Incorporation of High-Speed Shearing in the Fabrication of Whole Soybean Curd

Wang, Chenzhi; Li, Lin; Zhang, Qi; Raheem, Dele; Qin, Wen; Wu, Dingtao; Hu, Bin; Yang, Wenyu; Dong, Hongmin ; Vasanthan, Thava; Zhang, Qing

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Dear editor,

It is our pleasure to submit our manuscript entitled "Application of high speed shearing on fabrication of GDL-induced whole soybean curd: analysis of aggregation behaviors and microstructure" for consideration on ***Food and Bioprocess Technology***. No conflict of interest exists in this manuscript and all the authors listed have approved the submission and possible publication of this manuscript. I would like to declare that this study is an original research that has not been published elsewhere, and not under consideration by another journal, in part or in entirety. I am looking forward to hearing from you soon about the outcome.

Please be noted that this manuscript is the revised revision of FABT-D-19-00837. We've fully addressed the editor's concerns, cited the relevant papers published on FABT and compared their difference (the details are shown in Introduction, **line 94-107**). Briefly, common homogenization methods (e. g. high pressure homogenization, high energy wet media milling, micro-fluidization, high hydrostatic pressure technology) presented common drawbacks of high equipment cost and energy consuming. Commercial HSS equipment runs smoothly under atmospheric pressure and possesses advantages in energy-saving and effectiveness by contrast. Moreover, the relevant papers published on **FABT** mainly focused on artificial gels (e. g. TSK gel G2000 SWXL column; Surmi Gel). Our study mainly focused on a conventional soybean curd (tofu). Thus, obviously, our object of study is different. Application of HSS on micronization of okara fibers contributed to improve the utilization ratio of raw materials and increase the content of dietary fibers in an innovative whole soybean curd. To understand the modification mechanism of HSS, the aggregation behaviors of protein particles and the microstructure of obtained soybean curd

are fully investigated.

We hope that this manuscript will be taken into consideration.

Yours Sincerely,

Qing Zhang, Ph. D., E-mail: zhangqing@sicau.edu.cn (Q. Zhang);

College of Food Science, Sichuan Agricultural University.

29 **Application of high speed shearing on fabrication of GDL-induced whole soybean curd:**
30 **analysis of aggregation behaviors and microstructure**

31

32 Chenzhi Wang [a]†, Lin Li [a]†, Qi Zhang [a], Dele Raheem [b], Wen Qin [a], Dingtao Wu [a], Bin Hu [a],
33 Wenyu Yang [c], Hongmin Dong [d], Thava Vasanthan [d], Qing Zhang[a] *

34

35 [a] College of Food Science, Sichuan Agricultural University, No. 46, Xinkang Road, Ya'an 625014,
36 Sichuan, China.
37 [b] Northern Institute for Environmental and Minority Law (NIEM), Arctic Centre, University of
38 Lapland, Rovaniemi 96101, Finland
39 [c] Key Laboratory of Crop Ecophysiology and Farming System in Southwest, Ministry of
40 Agriculture/Sichuan Engineering Research Center for Crop Strip Intercropping System, Chengdu
41 611130, Sichuan, China.
42 [d] Department of Agricultural, Food and Nutritional Science, University of Alberta, Edmonton,
43 Alberta T6G 2P5, Canada.
44 *Correspondence to Qing Zhang (Q. Zhang). College of Food Science, Sichuan Agricultural
45 University, No. 46, Xinkang Road, Ya'an 625014, Sichuan province, China. E-mail:
46 zhangqing@sicau.edu.cn; Telephone/Fax: +86-0835-2882359.
47 †Author contribution statement: Chenzhi Wang and Lin Li contributed equally to this study.
48 E-mail addresses: cz.wang1995@gmail.com (Chenzhi Wang), 1510960056@qq.com (Lin Li),
49 3218776356 @qq.com (Qi Zhang), raheemdel@aim.com (Dele Raheem), qinwen@sicau.edu.cn
50 (Wen Qin), DT_Wu@sicau.edu.cn (Dingtao Wu), hubin1118@126.com (Bin Hu),

mssiyangwy@sicau.edu.cn (Wenyu Yang), hongmin1@ualberta.ca (Hongmin Dong), tv3@ualberta.ca (Thava Vasanthan).

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Abstract: This study evaluated the feasibility of high speed shearing (HSS) on fabrication of GDL-induced whole soybean curd (WSC). The smaller particle size of suspension was achieved by HSS. However, the Native and SDS-PAGE indicated that shearing-denaturation led to the content decrease of lipoxygenase in raw suspension and inhibited the formation of specific band 7 in heated suspension. At higher shearing time and speed, the α-helix, β-types structures, intrinsic tryptophan fluorescence intensity, gel strength and centrifugation water holding capacity (cWHC) of WSC gradually decreased. However, the surface hydrophobicity increased conversely. From structural aspect, WSC exhibited a typical aggregated type gel network that was filled and covered with okara fibers. At higher shearing time and speed, the distribution of soy protein particles became more aggregated. When okara was filtered, sheared and added back to soymilk to fabricate WSC, higher gel strength and cWHC of WSC were obtained. To sum up, application of HSS on fabrication of WSC provides a cost-efficient way to utilize okara in soy product industry.

