

Rheological Characteristics and Microstructure of Probiotic Soy Yogurt Prepared from Germinated Soybeans

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Summary

Soy yogurts (sogurts) were prepared from germinated soybeans [*Glycine max* (L.) Merrill] with different hypocotyl lengths. This study is mainly focused on the effects of soybean germination on the rheological characteristics and the microstructure of sogurt. Results show that, after soybean germination, sogurt had lower storage module (G') and loss module (G'') in the linear viscoelastic region than the sogurt from ungerminated soybean. Shear sweep test demonstrated that the flow behaviour index (n) of sogurt obviously rose from 0.225 to 0.241 ($p < 0.05$), while the yield stress (τ_0) and consistency coefficient (κ) decreased remarkably from 3.25 to 1.98 Pa and from 12.61 to 8.37 Pa·s ^{n} , respectively. Furthermore, the three-dimensional network of sogurt became more ordered and open, with the diameter of interspaces increased from 1–2 to 5–6 μm . Protein hydrolase system assays indicated that endopeptidase, aminopeptidase, dipeptidyl aminopeptidase and carboxypeptidase in sprouted seeds showed different activities towards synthetic substrates. It can be concluded that, after the limited proteolysis of soybean storage proteins by endopeptidases and exopeptidases in germinated soybeans, the rheological properties and microstructure of sogurts can be improved.

Key words: soybean germination, soy yogurt (sogurt), probiotics, rheological properties, microstructure, protein hydrolase

Introduction

Soybean is one of the widely consumed oil crops worldwide, with health benefits because it is rich in unsaturated fatty acids and in low cholesterol. Fermented soybean foods are especially popular in the Asian countries such as China, Japan and Indonesia. Soy yogurt is prepared from the fermented soymilk by lactic acid bacteria (LAB). Recently, there has been increased interest in the improvement of sogurt, *e.g.* by the addition of combined probiotics (1), prebiotic supplementation (2),

enhancement with γ -aminobutyric acid (3) and active isoflavone aglycone (4). Nevertheless, due to the unpleasant beany flavour, insufficient acidity, rigid and brittle gel structure (5), sogurt has not been generally accepted yet.

After sprouting, soybean is reported to be more nutritional with increased levels of polypeptides, free amino acids, isoflavones, dietary fibres, vitamins and minerals (6). At the same time, the anti-nutritional factors such as trypsin inhibitor, haemagglutinin and phytic acid are significantly reduced (7,8). Degradation of soybean pro-

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teins is recognized as one of the most complicated and important physiological and biochemical changes during seedling growth. Globular storage proteins β -conglycinin (7S) and glycinin (11S) constitute approx. 70 % of the total soybean proteins, the former is a trimeric glycoprotein consisting of α' , α and β subunits (9), the latter is a hexamer composed of six kinds of subunits, each one containing acidic and alkaline chains linked by a disulphide bond (10). It is generally thought that soy storage proteins are first partly cleaved by endopeptidases at specific bonds, and then further degraded by unrestricted proteases (11).

According to literature reports, there are extensive peptidases that are capable of degrading soy storage proteins during seed sprouting. Two endopeptidases were assayed by Bond and Bowles (12), one of which is a carboxyl endopeptidase, exhibiting optimum activity at acidic pH, while another is a neutral metalloendopeptidase, showing degrading activity towards the α' and α subunits of 7S and the acidic chains of 11S. Proteases G1 and G2 are also responsible for the hydrolysis of the acidic subunits of glycinin. The former is a legumain-like cysteine protease and it shows high specificity for cleavage sites, the latter is presumed to be a collection of enzymes (8). Moreover, protease C1 is a serine protease reported to be responsible for the cleavage of α' , α subunits of 7S globular protein in the N-terminal region (13). Protease C2, a papain-like cysteine protease that shows low specificity towards particular split site or sequence, can degrade all subunits of β -conglycinin and acidic polypeptides of glycinin (14). After enzymolysis, the generated polypeptides and oligopeptides can be further cleaved by exopeptidases, and the free amino acids are then released from the carboxylic or amino terminal domain. There are also some literature data on the activities of carboxypeptidases (8,15) and aminopeptidases (12,16,17) in soybean seedlings.

