Research Article
Protein Modifications after Foxtail Millet Extrusion: Solubility and Molecular Weight

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Abstract: With the aim of illustrating the effects of extrusion cooking on the solubility of proteins in foxtail millet and their molecular basis, foxtail millet was extruded at five barrel temperature profiles and feed moisture contents. The proteins of raw and extrudate samples were extracted with six solutions sequentially. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) of total protein and Starch Granule-Associate Protein (SGAP) was performed. Extrusion caused a significant decrease in globulin, setarin and glutelin fractions with a corresponding increase in SDS- and SDS+2-ME-soluble and residual fractions. Increasing extrusion temperature or moisture content all led to SDS-soluble fraction decrease, while SDS+2-ME-soluble fraction increase. SDS-PAGE demonstrated that disulfide bond cross-linking occurred among glutelin and with setarin subunits. Extrusion had a less pronounced impact on the 60 kDa SGAP than the other middle-high molecular weight subunits. It is the protein-protein interaction shift from electrostatic force to hydrophobic and/or hydrogen forces and covalent disulfide cross-links that contributed to the decreased solubility of protein in foxtail millet extrudates.

Keywords: Extrusion, foxtail millet, protein, SDS-PAGE, solubility

INTRODUCTION

Foxtail millet (*Setariaitalica*), rich in the minerals and phytochemicals (FAO, 2008), is a nutritious food ingredient in traditional diet, especially for people in Europe, Asia and Africa. Extrusion cooking can be utilized to manufacture various types of foods with the advantages of high productivity and energy efficiency, low cost and zero effluents, etc., (Guy, 2001). In China, foxtail millet is mainly consumed as gruel. Considering its less exploration for food usage, extrusion cooking may become an effective way for producing foxtail millet-based foods.

Although protein is the second major component of cereals, its type and concentration (Faubion and Hoseney, 1982) and modification (Li and Lee, 1996; Koh *et al*., 1996; Lee *et al*., 2006) plays an important role in the physical, functional and micro-structural properties of extrudates. Previous reports showed that extrusion can lead to decrease in solubility (Li and Lee, 1996, 1997; Racicot *et al*., 1981; Ummadi *et al*., 1995; Fischer, 2004). Osborne procedure (Osborne, 1924) is often utilized in cereal chemistry to extract cereal protein fractions sequentially using water, NaCl, ethanol and acid or alkaline solutions. The resulting protein fractions are called albumin, globulin, prolamin (termed setarin for millet) and glutelin, respectively.

Because of the bad solubility of the proteins of some cereals such as corn, sorghum and millet, Sodium Dodecyl Sulfate (SDS) and/or 2-Mercaptoethanol (2-ME) solutions were also used alone or in combination to get better protein fractionation (Kumar and Parameswaran, 1998; Monteiro *et al*., 1982). On accounting of the lowered solubility of extrudated protein, these more powerful extracting solutions will inevitably give more information on the protein solubility after extrusion.

Previous studies showed that extrusion can lead to disappearance/decrease and appearance/increase of some bands in SDS-PAGE patterns (Li and Lee, 1996; Koh *et al*., 1996; Ummadi *et al*., 1995; Fischer, 2004). More detail information, such as which subunits were cross-linked to form the newly appeared bands and the fate of the disappeared or decreased subunits, are unavailable. Additionally, a very small fraction of total cereal protein is bound to the starch granules and called Starch Granule-Associate Protein (SGAP). There is a lack of report on the effect of extrusion on SGAP.

The purpose of this study was to determine the effects of extrusion temperature and moisture content on protein solubility in water, NaCl, ethanol and NaOH, SDS and SDS+2-ME solutions and SDS-PAGE behavior of total protein and SGAP caused by extrusion of foxtail millet.

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Table 1: Extrusion conditions and their codes

Codes	Barrel temperatures (ºC)					Moisture contents (%)
	Zone I	Zone II	Zone III	Zone IV	Zone V	
C1	100	120	130	140	150	19
C2	100	120	140	150	165	19
C3	100	130	150	165	180	19
C4	100	130	160	185	195	19
C5	100	140	170	190	210	19
C6	100	130	150	165	180	15
C7	100	130	150	165	180	17
C8	100	130	150	165	180	21
C9	100	130	150	165	180	23

MATERIALS AND METHODS

Raw material: Foxtail millet with protein content (N×5.7) of 10.40% (dry base) was obtained from Hebei Academy of Agriculture and Forestry Sciences (Hebei Province, China).

Extrusion processing: Foxtail millet grain was extruded on a DSE-25 co-rotating twin-screw extruder (Brabender GmbH and Co., Germany) with screw diameter 25 mm, screw length 500 mm, die inner diameter 6 mm. The barrel is composed of one feed zone and five heating zones. The temperature of each zone can be controlled and recorded automatically.

Extrusion experiments were conducted at five temperature profiles and feed moisture contents (expressed as percentage of wet basis). For all extrusion runs, the feed rates were fixed at 15 rpm and screw speeds 140 rpm. Extrusion conditions and their codes are shown in Table 1.