Keywords: Whole soybean curd; High speed shearing; Protein subunits; Secondary structure

1. *Introduction*

Soybean curd (tofu) was widely consumed in Asian countries owing to its nutritious and cholesterol-free properties (Zhu, Wu, Saito, Tatsumi, & Yin, 2016). The conventional recipe for soybean curd making involves the soaking of soybean seeds, grinding with additional water, cooking for protein denaturation, filtration of okara (insoluble soybean pulp), renneting with coagulant and pressing for final shaping (Kawaguchi, Kita, Shinyashiki, Yagihara, & Fukuzaki, 2018). Traditionally, the filtered okara in is usually discarded directly or processed into animal fodder due to its coarse particle size and adverse effect on sensory and textural properties of soy products (Aravind et al., 2012). However, it is estimated that 47% of the soy materials remained in

82 okara (Liu, 1997). As a matter of fact, okara is a crucial source of dietary fibers (Li, Qiao & Lu,
83 2012), of which insoluble state occupies the dominant fraction (O'Toole, 1999). The intake of
84 dietary fibers has been confirmed to possess functional properties such as hypolipidimic and
85 hypoglycemic effects (Kim, Yu, Byun & Cha, 2016). To enhance the utilization rate of soybean
86 materials as well as increase the content of dietary fibers in soybean curd for human nutrition, an
87 innovative whole soybean curd (WSC) that eliminated the filtration step and retained the okara into
88 the soybean curd was developed (Joo and Lee, 2011). However, the obtained WSC exhibited
89 weakened gel strength and disrupted network due to the introduction of okara fibers and protein
90 denaturation by high temperature and pressure (Liu & Kuo, 2016; Liu, Chien & Kuo, 2013). Thus,
91 the modification of okara was consider as the core issue to fabricate whole soybean curd of high
92 quality. The methods for okara fibers modification could be broadly classified into three types,
93 namely, physical, chemical, and enzymatic methods (Mateos-Aparicio, Mateos-Peinado & Rupérez,
94 2010; Kasai et al., 2004; Chen et al., 2014b). Among these methods, physical methods (e. g. high
95 pressure homogenization, high energy wet media milling, micro-fluidization) were proved to be
96 economical (compared with enzymatic methods), environmental (compared with chemical methods)
97 and effective. Wang et al (2018) obtained wheat gluten gels with higher gel strength and water
98 holding capacity by application of high hydrostatic pressure technology; Zhou et al (2018) applied
99 high-pressure pretreatment (HPP) to fabricate chicken breast gels, the results revealed that HPP
100 significantly decreased the cooking loss and facilitated to form a dense gel network. However, these
101 technologies presented common drawback of high equipment cost and energy-consuming. Being
102 one of the physical methods, HSS allows the materials to be cut, compressed and folded in the shear
103 gap by the strong shearing, dispersing, impacting, and turbulent flow. Consequently, the materials

104 could be well micronized, emulsified, mixed and homogenized in a short time. Based on the above
105 advantages, HSS has been successfully applied in food industry for the degradation of chitosan,
106 disaggregation of pectin and structural modification of tomato fibers (Rong et al., 2011; Hua et al.,
107 2017; Chen et al., 2014a).

108 In China, soybean product industry produced about 280 million tons of okara every year (Li,
109 Qiao & Lu, 2012). Commercial HSS equipment runs smoothly under atmospheric pressure and
110 possesses advantages in energy-saving and effectiveness. Thus, it could be considered as a potential
111 technology to micronize okara fibers for fabrication of WSC. To evaluate the feasibility of HSS for
112 soybean curd making, soybean flour suspensions were prepared and sheared by a high speed
113 shearing homogenizer at different parameters. The sheared suspensions were then coagulated into
114 WSC. The aggregation behaviors, microstructure and textural properties of WSC were analyzed,
115 aiming to provide a potential cost-efficient way to expand further application of okara in soy
116 product industry.

117 **2. *Materials and methods***

118 2.1 *Materials*

119 Soybean seeds (variety: intercropping Nandou 12) were kindly provided by College of
120 Agronomy, Sichuan Agricultural University and used throughout this study. Soybean flours were
121 obtained by grinding of soybean seeds by a grinder (800-Y, X-Hardware^{TM}, Zhejiang, China) at
122 speed of 26000 rpm for 2 min. Food grade D-glucono-1, 5-lactone solution (GDL) was purchased
123 from Xingzhou, Food Co., Ltd. (Anhui, China).