Latest research has demonstrated that different soybean subunits within β -conglycinin (18) and glycinin (19) have different effects on gel properties of heat-induced and glucono- δ -lactone (GDL)-induced soy protein aggregations. Soy storage proteins are partly hydrolyzed after seed sprouting, thus, the network structure of lactic acid bacteria-coagulated soy protein gel may be affected as well. However, there is still little research related to this topic. Our previous research had indicated the improvement of the physicochemical, textural and sensory characteristics of sogurt made from soybean seedlings (20). The object of this work is to further study the effects of germination on the rheological properties and microstructure of sogurt.

Materials and Methods

Materials and chemicals

Soybean [*Glycine max* (L.) Merrill] was purchased from a local supermarket (Guangzhou, PR China). Skimmed milk powder, with protein content of $w=32.7\%$, was purchased from Fonterra Co., Ltd (Tauranga, New Zealand). Substrates for the assay of peptidase activities were purchased from Sigma-Aldrich, Inc. (Schnelldorf, Germany) and Chem-Impex International, Inc. (Wood Dale, IL, USA). Other reagents were analytically pure.

Lactobacillus bulgaricus AS1.1482 and *Streptococcus thermophilus* IFFI 6038 were provided by Microbiological Culture Collection Center (Guangdong, PR China). *Lactobacillus helveticus* B02 was obtained from Chr. Hansen Food Ingredients Co., Ltd. (Tianjin, PR China). De Man, Rogosa and Sharpe (MRS) broth was used for the incubation of stock culture, while the reconstituted skimmed milk (RSM) ($w=12\%$) was used for subculture. After sterilization, the media were inoculated and then incubated in an ordinary incubator at 37 °C for 12–15 h, followed by refrigeration at 4 °C until further assays.

Sogurt preparation

Sogurt was prepared as described before (20). Briefly, after surface-sterilization, soybeans were incubated at 25 °C with 80 % relative humidity in the dark until the hypocotyl length reached 3 or 6 cm. After peeling, the seeds were homogenized with double distilled water at 85 °C and a ratio of 1:10 (m/V). The resulting slurries were filtered to yield soymilk, and then mixed with reconstituted skimmed milk ($w=12\%$) at a ratio of 7:3 (by volume). Next, the mixed media were supplemented with 8 % (m/V) sucrose and then sterilized at 100 °C for 20 min. After cooling to 42 °C, they were inoculated with the combined strain starters *Lactobacillus bulgaricus* AS1.1482, *Streptococcus thermophilus* IFFI 6038 and *Lactobacillus helveticus* B02 ($\psi=5\%$, at a volume ratio of 1.5:1.5:2), followed by 4 h of incubation at 42 °C. Sogurt was subsequently removed and refrigerated at 4 °C for further assay. In this work, soy yogurt samples prepared from the soaked beans without germination were labelled S_0 sogurt, while samples prepared from soybean seeds with 3- and 6-cm hypocotyls were labelled S_3 and S_6 sogurt, respectively. As a reference, reconstituted skimmed milk yogurt (RSMY) was prepared as well, by fermenting RSM using the same procedure.

Measurement of rheological properties

The rheological properties of specimens were analyzed with a rheometer (AR550, TA Instruments-Waters LLC, New Castle, DE, USA), using the method of Donkor *et al.* (21) with minor modifications. A 40-mm stainless steel parallel plate with a gap of 1 mm was used to measure the samples at (25.0 ± 0.5) °C with two repetitions. Strain sweep (0–50 %) was first operated at 1 Hz to determine the linear viscoelastic range. Specimens were subjected to frequency sweep ranging from 0.1 to 10 Hz at 0.5 % constant strain, then followed by a shear sweep with increasing shear rate from 0 to 500 per s in 180 s. Herschel-Bulkley model was used to obtain the flow behaviour parameters. The flow model is expressed by the following equation:

$$\tau = \tau_0 + \kappa \cdot \dot{\gamma}^n \quad /1/$$

where τ is the shear stress, τ_0 is the yield stress, κ is the consistency coefficient, $\dot{\gamma}$ is the shear rate and n is the flow behaviour index.