Samples preparation: The extrudate samples were oven-dried at 40°C for 24 h. The dried extrudates and foxtail millet grain were ground to pass a 70-mesh sieve. The resulting powders was defatted by stirring the flour for 2 h with petroleum ether (bp 60-80°C) and allowed to stand overnight to remove un-complexed lipids. The defatted flour was air-dried at 40°C after removing the solvent. The moisture content of each sample was measured according to AOAC method 925.10 (AOAC, 1990) just before protein extraction.

Protein fractionation and determination: After extracting protein fractions from raw and extruded samples using water, 5% (w/v) NaCl, 70% (v/v) ethanol, 0.1N NaOH sequentially according to the Osborne procedure (Osborne, 1924), still considerable protein was found in the residues. This observation prompted us to use much powerful extracting solutions of 1% (w/v) SDS, 1% (w/v) SDS+2% (v/v) 2-ME successively. The protein fractionating procedure is described as following:

Step 1: The defatted sample powder (3 g) was dispersed in 35 mL of water within a triangular flask under gentle magnetic stirring to prevent powder agglomeration.

Step 2: The resulting mixture was transferred to a 50 mL scaled centrifuge tube. Five milliliters of water was used to wash the triangular flask twice and transferred into the centrifuge tube. Additional water was added up to 50 mL.

Step 3: After shaking at room temperature for 10 h, the mixture was centrifuged at 3800×g for 40 min to get supernatant for protein content determination. In this case, the protein in supernatant was water-soluble fraction.

Step 4: Before extracting each of the following protein fractions, 30 mL of the former extracting solution was used to extract the remaining protein fraction in the sediment from step 3 for 1 h twice, followed by 30 mL of water washing three times to remove the former extracting solution. The resulting five supernatants were all discarded after centrifugation. After adding water to the centrifuge tube up to 15 and 35 mL of 7.14% (w/v) NaCl solution, anhydrous ethanol, 0.14 N NaOH, 1.43% (w/v) SDS, 1.43% (w/v) SDS+2.86% (v/v) 2-ME solutions was added and repeat step 3 to extract NaCl-, ethanol-, NaOH-, SDS- and SDS+2-ME-soluble fractions, respectively.

Thirty five milliliters of each supernatant from step 3 were concentrated and its protein was determined by a Kjeltec® 2300 Analyzer Unit (Foss Tecator AB, Sweden) using a protein conversion factor of 5.7. The protein content of each fraction in 3 g sample was calculated as the measured value multiplied by 50/35. The extracting process and protein determination were conducted in triplicate for each sample.

Starch isolation and purification: In order to identify the 60 kDa SGAP in the SDS-PAGE pattern of total protein, foxtail millet starch was isolated using the procedure of Zheng *et al.* (1998) with following modifications:

- After passing through a 100-mesh sieve, the foxtail millet flour slurry was homogenized in a colloid mill three times followed by passing a 200-mesh sieve.
- In order to separate protein from starch, after steeping in 0.2% (w/v) NaOH solution and

centrifuging the flour slurry mixture, the brown protein layer, yellow mud-like layer and fine fiber layer at the top of the resulting sediment were all scraped off.

- After adjusting the pH of the starch slurry to 7.0, the sediment was washed twice with water to remove NaCl.

The obtained crude starch was purified for 7 h under intermittent stirring. Six solutions of 1% (w/v) SDS, 2% (w/v) SDS, 1% (w/v) SDS+2% (v/v) 2-ME, 2% (w/v) SDS+2% (v/v) 2-ME, 1% (w/v) SDS+5% (v/v) 2-ME and 2% (w/v) SDS+5% (v/v) 2-ME were used to ensure to remove the residual protein completely in one stroke. The resulting starches were designated as PS1, PS2, PS3, PS4, PS5 and PS6, respectively.

SDS-PAGE: The procedure of Laemmli (1970) was applied for SDS-PAGE using a AE6450 electrophoresis apparatus (ATTO, Japan). For SDS-PAGE of total protein, defatted samples of 50 mg each were extracted with 1 mL of sample buffer. The mixtures were intermittently shaken at room temperature for 10 h and centrifuged at 18,000×g for 15 min to obtain supernatants for SDS-PAGE. For SDS-PAGE of SGAP, 200 mg starch was used and extracted for 2 h. All runs were carried out at a constant current of 12.5 mA/gel.

Quantity One® (version 4.62, Bio-Rad Laboratories, USA) was used to estimate molecular weight and intensity profile along the lanes in the SDS-PAGE photograph. After subtracting lane background, the intensity data were exported to Microsoft® EXCELL (version 2003, Microsoft Corp., USA) and plotted.

Statistical analysis: Statistical analysis were carried out by SAS® (version 8.01, SAS Institute, USA) and Duncan multiple-range test was applied at a significance level of $p < 0.05$.