2.2 *Fabrication of soybean flour suspension, okara-filtered curd (OFC) and whole soybean curd (WSC)*

Soybean flour suspension and soybean curds were fabricated according to a previous method by Joo, Kim & Lee (2011) with slight modification. Briefly, 26.67 g of soybean flours and 200 mL of distilled water were mixed in a 250 mL beaker and subsequently stirred at 700 rpm for 2 min to obtain soybean flour suspension. The suspension was then subjected to high speed shearing process (details were introduced in section 2.3). The sheared suspension was heated to 95 °C and maintained for 5 min. A 300-mesh sieve was applied to remove the okara in the heated suspension. Subsequently, 150 mL of the filtrate was loaded into a 250 mL beaker and coagulated with D-glucono-1, 5-lactone solution (GDL, 0.4%, w/v). The mixture was incubated at 55 °C for 30 min to form OFC. For fabrication of WSC, the sole discrepancy was that the filtration step was omitted. Meanwhile, WSC without HSS treatment was set as the control group.

2.3 *High speed shearing (HSS) of soy flour suspension*

To figure out the effect of shearing time on formation of soybean curd, three copies of 150 mL soybean flour suspensions were loaded into three 250 mL beakers and subsequently sheared by a high speed shearing homogenizer (AD500S-H, Angni^{TM} Instruments, Shanghai, China) at speed of 15000 rpm for 2, 10 and 20 min, respectively. An ice-water-bath was applied to cool the beakers during the shearing process. To analyze the effect of shearing speed on formation of soybean curd, another three copies of 150 mL soybean flour suspension were loaded into three 250 mL beakers and then sheared at speed of 2000, 10000, 20000 rpm for 10 min under the ice bath condition.

2.4 *Particle size distribution of soy flour suspension*

A drop of soybean flour suspension was added into a laser scattering particle analyzer

(Rise-2006, Rise science™, Jinan, China). The median diameters (D_{50}), surface area to volume ratio (S/V, m^2/cm^3) were determined at refractive index of 1.590 and absorption of 0.001.

2.5 *SDS-PAGE and Native-PAGE analysis of soybean flour suspension and soybean curd*

Protein subunit composition was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Briefly, 1 g of lyophilized soybean flour suspension (or soybean curd) was dissolved in the loading buffer (composed of 0.15 mol/L Tris-HCl, 4% SDS, 5% β-mercaptoethanol) and centrifugated at 8000×g for 10 min. The clear supernatant extract was diluted to concentration of 2 mg/mL (in soluble protein content). The loading buffer was boiled for 5 min and subsequently loaded into an electrophoresis apparatus (Mini-PROTEAN Tetra, Bio-Rad®, CA, USA.) for analysis. SDS-PAGE was conducted at constant voltage of 120 V using a 5% of stacking gel and a 12% of acrylamide separating gel (AR0138, Boster Biotech™ Ltd., Beijing, China). A pre-stained molecular marker (11-245 kDa, Tiangen Biotech, Beijing, China) was applied to estimate the molecular weight of protein subunits. The gels were stained with coomassie bright blue and analyzed by Quantity one 4.6.2 (Bio-Rad®, CA, USA) for intensity analysis.

The native electrophoresis (Native-PAGE) was conducted without addition of β-mercaptoethanol and SDS, the other procedures were the same as SDS-PAGE.

2.6 *SEM and TEM analysis of soybean curd*

Scanning electron microscope (SEM) and transmission electron microscopy (TEM) analyses were conducted based on the method of Noh, Park, Pak, Hong & Yun (2005). Briefly, the soybean curd was sliced, rapidly frozen (-80 °C) and sputter-coated with a layer of Pb (5 nm) and placed inside a scanning electron microscope (LE, Phenom®, Shanghai, China). The SEM images were

168 taken at an accelerating voltage of 10 kV. For TEM analysis, the curd cubes were serially
169 dehydrated in series of ethanol aqueous solutions with gradient concentration (60%, 70%, 80%,
170 90%, 100%) for 10 min respectively. The dehydrated samples were immersed in isoamyl acetate
171 for 1 h, sliced by an ultramicrotome (Sapernova, Leica®, Austria) and observed by a transmission
172 electron microscope (JEM-2100F, JEOL®, Japan).

173 2.7 *Fourier transform infra-red (FT-IR) spectroscopy of soybean curd*

174 The lyophilized soybean curds were ground into powders and pressed with KBr powder
175 (1:100, w/w) into pellets for analysis. A Nicolet 5700 spectrometer (Thermo Fisher Scientific®,
176 USA) was applied to determine the absorbance within the frequency of 4000-400 cm^{-1} at a
177 resolution of 4 cm^{-1}. The data of the Amide I band (1600-1700 cm^{-1}) were analyzed by Peak Fit 4.
178 12 (SYSTAT®, USA) to estimate the relative content of secondary structures of proteins. The
179 frequencies assigned to secondary structures were: a-helix turns (1648-1660 cm^{-1}), β-sheet
180 (1612-1641 cm^{-1}), β-turns (1662-1684 cm^{-1}), random coil (1640-1650 cm^{-1}) (Zhang & Hua, 2007).