Microstructure observation

The microstructures of the specimens were observed by scanning electron microscope (SEM) following the method of Supavitpatana *et al.* (22). After critical point drying by liquid carbon dioxide, specimens were sub-

sequently fractured at room temperature, placed on aluminium stubs, sputter-coated with platinum for 150 s using a sputter coater and then observed by a scanning electron microscope (S-3000N, Hitachi, Tokyo, Japan) at 20 kV.

Free amino acid analysis

HPLC analysis for the evaluation of free amino acids (FAAs) was carried out according to the previous report (20), using a Waters HPLC system fitted with a PicoTag[®] column (Waters Corp., Milford, MA, USA). By comparison with known standards, quantitative data of FAAs were obtained.

Crude enzyme extraction

Crude enzyme extraction was obtained using the method of Wilson *et al.* (8) with some modifications. In brief, germinated seeds were harvested and homogenized with ice-cold 50-mM phosphate buffer solution (PBS), pH=7.0, at a rate of 5 mL of buffer per g of soybean. Extracts were filtered and centrifuged (15 000×g, 30 min, 4 °C; Hitachi CR 22G). Then the supernatant was further filtered through 0.45- μ m filter membrane and stored at -20 °C until further use. In this work, crude enzyme extract prepared from the soaked beans without germination was labelled E₀, while those from soybeans with 3- and 6-cm hypocotyls were labelled E₃ and E₆, respectively. Protein concentration of the crude enzyme extract was assayed using the Bradford method with duplicate analyses (23). The bovine serum albumin (BSA) was used as standard.

Protein hydrolase system assay

Protease activity assay was performed as described by Li *et al.* (24). Briefly, the substrate casein was dissolved to a final concentration of 2 % (*m/V*) with lactate buffer (pH=3.0), phosphate buffer (pH=7.0) and borax/sodium hydroxide buffer (pH=10.0). Solution containing 1 mL of enzyme extract and 1 mL of substrate reacted at 40 °C for 24 h, and then the reaction was ended by the addition of 2 mL of 0.4 M trichloroacetic acid (TCA). To 1-mL filtrate aliquots, 5 mL of Na₂CO₃ (0.4 M) and 1 mL of Folin-Ciocalteu reagent were added, and then incubated at 40 °C for 20 min. The absorbance of the mixed solution was monitored at 660 nm. Enzyme blanks were also monitored as reference. The protease activity was defined as the amount of enzymes that release 1 μ g of tyrosine per day per mg of casein at 40 °C.

Endopeptidase, aminopeptidase and dipeptidyl aminopeptidase activities were assayed by synthetic substrates with minor modifications (24,25). The substrates used for endopeptidase activity assays were as follows: N-benzoyl-Gly-Pro-Arg-*p*-nitroaniline (NBZ-Gly-Pro-Arg-pNA), NBZ-Glu-pNA, NBZ-Phe-pNA and NBZ-Asp-pNA. Leu-pNA, Val-pNA, Ala-pNA and Glu-pNA were prepared for aminopeptidase activity evaluations and Gly-Phe-pNA for dipeptidyl aminopeptidase measurements. All of the substrates were dissolved in methanol to final concentrations of 16.4 mM for endopeptidase, 16 mM for aminopeptidase and 4 mM for dipeptidyl aminopeptidase. The reaction solution, consisting of 100 μ L of enzyme extract, 100 μ L of substrate solution and 2.8 mL of PBS (100 mM, pH=7.0) reacted at 37 °C for 6 h,

then the released *p*-nitroaniline was monitored at 410 nm to measure the absorbance. Different concentrations of the extract were repeated and the enzyme blanks were also monitored as reference. The activity was considered as μ mol of substrate hydrolyzed per day per mg of protein.