RESULTS AND DISCUSSION

Protein extracting duration: Extrusion processing can denature protein then change its solubility. The extracting time for extrudated wheat protein in previous reports varied from two (Ummadi *et al*., 1995) to 24 h (Fischer, 2004). Actually, extracting time depends on the nature of raw material and solvent, extracting temperature, material/solvent ratio, stirring or not, etc. For determining the extracting duration for extrudated foxtail millet protein, Three gram of C3 extrudate was extracted with 50 mL of 5% NaCl and 1% SDS solutions. The nitrogen contents in the supernatants after different durations were determined and presented in Fig. 1.

Statistic analysis suggests that when extracting time was 8 h or longer, the nitrogen contents in supernatants after 5% NaCl and 1% SDS extraction did not increase further significantly ($p < 0.05$ and $p < 0.01$, respectively). Extraction duration of 10 h was adopted in this study for protein fractionation.

Protein solubility: Seven protein fractions of the native foxtail millet grain and its extrudates produced under different extrusion temperatures and moisture contents are shown in Table 2 and 3, respectively. In raw materials, protein was present in the highest amount in the setarin fraction, followed by the glutelin fraction, SDS-soluble fraction and SDS+2-ME-soluble fraction.
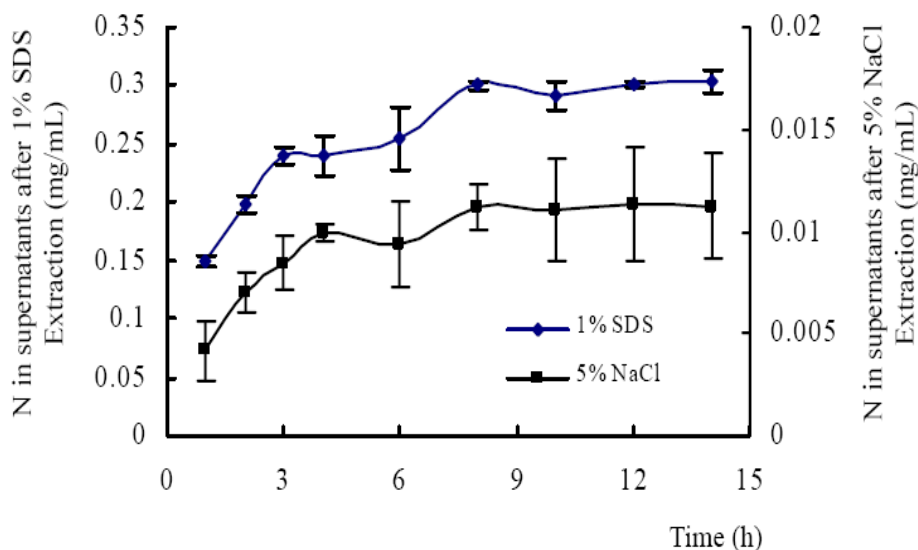

Fig. 1: Nitrogen content (N) in the supernatants of extrudate C3 after extracting with 1% SDS and 5% NaCl solutions for different durations

Table 2: Protein fractions of raw and extruded foxtail millet at five barrel temperature profiles[1]

Protein fractions	C0	C1	C2	C3	C4	C5
Water-soluble	2.85±0.08b	2.14±0.02c	2.06±0.07c	2.35±0.23c	2.81±0.29b	3.21±0.06a
NaCl-soluble	2.69±0.26a	0.44±0.08d	0.54±0.05cd	0.73±0.08bc	0.79±0.09b	0.83±0.10b
Ethanol-soluble	45.64±0.76a	3.65±0.44d	2.85±0.35e	4.08±0.03d	6.05±0.52c	9.27±0.38b
NaOH-soluble	21.08±0.15a	6.73±0.87b	5.58±0.10c	5.09±0.09c	4.17±0.03d	4.22±0.30d
SDS-soluble	16.85±1.26f	61.79±0.33a	59.03±1.07b	48.09±0.76c	39.44±0.96d	29.54±2.32e
SDS+2-ME-soluble	7.13±0.34f	15.61±0.89e	17.02±0.92d	22.65±0.96c	28.05±0.65b	30.64±0.26a
Residual protein[2]	3.76±2.09e	9.64±2.00d	12.93±1.85cd	17.02±2.12bc	18.70±2.54ab	22.29±3.11a

[1]: Each value is the weigh percentage of total dry protein; and expressed as an average of triplicate measurements±standard deviation; Values followed by the same letter in the same row are not significantly different ($p<0.05$); [2]: Residual protein was calculated by the difference between total protein and the sum of protein fractions; The total protein was estimated from the protein content 10.40% assuming extrusion processing did not alter it

Table 3: Protein fractions of raw and extruded foxtail millet at five moisture contents[1]