181 2.8 *Texture and centrifugation water holding capacity (cWHC) of soybean curd*

182 Texture of soybean curd was determined according to the standard of Gelatin Manufacturers
183 Institute of America (GMIA, 2013). A beaker containing the soybean curd was placed onto the
184 platform of a TA-XT Plus Texture Analyzer (Stable Micro System®, Surrey, UK) and compressed
185 by a P/0.5 probe. The test settings follow below: pre-test speed: 1.0 mm/s; test speed: 1.0 mm/s;
186 post-test speed: 10 mm/s; compression distance: 4 mm. The gel strength was defined as the strains
187 (g) that the probe detected at compressed distance of 4 mm. cWHC was determined as per the
188 method of Salvador et al. (2009) with slight modification. A 5 g of curd sample was cut from the
189 central position of the soybean curd and then loaded into a centrifuge tube that filled with

absorbent cotton at the bottom. The tube was centrifuged at speed of 10000 rpm for 10 min. cWHC was calculated as the percentage of centrifugated curd to the original weight.

2.9 *Surface hydrophobicity (H_0) of proteins in soybean curd*

The surface hydrophobicity (H_0) of soybean curd was determined as described by Achouri, Boye, Yaylayan & Yeboah (2005) with slight modification. The lyophilized soybean curds were ground into powders and stirred with 0.1 mol/L phosphate buffer (pH=7.0) at 1200 rpm for 30 min. The sample was subsequently centrifugated at 8000×g for 10 min. The clear supernatant extract was serially diluted to concentrations of 0.2, 0.1, 0.05, 0.025, and 0.0125 mg/mL (in soluble protein content). 20 μL of 8-anilino-1-naphthalenesulfonicacid (ANS, 8 mmol/L) solution was mixed with 4 mL of the sample solution and immediately detected by a fluorescence spectrometer (Lumia, Thermo scientific®, Massachusetts, USA) at wavelengths of 365 nm (excitation) and 484 nm (emission) to measure the fluorescence intensity. The index of H_0 was defined as the initial slope of fluorescence intensity as calculated by linear regression.

2.10 *Intrinsic tryptophan fluorescence intensity of proteins in soybean curd*

The lyophilized soybean curds were ground into powders, dissolved in phosphate buffer (0.1 mol/L, pH=7.0) and diluted to concentration of 0.1 mg/mL (in soluble protein content). A fluorescence spectrometer (Lumia, Thermo scientific®, Massachusetts, USA) was applied to determine the intrinsic emission fluorescence spectra of the solution. The excitation wavelength was set at 290 nm and the emission spectrum was recorded within the wavelength of 400 to 600 nm at a slit width of 5 nm (Li, Bao, Xu & Chi, 2015).

2.11 *Statistical analysis*

All experiments were performed in triplicate and the results were expressed as mean and

212 standard deviations (SD). Statistical analyses of different samples were performed using Duncan's
213 multiple range test (DMRT, $p<0.05$) using SPSS. 16.0 (IBM®, Chicago, USA). The significant
214 difference of the same groups before and after heating process was performed using t-test at
215 95% confidence level ($p<0.05$).

216 **3. *Results and discussion***

217 3.1 *Physicochemical properties of soy flour suspension*

218 3.1.1 Particle size distribution

219 Particle size distribution of soybean flour suspension treated with HSS was exhibited in Fig.
220 1. The related median diameters (D_{50}) and surface area to volume ratio (S/V) were presented in
221 Table 1. Values for the percentage of diameter below 10 μm, median diameter (D_{50}) and surface
222 area to volume ratio (S/V) in raw okara-filtered suspension (Fig. 1-E) were 100%, 1.87±0.05 μm
223 and 1.71±0.02 m^2/cm^3, which verified to 1.80%±0.02, 122.92±2.59 μm and 0.05±0.00 m^2/cm^3
224 after the heating process. Thermal denaturation of soybean proteins in liquid system was
225 accompanied with exposure of hydrophobic groups and self-association of proteins, resulting into
226 the formation of aggregations with larger diameters (Kohyama, Sano & Doi, 1995; Nishinari,
227 Fang, Guo & Phillips, 2014). Moreover, Guo et al. (2012) pointed out that the polypeptide chains
228 of soy protein would unfold during heating and the size of β-conglycinin and glycinin increased
229 with heating temperature consequently. Unsurprisingly, the percentage of diameter below 10 μm
230 and D_{50} in control group verified to zero and 165.12±3.30 μm with the introduction of okara.
231 Thermal process showed significantly effect on D_{50} as well, which increased to 185.47±3.30 μm.
232 On the one hand, this increase was attributed to the aggregation of proteins. On the other hand,
233 okara fibers absorbed water and expanded during thermal process. Depending on decreased D_{50}

and increased surface area to volume ratio, HSS effectively reduced the particle size of soybean flour suspension. Thus, HSS process, more or less, disintegrated the materials and destroyed the component cell layers of soybean seed.