Assay of carboxypeptidase activities was performed using methods of Macedo *et al.* (25) and Folkertsma and Fox (26) with slight modifications. The following substrates were obtained for the research: N-carbobenzoxy-Ala-Tyr (NCBZ-Ala-Tyr), NCBZ-Ala-Phe, NCBZ-Ala-Leu, NCBZ-Glu-Phe and NCBZ-Trp-Leu, which were dissolved in potassium phosphate buffer (10 mM, pH=7.0) to a final concentration of 2.5 M. The mixed solution that consisted of 200 μ L of enzyme extract and 1.8 mL of substrate solution was incubated at 37 °C for 6 h. Aliquots of 50 μ L were mixed with Cd-ninhydrin reagent (2 mL) and incubated at 84 °C for 5 min. The absorbance of the solution was monitored at 507 nm. Different concentrations of the extract were repeated and the enzyme blanks were also monitored as reference.

Statistical analysis

Data were expressed as mean values \pm standard deviation (S.D.). The analyses of variance were performed using ANOVA and the significant differences were analyzed by Duncan's test with a 95 % confidence level.

Results and Discussions

Viscoelastic rheological properties

The elastic and viscous properties of a fluid are represented by two critical viscoelastic parameters, namely storage (G') and loss (G'') modulus, respectively (27). RSMY and sogurts prepared in this work exhibited high frequency sensitivity in the linear viscoelastic region from 0.1 to 10 Hz (Fig. 1), *i.e.* G' and G'' values rose progressively with the increasing frequency. This could be attributed to higher molecular mobility at higher frequency

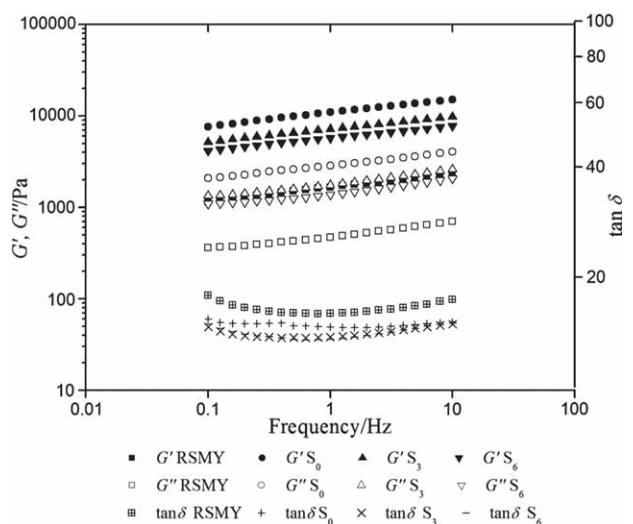


Fig. 1. Frequency sweeps of RSMY and sogurt. G' =storage modulus, G'' =loss modulus, $\tan \delta$ =dissipation factor, RSMY=reconstituted skimmed milk yogurt; S₀, S₃ and S₆ indicate soy milk from soybeans without any or with different hypocotyl lengths

(28). As illustrated in Fig. 1, G' value was remarkably higher than G'' value over the entire frequency sweep, demonstrating the gel-like rheological behaviour of all samples (21). Interestingly, G' and G'' values of sogurts obtained from germinated soybeans were obviously lower than those of the ungerminated one, and these values gradually decreased with the increase of hypocotyl length. The reduced elastic and viscous properties might be caused by the improvement of protein micelle networks of sogurt (Fig. 2). Thus, the network structures of the S_3 and S_6 sogurt were more loose with lower G' values (29). By comparing the dissipated energy with the stored energy during each oscillation, the loss factor, namely $\tan \delta$, is represented as the ratio of G'' and G' (27). The loss factors of all specimens gradually decreased below the frequency of 1 Hz and then increased to 10 Hz (Fig. 1), demonstrating that the lost energy is reduced as the stored energy increased at low frequency (30), and *vice versa* at high frequency.