Protein fractions	C0	C6	C7	C3	C8	C9
Water-soluble	2.85±0.08b	2.21±0.13b	2.68±0.51b	2.35±0.23b	2.38±0.28b	2.50±0.04b
NaCl-soluble	2.69±0.26a	0.76±0.02b	0.70±0.09b	0.73±0.08b	0.80±0.06b	0.77±0.08b
Ethanol-solubale	45.64±0.76a	4.07±0.13b	3.90±0.01c	4.08±0.03bc	4.32±0.33bc	4.66±0.03b
NaOH-soluble	21.08±0.15a	5.47±0.29b	5.12±0.08c	5.09±0.09c	5.27±0.06bc	5.30±0.02bc
SDS-soluble	16.85±1.26f	53.01±0.62a	52.32±3.01a	48.09±0.76b	48.24±2.19b	47.97±1.69b
SDS+2-ME-soluble	7.13±0.34f	16.43±0.09c	20.26±1.22b	22.65±0.96a	22.52±0.81a	23.22±1.59a
Residual protein[2]	3.76±2.09e	18.05±0.50a	15.02±0.90a	17.02±2.12a	16.47±3.58a	15.57±3.33a

[1]: Each value is the weigh percentage of total dry protein and expressed as an average of triplicate measurements±standard deviation; Values followed by the same letter in the same row are not significantly different ($p<0.05$); [2]: Residual protein was calculated by the difference between total protein and the sum of protein fractions; The total protein was estimated from the protein content 10.40% assuming extrusion processing did not alter it

The results indicate that setarin was the first principal protein of foxtail millet, which is in general agreement with the findings of most previous reports (Danno and Natake, 1980; Takumi *et al.*, 2001; Kamara *et al.*, 2009). Our results about albumin and globulin are lower than that of Danno and Natake (1980) but very close to the data of Takumi *et al.* (2001) and Kamara *et al.* (2009).

Statistical analysis (Table 2 and 3) shows that, extrusion processing at the five temperature profiles and moisture contents all caused a significant decrease in NaCl-, ethanol- and NaOH-soluble fractions with a accompanying increase in the SDS-, SDS+2-ME-soluble and insoluble fractions. Ethanol-soluble protein had a most marked decrease after extrusion. More than 60% of total protein became extractable only by SDS and SDS+2-ME solutions. Even so, still more than 9.5% of total protein was insoluble. Our results are consistent with the previous finding of Ummadi *et al.* (1995) on semolina, in which a marked decrease in globulin, gliadin and glutenin fractions with a concomitant increase in the insoluble fraction after extrusion was reported. An interesting finding was that the water-soluble fraction in the extrudate at a zone V temperature of 210°C was even higher than that in control. Probably, because of the very high extrusion temperature, protein degradation might have occurred and resulted in water-soluble protein fragments. If this was the case, the water-soluble fraction in extrudate should be the sum of the albumin part not affected by extrusion and the protein fragments from degradation. This assumption can also explain, at least partially, why the decrease of water-soluble protein after all extrusion runs was much smaller than that of NaCl-, ethanol- and NaOH-soluble fractions. Indeed, Selling (2010)

recently reported that zein chain cleavage begin when extrusion temperature is above 180°C.

Basically, different extracting solutions dissolve protein by disrupting different kinds of molecular interactions. So, the solubility in different solutions will reflect the type and strength of the interactions among protein molecules. Alkaline solution dissolves protein by giving a higher electrostatic repulse force among protein molecules when pH value is far away from isoelectric point. While ethanol solution dissolves protein through lowering the dielectric constant of the solution medium then gives a higher electrostatic repulse force. So, the decrease of ethanol- and NaOH-soluble proteins in extrudates indicates that the contribution of electrostatic interaction to the total forces holding the structure of protein aggregates decreased after extrusion. SDS disrupts the hydrogen bonds and/or hydrophobic interactions, then dissolves protein. Marked increase of SDS-soluble protein after extrusion suggests that the denatured protein aggregated mainly through hydrogen bonds and/or hydrophobic interactions in extrudates. Chemical compound 2-ME dissolves protein through cleaving disulfide bonds. The SDS+2-ME-extractable proteins in all extrudates were higher than that in raw sample, indicating that extrusion has caused disulfide cross-linking between protein molecules. Li and Lee (1996) reported that disulfide cross-linking is the major form of covalent linking in wheat flour protein after extrusion. Different from Li and Lee (1996), still considerable amount of protein was observed after extracting with SDS+2-ME solution in this research. The SDS+2-ME-insoluble protein might aggregate through the covalent bonds other than disulfide cross-links. Another possible reason is its

complex with gelatinized starch. Recently, De Pilli *et al.* (2012) suggested that it is important to consider all components present in the extruded food in order to study biopolymer modifications that occur during extrusion processing. It will be interesting to explore the molecular interactions that contribute to the insolubility of the residual protein fraction.

Although water-, NaCl- and ethanol-soluble fractions decreased after extrusion, higher temperature gave more water-soluble fraction when zone V temperature ≥180°C and finally more than control, more NaOH-soluble fraction when zone V temperature ≤195°C and more ethanol-soluble when zone V temperature ≥165°C. The SDS-soluble protein increased after extrusion, but decreased with temperature increase. In contrast, both extrusion (compared with before extrusion) and increasing temperature had a same-direction effect on the SDS+2-ME-soluble and residual protein fractions, which increased after extrusion and increased further with extrusion temperature increase. Previous reports did not give a consistent conclusion about the impact direction of temperature on protein solubility. Li and Lee (1996) reported that water and NaCl-soluble fractions of extrudated wheat flour increased with die temperature increasing from 160 to 185°C. While Ummadi *et al.* (1995) demonstrated that higher extrusion temperature (96°C, compared with 50°C) led to reduced water-, NaCl- and ethanol-solube fractions in extrudated semolina.