3.1.2 Protein subunits composition

The effects of HSS and thermal process on the composition of soy protein subunits in soybean flour suspension were assessed by Native-PAGE and SDS-PAGE. As exhibited in Fig. 2-A, six major bands were separated in raw soybean flour suspension by Native-PAGE. Native-PAGE was conducted under non-reducing and non-denaturing conditions. Thus, the soy proteins' secondary structure and native charge density were well maintained. The data listed in Table 2 showed that the intensities of bands 1 to 6 decreased with increased shearing time and speed, revealing that HSS modified the charge to mass ratios of soy protein subunits and led to their shearing-denaturation. Thermal process was the central pre-procedure for curd making as well as the elimination of off-flavour in soymilk. The Native-PAGE profiles of heated soybean flour suspension exhibited in Fig. 2-B indicated that the images of band 1, 2, 3, and 5 almost disappeared in the gel. It should be attributed to thermal-induced denaturation, glycosylation and aggregation of storage proteins and whey fraction (Ingrassia, Palazolo, Risso & Wagner, 2017).

Generally, soybean proteins were mainly composed of storage globulin and whey fractions (Li et al., 2014). Among storage globulin, β-conglycinin (7S) and glycinin (11S) constituted more than 80% of contents and were regarded as the main fraction of storage proteins in soybean seeds (Saio, Kamiya & Watanabe, 1969). Soy whey proteins only made up 9%-15.3% contents of soybean proteins and were mainly classify into lipoxygenase (LOX, 102 kDa), β-amylase (61.7 kDa), lectin (33 kDa), Kunitz trypsin inhibitor (KTI, 20 kDa) and Bowman-Birk trypsin inhibitor

(BBI, 7.9 kDa) (Li et al., 2014). SDS-PAGE achieved separation of protein subunits in raw suspension by mass (Fig. 2-C). The storage proteins and whey fractions of raw soybean flour suspension were separated and corresponded to β-conglycinin (7S), glycinin (11S), LOX and Kunitz trypsin inhibitor (KTI), respectively. Being the major culprits for the generation of off-flavour in soy product, the bands of LOX exhibited decreased intensity especially in group 3, 4, 5 and 6. Therefore, HSS disintegrated LOX partly and decrease their content. The SDS-PAGE profiles of heated suspension were presented in Fig. 2-D, whose corresponded band intensities were exhibited in Table 3. Obviously, the band intensity of LOX almost disappeared in group 3 (10 min, 20000 rpm) and group 6 (15000 rpm, 20 min). Thus, increased shearing time and speed led to the content decrease of LOX in heated suspension. Combined with the intensity of LOX in raw suspension, we assumed that HSS unfolded LOX partly in raw suspension. Thus, the sheared LOX was easier to denature completely in thermal process.

It was worth noting that a specific band 7 whose molecular weight was about140 kDa was observed in heated suspension. However, this new band almost disappear in group 3 (10 min, 20000 rpm) and group 6 (15000 rpm, 20 min). As we discussed earlier, thermal-denatured proteins usually self-associated or interacted with other molecules to form aggregations. But the HSS-treated proteins exhibit different aggregation behaviors and failed to form the specific aggregations (like band 7) in thermal process.

3.2 *Quality evaluation of soybean curd*

3.2.1 Secondary structure, surface hydrophobicity (H_0), intrinsic tryptophan fluorescence and protein subunits composition

The FTIR spectra of proteins in soybean curds that fabricated from sheared suspension were

exhibited in Fig. 3-C. The relative contents of secondary structures and surface hydrophobicity of proteins in soybean curd were exhibited in Table 4. Obviously, the control group lost evidently the α-helix and β-types (β-sheet and β-turns) structures with a concomitant increase in unordered random coil. The surface hydrophobicity (H_0) of control group increased notably as well. These results implied that the introduction of okara made the secondary structure of soybean protein more unordered and exposed more hydrophobic groups. In comparison with the control group, the α-helix and β-types (β-sheet and β-turns) structures of all experimental groups (sheared WSC) decreased gradually with increased shearing time and speed. Moreover, the surface hydrophobicity (H_0) of experimental groups increased notably as well. As indicators of ordered structures, α-helix and β-sheet of proteins are usually buried in the interior of polypeptide chains, which unfolded during the thermal treatment (Bu et al., 2015). Combined with the results that we discussed in protein subunits composition, we held that HSS process unfolded the soybean proteins partly, thus more β-types (β-sheet and β-turns) structures and hydrophobic groups exposed to the environment during the thermal process.