Flow behaviour

Flow behaviour parameters of RSMY and sogurts are summarized in Table 1. Values of the correlation coefficients (R^2) are all greater than 0.995, indicating that the data obtained in this work fitted well to the Herschel-Bulkley model. The flow behaviour index (n) demonstrates the deviation from Newtonian flow ($n=1$). It is a pseudoplastic fluid when $n < 1$, and a dilatant fluid when

$n > 1$ (30). As shown in Table 1, all of the specimens exhibited pseudoplastic fluid properties, as n values were well lower than 1. After soybean germination, the flow behaviour index (n) of sogurt dramatically rose from 0.225 to 0.241 ($p < 0.05$). Higher n value of sogurt prepared from germinated seeds indicated the less shear-thinning behaviour during shear sweep, which might be caused by the decrease of the length of molecular chains and the cross-linking of protein micelles.

On the contrary, the consistency index (κ) and yield stress (τ_0) of soy yogurt decreased remarkably from 12.61 Pa·s ^{n} and 3.25 Pa (in S_0 sogurt) to 8.37 Pa·s ^{n} and 1.98 Pa (in S_6 sogurt), respectively. Harte *et al.* (31) suggested that the yield stress values of yogurt have close correlation with the sensory firmness as perceived by a trained panel. The reduction of τ_0 values in S_3 and S_6 sogurt (Table 1) was in accordance with the softer sensory characteristics (20). Furthermore, apparent viscosity of sogurt decreased as well, from 0.63 (S₀ sogurt) to 0.46 Pa·s (S₆ sogurt) at 22 s⁻¹, and from 0.23 (S₀ sogurt) to 0.17 Pa·s (S₆ sogurt) at 100 s⁻¹ ($p < 0.05$). Lamsal *et al.* (32) reported that the values of consistency index and apparent viscosity were reduced with the progression of the degree of hydrolysis of soybean isolated protein. Previous study indicated that, as hypocotyl length increased, the degree of proteolysis of soy storage protein was enhanced (20), which should be responsible for the improvement of flow behaviour of sogurt.

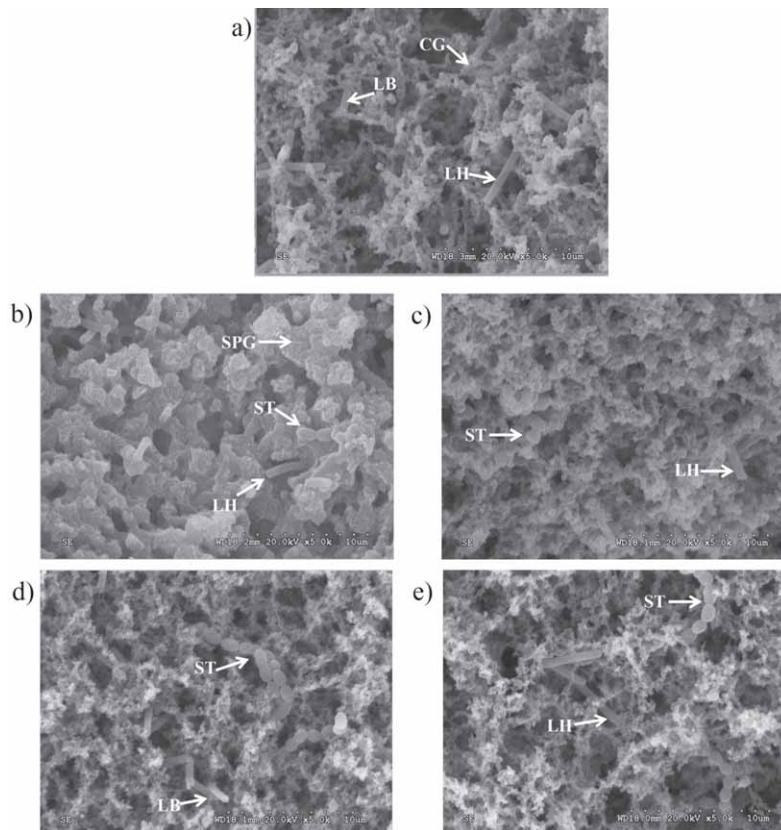


Fig. 2. Microstructure of: a) reconstituted skimmed milk yogurt (RSMY), b) sogurt from ungerminated soybeans (100 % soymilk), c) sogurt from ungerminated soybeans (70 % soymilk+30 % RSM; S_0), d) and e) sogurt from germinated soybeans (mixed soymilk from soybeans with different hypocotyl lengths; S_3 and S_6 , respectively).