There was no significant difference between the values of water-, NaCl-soluble and residual proteins for all moisture levels (Table 3). Increasing moisture content had a similar direction but smaller magnitude of impact on the SDS- and SDS+2-ME-soluble fractions than increasing temperature did. Our results on the variation of NaCl-, ethanol- and NaOH-soluble fractions with moisture content are not in agreement with the reports of Racicot *et al.* (1981) on corn meal extruded at 140°C.

It is difficult to give a theoretical explanation for the dependence of protein solubility on extrusion temperature or moisture content. But, we can make sense of the above discrepancy from a viewpoint of kinetics. Actually, many changes, such as protein insolubilization, occurring in an extruder should be treated as kinetic processes. The residence time of materials in an extruder is very short, normally less than 2 min. Then protein insolubilization process may have not reach equilibrium before the materials is extruded out of the die. The reaction extent of protein solubility decrease is a function of reaction rate and time (the residence time in an extruder). The reaction rate in an extruder depends on temperature, moisture content and shear effect (Zhao *et al.*, 2011a). Raising temperature will increase the reaction rate of protein insolubilization. At the same time, it will also decrease the melt viscosity and result in lower shear effect, which on the contrary will decrease the reaction rate. In addition, lowering viscosity will also alter residence
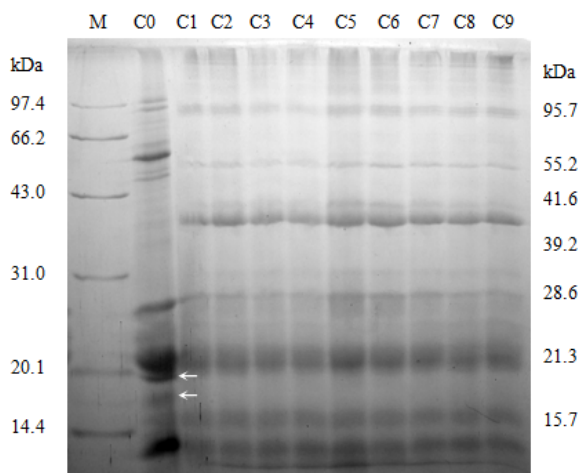

Fig. 2: Non-reducing SDS-PAGE patterns of total protein in foxtail millet and its extrudates
M: Marker; C0: Raw sample; Zone V temperature (°C) and moisture content: C1 = 150 and 19%, C2 = 165 and 19%, C3 = 180 and 19%, C4 = 195 and 19%, C5 = 210 and 19%, C6 = 180 and 15%, C7 = 180 and 17%, C8 = 180 and 21%, C9 = 180 and 23%
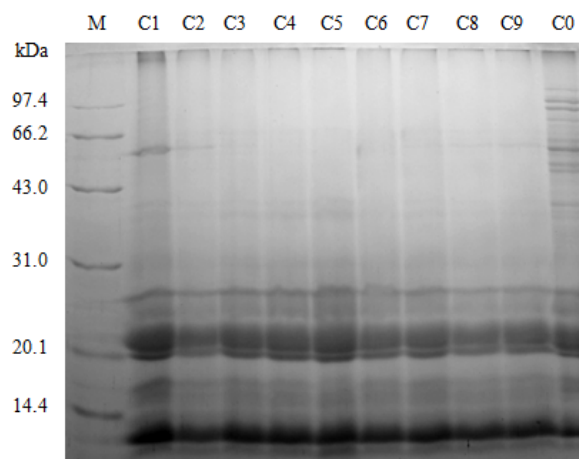

Fig. 3: Reducing SDS-PAGE patterns of total protein in foxtail millet and its extrudates
M: Marker; C0: Raw sample; Zone V temperature (°C) and moisture content: C1 = 150 and 19%, C2 = 165 and 19%, C3 = 180 and 19%, C4 = 195 and 19%, C5 = 21 and 19%, C6 = 180 and 15%, C7 = 180 and 17%, C8 = 180 and 21%, C9 = 180 and 23%

time. So, the same factor can favor a reaction in one way and retard it in another way. The final result is the combination of these two opposite effects. Same as temperature, moisture content variation also has two opposite effects on the rate of protein insolubilization. Higher moisture content will favor the free movement then configuration transform of the protein macromolecules and resulting in a higher denature rate. At the same time, higher moisture content will give lower viscosity, then lower shear force, which will decrease the shear effect on protein insolubilization. So,

it appears that both extrusion temperature and moisture content, especially when varied in a broader range, will not give a one-way effect on protein solubility, as manifested by the case of ethanol-soluble in this study.

SDS-PAGE analysis: The SDS-PAGE patterns of total proteins in the foxtail millet control and extrudates under reducing and non-reducing conditions are shown in Fig. 2 and 3, respectively.