The determination of intrinsic fluorescence was carried out to evaluate the polarity of the environment around Tryptophan (Trp) residues and detect the tertiary structure of proteins, which was conducted in view of the fact that Trp residues were sensitive to the subtle differences in the environment around (Vetri & Militello, 2005). Fig. 3-A/B showed the intrinsic fluorescence emission spectra of soybean curds that fabricated from sheared suspension. The maximum emission wavelength (λ_{max}) of okara-filtered group was observed at 442.25 nm, which slightly decreased to 440.45 nm in control group. With respect to HSS-treated samples, the λ_{max} shifted to 438.26 nm (15000 rpm, 2 min), 438.13 nm (15000 rpm, 10 min), 437.92 nm (15000 rpm, 20 min),

438.46 nm (10 min, 2000 rpm), 436.61 nm (10 min, 10000 rpm) and 435.45 nm (10 min, 20000 rpm), respectively. Moreover, the fluorescence intensities of HSS-treated samples were detected to decline as well. These blue shifts of λ_{max} implied that the Trp residues of sheared samples located into a more hydrophilic environment due to protein aggregation. It is common knowledge that the hydrophobic groups and most of chromophore groups buried inside the structure of native soybean proteins and unfolded during thermal process, resulting into the red shift of λ_{max} (Li et al., 2019). However, the unfolded proteins then aggregated into particles with addition of coagulants and the λ_{max} decreased consequently. Combined with the results of secondary structure and surface hydrophobicity, we believed that HSS process increased the exposure degree of hydrophobic groups in soybean proteins, which more tended to aggregate into particles and caused blue shift of λ_{max}. The decreased fluorescence intensities of HSS-treated samples were in coincident with the study of Pallares et al. (2004), which reported that the unfolded proteins would expose more chromophore groups to the solvent and caused the decrease of fluorescence intensity consequently.

The SDS-PAGE profiles of soybean curds that fabricated from HSS-treated suspension were presented in Fig. 3-D. The obvious difference of images between the heated suspension (Fig. 2-D) and soybean curd was the absence of LOX, 11S, KTI and intensity decrease of band 7 and 7S. Generally, in a suspension system, the hydrophobic groups of protein molecules were negatively charged, buried inside the native polypeptide chains and unfolded during thermal process. The introduced GDL (cations) neutralized the net charge on the surface of soy protein and the protein subunits aggregated into particles as a consequence of hydrophobic interaction (Guo & Ono, 2005). There was no significant difference in protein subunits composition between okara-filtered

322 and control groups. However, HSS-treated samples presented different protein subunits
323 composition, reflexing in decreased intensity of 7S and Band 7. As we discussed in secondary
324 structure and intrinsic fluorescence, HSS-treated soybean curd exposed more hydrophobic groups
325 and exhibited different aggregated behaviors. Thus, we assumed that the HSS-treated protein
326 subunits more tended to aggregated into particles, resulting into the decrease intensity of 7S and
327 Band 7.

328 3.2.2 Microstructure, texture and centrifugation water holding capacity (cWHC)

329 The microstructure of soybean curd was clearly characterized by SEM (Fig. 4). The scanning
330 electron micrographs of okara-filtered curd exhibited a relatively smooth and dense surface.
331 Although the suspension had been filtered, the microfibril bundles (Hua et al., 2017) were also
332 detected in the curd system. Compared with the okara-filtered curd, the control group exhibited a
333 complex structure. Okara fibers were observed to fill inside and cover on the surface of gel
334 network. In a zoomed-in observation (Fig. 4-b), a typical honeycomb like and hollow columnar
335 structure of parenchyma was detected (Qutob et al., 2008). However, the microstructures of
336 HSS-treated curds (Fig. 4-C and D) exhibited a looser and less-connected protein network, it was
337 attributed to shearing denaturation of soybean proteins in raw suspension as reported by Liu &
338 Kuo (2016). Moreover, parenchyma was effectively disintegrated, whose diameter significantly
339 decreased to about 80-100 μm.

340 The aggregation behaviors of soybean curds were analyzed by transmission electron
341 microscope and the micrographs were shown in Fig. 5 (a-d). Different from common honey-comb
342 structure, typical aggregated type gel networks (Hermansson, 1985) were detected in our
343 GDL-induced soybean curds. The protein aggregates in okara-filtered curd (Fig. 5-a) exhibited a

344 uniform distribution. However, the introduced okara (Fig. 5-b) aggregated with the proteins to
345 form larger particles. The anionic groups (-OH) on the surface of dietary fibers could reduce
346 protein-protein interaction and caused uneven distribution (Liu & Kuo, 2016). When HSS
347 treatment was applied (Fig. 5-c and d), the distribution of soy proteins became more aggregated,
348 and the gaps (the blank parts of the micrograph) between the protein networks became larger. This
349 was in accordance with results from microstructure of soybean protein isolate gels treated with
350 HSS obtained by Bi et al. (2018). As we discussed before, HSS-treated soybean proteins exhibited
351 higher surface hydrophobicity (H_0) in suspension and lower fluorescence intensity in soybean curd,
352 which well supported the fact that HSS-treated soybean curd exhibited a more aggregated and
353 less-connected gel network.

