulsifying or foaming enhancers. These results could be a starting point to increase the use of pearl millet as a gluten free cereal particularly its alcohol soluble proteins: pennisetin for food, feed and non food applications for celiac patients.

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