Comparison of the non-reducing SDS-PAGE patterns of the raw with these of the extruded samples (Fig. 2) shows that the bands of Mw 28.6, 21.3, 15.7 and 13.4 kDa, respectively were present both before and after extrusion. All the bands of Mw more than 30 kDa in control sample disappeared after extrusion. The 18.1 and 20.1 kDa bands (indicated by white arrows) in lower molecular weight region also disappeared. Instead, one intense band at Mw of 39.2 kDa and three less intense but still visible bands at Mw of 95.7, 55.2 and 41.6 kDa, respectively appeared after extrusion. As for the 95.7 kDa subunit, there was a band in the control sample whose mobility was almost equal to that of the 95.7 kDa band. After reduction, as showed in Fig. 3, the 95.7 kDa band disappeared while the band in control sample remained. This indicates that the 95.7 kDa band was newly formed by extrusion but not the already existed band in raw foxtail millet.

Based on the molecule weight estimates of the disappeared and decreased bands in raw sample and of the newly formed bands in extrudates, we consider that the 39.2 kDa band was the result of one 18.1 kDa subunit cross-linking with one 21.3 kDa subunit; while the 41.6 kDa bond was the result of one 20.1 kDa and one 21.3 kDa subunit; the 55.2 kDa bond was the result of one 13.4 kDa, one 20.1 kDa and one 21.3 kDa subunit; part of the new formed 55.2 and 39.2 kDa proteins polymerized further to form the 95.7 kDa band. After reduction with 2-ME, the 95.7, 55.2 and 39.2 kDa, respectively bands all disappeared (Fig. 3). This observation demonstrates that disulfide bonds contributed to the cross-linking. The Above assumptions are also supported by the intense increase

of the 13.4 and 21.3 kDa, respectively bands and the reappearance of the 18.1 kDa band after reduction. It is difficult to distinguish between the two bands of 15.7 and 18.1 kDa, respectively in Fig. 3 visibly. We got intensity profile along the lanes with the help of Quantity One software. The results are presented in Fig. 4. It clearly indicated that the 18.1 kDa band appeared again after reduction.

The 18.1 kDa band is the subunit of gultenin, while the 21.3 and 13.4 kDa, respectively bands are the subunits of setarin (Takumi *et al.*, 2001; Zhao *et al.*, 2011b). It means that the extrusion-induced disulfide bond cross-linking occurred among glutelin subunits and with setarin subunits. Indeed, setarin and glutelin contain a number of cysteine residues (Monteiro *et al.*, 1982), which makes the disulfide cross-linking possible. Previous reports on wheat flour suggested that gliadin-like polypeptides are highly susceptible to modification during extrusion (Koh *et al.*, 1996).

Chanvrier *et al.* (2007) found that some subunits of wheat flour protein, which disappeared in non-reducing SDS-PAGE after extrusion, reappeared in reducing SDS-PAGE. In contrast, Ummadi *et al.* (195) reported the presence of new bands in the reducing SDS-PAGEs of albumin, glutenin, insoluble proteins after extrusion, while no new bands appearance in their corresponding non-reducing SDS-PAGEs. New bands appearance accompanying with some bands disappearance in non-reducing SDS-PAGE and the newly formed bands disappearance again after reduction, as found in present study, is more explainable.

The non-reducing SDS-PAGE patterns exhibited no difference among extrudates. The reducing SDS-PAGE patterns of all extudates were also similar except that the 60 kDa band remained visible for the extrudate produced at zone V temperature 150°C. Further density scanning (Fig. 4) showed that the 60 kDa band also appeared in the extrudates when zone V temperature was 165°C or moisture content was 19 and 21%, respectively. The band became fainter and fainter with barrel temperature increase or moisture content decrease and finally disappeared.
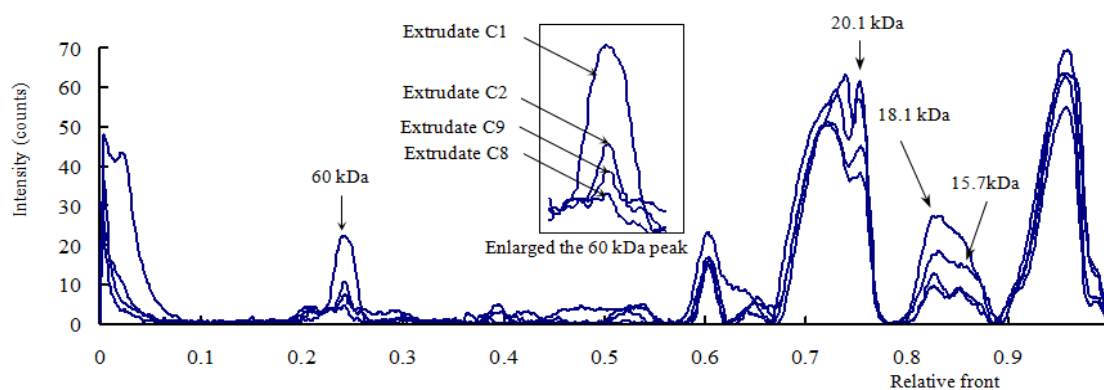