354 Being one of the critical evaluating indicators of curd quality, the gel strength of soybean
355 curds treated with HSS was shown in Fig. 5 (A, B). Obviously, the control group exhibited a
356 higher gel strength than okara-filtered group. Soy protein was the main component to form the gel
357 network (Kohyama, Sano & Doit, 1995). Okara commonly contains 20%-30% of crude proteins
358 and 40%-60% of fibers (de Figueiredo et al., 2018). Thus, the introduction of okara increased the
359 solid content and protein concentration in suspension and strengthened the gel network of soybean
360 curd. Numerous studies have confirmed the strong correlation between the microstructure and
361 texture of soybean curd (Yasir, Sutton, Newberry, Andrews & Gerrard, 2007; Wilkinson,
362 Dijksterhuis & Minekus, 2000). The increased gel strength of control group was also ascribed to
363 the difference in microstructure. The insertion of okara into protein network may induce to form
364 an increasingly complex and harder gel network. Moreover, the gel strength of whole soybean
365 curd declined with increased shearing speed and time, implying that the obtained soybean curds

366 were of inferior strength and elasticity. From structural aspect, the application of HSS destroyed
367 the compact internal gel network of the gel system, thus lowering the fracture resistance of the
368 curd system.

369 cWHC indicates the ability of the gel matrices to retain water molecules through capillary
370 effects, which reflected the spatial structure of the curd as well (Zhu et al., 2016). The cWHC of
371 HSS-treated whole soybean curd were presented in Fig. 5-C/D. Obviously, cWHC of control
372 group significantly declined in comparison with okara-filtered group. With application of HSS,
373 cWHC declined continuously with increased shearing speed and time. Thus, the decreased cWHC
374 indicated that the introduction of okara and application of HSS weakened the ability of soybean
375 curd to immobilize water within the gel network. From a structural aspect, the decreased cWHC
376 was in line with the microstructure of soybean curd (Li et al., 2019). In a curd system, water
377 molecules could either be bound to functional groups or hold in the pores of curd network (Shen
378 & Kuo, 2017). Owing to the introduced okara and applied HSS process, the gaps between the
379 protein networks were larger. The curd networks became looser and less-connected, thus less
380 water could be bound with the system.

381 *3.3 Addition of HSS-treated okara on texture and cWHC of WSC (supplementary experiment)*

382 Considering that the direct shearing of soybean flour suspension denatured the soybean
383 proteins partly and brought adverse effect on gel strength and cWHC of obtained WSC. We
384 conducted supplementary experiment that filtered and sheared the okara and add them back to the
385 soymilk to fabricate WSC. Briefly, soybean flours and distilled water were mixed at ratio of 1:7.5
386 (w/v) and subsequently stirred at 700 rpm for 2 min to obtain soybean flour suspension. The
387 obtained suspension was then heated to 95 °C and maintained for 5 min. A 300-mesh sieve was

388 applied to obtain the okara and filtrate. The filtrated okara was then mixed with distilled water at
389 ratio of 1:50 (w/v) and subsequently mixed at 700 rpm for 2 min. Three copies of the mixture
390 were sheared by a high speed shearing homogenizer (AD500S-H, Angni^{TM} Instruments, Shanghai,
391 China) at speed of 15000 rpm for 2, 10 and 20 min under the ice bath condition. Moreover,
392 another three copies of the mixture were sheared at speed of 2000, 10000, 20000 rpm for 10 min
393 under the ice bath condition as well. The sheared mixtures were then lyophilized and added into
394 the filtrate at ratio of 20% (w/v) and coagulated into soybean curd (The coagulation of soybean
395 curd, determination of gel strength and cWHC were conducted as the methods we discussed
396 before). The correlation between shearing speed, shearing time, gel strength and cWHC were
397 analyzed by Pearson's correlation test (Huang et al., 2010) at significant level of 95% (p<0.05)
398 using SPSS. 16.0 (IBM®, Chicago, USA). As expected, the results shown in Table 5 indicated that
399 shearing speed and time were positively correlated with gel strength and cWHC, of which
400 significant difference (p<0.05) was observed between shearing speed and cWHC. Moreover, the
401 gel strength also showed positive correlation with cWHC. These results were in accordance with
402 the study of Ullah et al. (2019) which reported that high-energy wet milling of dietary fibers
403 enhanced the WHC and texture of okara-contained tofu. On the one hand, filtration of okara avoid
404 the denaturation of filtrate (soybean protein) by high temperature and shearing force. On the other
405 hand, micronized okara contributed to form an ordered gel network, which showed higher
406 resistance to destructive compressing and better retention capacity of water.