Fig. 4: Intensity profile of lane C1, C2, C8 and C9 in the reducing SDS-PAGE
Zone V temperature (°C) and moisture content: C1 = 150 and 19%, C2 = 165 and 19%, C8 = 180 and 21%, C9 = 180 and 23%
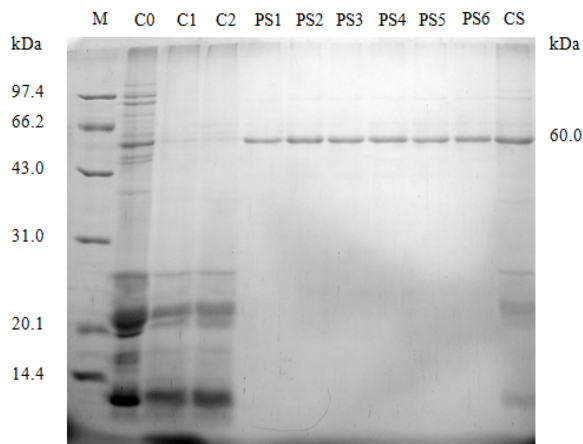
Fig. 5: Reducing SDS-PAGE patterns of total protein in foxtail millet (C0) and extrudates C1, C2 (zone V temperature (°C) and moisture content = 150 and 19%, 165 and 19%, respectively) and SGAP in raw Starch (S) and purified starches (purifying solution for PS1 = 2% SDS+5% 2-ME; PS2 = 2% SDS+2% 2-ME; PS3 = 2% SDS; PS4 = 1% SDS+5% 2-ME; PS5 = 1% SDS+2% 2-ME; PS6 = 1% SDS). M = marker

Our further research showed that the 60 kDa subunit is the SGAP of foxtail millet. The reducing SDS-PAGEs of crude and purified starches and C1, C2 extrudates were simultaneously performed for identifying the 60 kDa band in Fig. 3 as the 60 kDa SGAP in foxtail millet starch. Results are presented in Fig. 5. Aside the 60 kDa subunit, the starch without purification still contained sensible amount of setarin. Solution with a concentration of 1% SDS was powerful enough to remove the residual setrain protein, but had no impact on the amount of the 60 kDa protein. This observation demonstrates that 1% SDS cannot extract the 60 kDa subunit at room temperature (starch purification condition). Only when the starch was fully gelatinized (sample preparation condition for SDS-PAGE), can the 60 kDa subunit be extracted. Based on this observation, the 60 kDa subunit should be starch granule-associated protein (Baldwin, 2001).

As can be seen from Fig. 2 and 3, the 60 kDa SGAP in fully gelatinized starch of raw samples was soluble in SDS and SDS+2-ME solutions. After extrusion, it became soluble only in SDS+2-ME solution. This suggests that the solubility of SGAP was lowered after extrusion. For all extrudates, all the bands in middle-high molecular weight region except the 60 kDa band became invisible in Fig. 4, indicating that extrusion had a less pronounced impact on the 60 kDa SGAP than the other subunits. It is assumed that its association with starch granules had protected the SGAP from the thermal and shearing treatments in the extruder and remained a relatively higher solubility. With temperature increase or moisture content decrease, the thermal and shearing treatments were so harsh that the association cannot protect it.

The importance of the 60 kDa SGAP in the stickiness of cooked rice (Hamaker *et al.*, 1991) and the rheological properties of maize starch pastes (Han *et al.*, 2002) has been declared. Maybe, the 60 kDa SGAP and its modification also play some roles in determining the properties of starch-based extrudates.

CONCLUSION

Extrusion processing of foxtail millet caused a significant decrease of protein solubility as manifested by a decrease of globulin, setarin and glutelin fractions with a accompanying increase in SDS- and SDS+2-ME-soluble and residual fractions. The SDS-soluble fraction decreased, while the SDS+2-ME-soluble fraction increased significantly with extrusion temperature. Compared with increasing temperature, increasing moisture content had a similar trend but smaller magnitude of impact on the solubility of SDS- and SDS+2-ME-soluble fractions.

Extrusion led to some bands disappearance accompanying with new bands appearance in non-reducing SDS-PAGE and the new formed bands disappearance in reducing SDS-PAGE. Setarin and glutelin subunits involved in the formation of the new bands through disulfide cross-linking. Extrusion had a less pronounced impact on the 60 kDa SGAP than on the other middle-high molecular subunits. These findings could be helpful for controlling product properties of extruded cereals by adjusting protein fractions or selecting suitable cereal variety with proper protein subunit composition.

The decreased protein solubility reflected the interaction shift from electrostatic force to hydrophobic and/or hydrogen forces and covalent cross-linking occurring after extrusion. It will be interesting to explore the molecular interactions that contribute to the insolubility of the residual protein fraction.

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REFERENCES

AOAC, 1990. Official Method of Analysis. AOAC Intl. 15th Edn., Method 925.10 (19). Association of Official Analytical Communities, Washington, DC, USA.

Baldwin, P.M., 2001. Starch granule-associated proteins and polypeptides: A review. Starch, 53(10): 475-503.

Chanvrier, H., S. Uthayakumaran and P. Lillford, 2007. Rheological properties of wheat flour processed at low levels of hydration: Influence of starch and gluten. J. Cereal Sci., 45(3): 263-274.

Danno, G. and M. Natake, 1980. Isolation of foxtail millet proteins and their subunit structure. Agric. Biol. Chem. Tokyo, 44(4): 913-918.

De Pilli, T., A. Derossi, R.A. Talja, K. Jouppila and C. Severini, 2012. Starch-lipid complex formation during extrusion-cooking of model system (rice starch and oleic acid) and real food (rice starch and pistachio nut flour). Eur. Food Res. Technol., 234(3): 517-525.

FAO, 2008. Sorghum and Millet in Human Nutrition. Food and Agriculture Organization of the United Nations, Rome, Italy.

Faubion, M.J. and R.C. Hoseney, 1982. High-temperature short-time extrusion cooking of wheat starch and flour. II. Effect of protein and lipid on extrudate properties. Cereal Chem., 59(6): 533-537.

Fischer, T, 2004. Effect of extrusion cooking on protein modification in wheat flour. Eur. Food Res. Technol., 218(2): 128-132.

Guy, R., 2001. Extrusion Cooking Technologies and Applications. Wood head Publishing Ltd., Cambridge, England, pp: 1-2.

Hamaker, B.R., R.V.K. Grifin and K.A.K. Moldenhauer, 1991. Potential influence of a starch granule-associated protein on cooked rice stickiness. J. Food Sci., 56(5): 1327-1329.

Han, X.Z., O.H. Campanella, H. Guan, P.L. Keeling and B.R. Hamaker, 2002. Influence of maize starch granule-associated protein on the rheological properties of starch pastes. Part I. Large deformation measurements of paste properties. Carbohyd. Polym., 49(3): 315-321.

Kamara, M.T., Z.H. Ming and Z. Kexue, 2009. Extraction, characterization and nutritional properties of two varieties of defatted foxtail millet flour (*Setaria italica* L.) grown in China. Asian J. Biochem., 4(3): 88-98.

Koh, B.K., M.V. Karwe and K.M. Schaich, 1996. Effect of cysteine on free radical production and protein modification in extruded wheat flour. Cereal Chem., 73(1): 115-122.

Kumar, K.K. and K.P. Parameswaran, 1998. Characterisation of storage protein from selected varieties of foxtail millet (*Setaria italica* (L) Beauv). J. Sci. Food Agr., 77(4): 535-542.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227(5259): 680-685.

Lee, K.M., S.R. Bean, S. Alavi, T.J. Herrman and R.D. Waniska, 2006. Physical and biochemical properties of maize hardness and extrudates of selected hybrids. J. Agr. Food Chem., 54(12): 4260-4269.

Li, M. and T.C. Lee, 1996. Effect of extrusion temperature on solubility and molecular weight distribution of wheat proteins. J. Agr. Food Chem., 44(3): 763-768.

Li, M. and T.C. Lee, 1997. Relationship of the extrusion temperature and the solubility and disulfide bond distribution of wheat proteins. J. Agr. Food Chem., 45(7): 2711-2717.

Monteiro, P.V., T.K. Virupakshaa and D.R. Rao, 1982. Proteins of Italian millet: Amino acid composition, solubility fractionation and electrophoresis of protein fractions. J. Sci. Food Agr., 33(11): 1072-1079.

Osborne, T.B., 1924. The Vegetable Proteins. Longmans Green & Co., New York, pp: 25-35.

Racicot, W.F., L.D. Satterlee and M.A. Hanna, 1981. Interaction of lactose and sucrose with cornmeal proteins during extrusion. J. Food Sci., 46(5): 1500-1506.

Selling, G.W., 2010. The effect of extrusion processing on zein. Polym. Degrad. Stabil., 95(12): 2241-2249.

Takumi, K., H. Imai and K. Yao, 2001. Characterization of the millet storage protein: extractive efficiency, SDS-PAGE profile, amino acid composition and N-terminal sequence. J. Okayama Coll., 24(10): 25-35.

Ummadi, P., W.L. Chenoweth and P.K.W. Ng, 1995. Changes in solubility and distribution of semolina proteins due to extrusion processing. Cereal Chem., 72(6): 564-567.

Zhao, X., Y. Wei, Z. Wang, F. Chen and A.O. Ojokoh, 2011a. Reaction kinetics in food extrusion: Methods and results. Crit. Rev. Food Sci., 51(9): 835-854.

Zhao, X., Y. Wei and B. Zhang, 2011b. Physicochemical properties of foxtail millet protein. Cereal Feed Ind., 30(7): 34-37 (In Chinese).

Zheng, G.H., F.W. Sosulskib and R.T. Tyler, 1998. Wet-milling, composition and functional properties of starch and protein isolated from buckwheat groats. Food Res. Int., 30(7): 493-502.