407 **4. *Conclusions***

408 High speed shearing (HSS) of soybean flour suspension significantly affected the
409 composition of protein subunits and microstructural properties of GDL-induced whole soybean

curd. The smaller particle size of HSS-treated suspension was demonstrated by decreased median diameter. However, the soybean proteins in raw suspension shearing-denatured and presented content decrease in LOX. In heated suspension, HSS-treated proteins exhibit different aggregation behaviors and failed to form the specific band 7. HSS of soybean flour suspension affected the properties of induced soybean curd as well. At higher shearing time and speed, the α-helix, β-types structures, intrinsic tryptophan fluorescence intensity, gel strength and cWHC of whole soybean curd (WSC) gradually decreased. Conversely, the surface hydrophobicity (H_0) of WSC increased. From SEM analysis, WSC exhibited a complex gel network that filled and covered with okara fibers. When HSS was applied, the microstructure became looser and less-connected, leading to weakened gel strength and decreased cWHC. TEM analysis confirmed the aggregated type gel networks of GDL-induced WSC. At higher shearing time and speed, the distribution of soy proteins particles became more aggregated. When okara was filtered, sheared and add back to the soymilk to fabricate WSC, the shearing speed and time were positively correlated with gel strength and cWHC. To conclude, it seems impossible to shear the okara in suspension and at the same maintain the native structure of soybean protein. Therefore, the alternative method is to separate and micronize okara individually and add them back to soymilk to obtain WSC of high quality. Further investigations on comprehensive processing of dietary fibers in soybean curd should be conducted.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Figure captions

Fig. 1. Particle size distribution of soybean flour suspension treated with HSS. (A: raw suspension that sheared at speed of 2000, 10000, 20000 rpm for 10 min; B: cooked suspension that sheared at speed of 2000, 10000, 20000 rpm for 10 min; C: raw suspension that sheared at 15000 rpm for 2, 10 and 20 min; D: cooked suspension that sheared at 15000 rpm for 2, 10 and 20 min; E: okara-

561 filtered suspension).

562 **Fig. 2.** Native-PAGE (A, B) and SDS-PAGE (C, D) profiles of protein subunits extracted from
563 raw (A, C) and cooked (B, D) soybean flour suspension that treated with HSS (M: marker; C:
564 control group, untreated with HSS; 1: 10 min, 2000 rpm; 2: 10 min, 10000 rpm; 3: 10 min, 20000
565 rpm; 4: 15000 rpm, 2 min; 5: 15000 rpm, 10 min; 6: 15000 rpm, 20 min; HSS: high speed
566 shearing).

567 **Fig. 3.** Intrinsic emission fluorescence spectra (A, B), FTIR spectra (C) and SDS-PAGE profiles
568 (D) of soybean curds that fabricated from raw suspension treated with HSS (O: okara-filtered
569 soybean curd; C: control group, whole soybean curd untreated with HSS; 1: 10 min, 2000 rpm; 2:
570 10 min, 10000 rpm; 3: 10 min, 20000 rpm; 4: 15000 rpm, 2 min; 5: 15000 rpm, 10 min; 6: 15000
571 rpm, 20 min). Abbreviations: Okara-filtered, okara filtered soybean curd; Control group, whole
572 soybean curd that fabricated from soybean flour suspension untreated with HSS; 10 min+2000,
573 10000 and 20000 rpm, whole soybean curd that fabricated from soybean flour suspension sheared
574 for 10 min at speed of 2000, 10000, 20000 rpm; 15000 rpm+2, 10 and 20 min, whole soybean
575 curd that fabricated from soybean flour suspension sheared at 15000 rpm for 2, 10 and 20 min.

576 **Fig. 4.** Scanning electron micrographs (SEM) of soybean curd fabricated from soybean flour
577 suspension treated with HSS. (A, a) Okara-filtered soybean curd. A: 500×, a: 1000×. (B, b)
578 Control group, whole soybean curd that fabricated from suspension untreated with HSS. B: 500×,
579 b: 1000×. (C, c) Whole soybean curd that fabricated from suspension sheared at speed of 20000
580 rpm for 10 min. C: 500×, c: 1000×. (D, d) Whole-soybean curd that fabricated from suspension
581 sheared at 15000 rpm for 2 min. D: 500×, d: 1000×.

582 **Fig. 5.** Transmission electron micrographs (TEM) of soybean curd (a: Okara-filtered soybean curd;

583 b: whole soybean curd that fabricated from suspension untreated with HSS; c: Whole soybean
584 curd that fabricated from suspension sheared at speed of 20000 rpm for 10 min; d: Whole soybean
585 curd that fabricated from suspension sheared at 15000 rpm for 2 min); (A, B) Gel strength of
586 soybean curd; (C, D) Centrifugation water holding capacity (cWHC, %) of soybean curd.