Labelled arrows: CG=casein granules, SPG=soy protein granules, ST=*Streptococcus thermophilus* IFFI 6038, LH=*Lactobacillus helveticus* B02, LB=*Lactobacillus bulgaricus* AS1.1482

Table 1. Rheological parameters of RSMY and sogurt

Sample	Herschel-Bulkley model				Apparent viscosity/(Pa·s)	
	τ_0 /Pa	κ /(Pa·s ⁿ)	n	R ²	at 22 s ⁻¹	at 100 s ⁻¹
RSMY	(1.17±0.06) ^a	(6.49±0.20) ^a	(0.288±0.00) ^d	0.995	(0.33±0.02) ^a	(0.13±0.01) ^a
S ₀	(3.25±0.18) ^d	(12.61±0.67) ^d	(0.225±0.00) ^a	0.999	(0.63±0.05) ^c	(0.23±0.02) ^c
S ₃	(2.61±0.26) ^c	(10.03±0.37) ^c	(0.234±0.00) ^b	0.998	(0.52±0.02) ^b	(0.19±0.00) ^b
S ₆	(1.98±0.01) ^b	(8.37±0.03) ^b	(0.241±0.00) ^c	0.998	(0.46±0.00) ^b	(0.17±0.00) ^b

Values are mean±standard deviations, $N=2$; different letters in superscript in the same column represent significantly different ($p<0.05$) values; RSMY=reconstituted skimmed milk yogurt; S₀, S₃ and S₆ indicate soymilk from soybeans with different hypocotyl lengths; τ_0 =yield stress, κ =consistency coefficient, n =flow behaviour index, R²=correlation coefficient

Microstructure of yogurts

The microstructures of RSMY and sogurts were observed using scanning electron microscope (SEM) at magnification of 5000. As shown in Fig. 2, the microstructures of all specimens were well-defined three-dimensional networks filled with combined probiotics of *Streptococcus thermophilus* IFFI 6038 and two kinds of *Bacillus* (arrows labelled in Fig. 2). According to the research in our laboratory, the number of viable lactic acid bacteria in all sogurt specimens was about 8.60–8.68 log CFU/mL after the first day of cold storage at 4 °C, and was still around 6.94–6.57 log CFU/mL after 3 weeks of cold storage (data not shown). It can be indicated that the probiotic strains used in this work can colonize in a good number before exerting their health benefits to the host (33).

The protein gel of RSMY is a honeycomb-like aggregation of casein particles containing interspaces of different sizes (Fig. 2a), while that of sogurt is a compact and rigid network of soybean protein macroaggregates (Fig. 2b). Notably, after supplementation with RSM ($\psi=30\%$), the whole protein network of S₀ sogurt was more organized and open, having diameter of interspaces at around 1–2 μm (Fig. 2c). In addition, as soybean hypocotyl length increased, larger, more open and ordered interspaces of sogurt gel network were discernible, and the diameter of sogurt network interspaces remarkably increased from 3 or 4 μm in S₃ sogurt (Fig. 2d) to 5 or 6 μm in S₆ sogurt (Fig. 2e) as well. As germination continued, the α' , α subunits of 7S and acidic chains of 11S were degraded gradually (20); thus, the molecular structure of soybean storage protein was more open and the cross-linking of protein micelles in sogurt was therefore reduced. Nevertheless, as hypocotyls reached 6 cm, the relatively over-degraded soybean protein subunits resulted in weaker linkage of soy protein micelles (Fig. 2e). Thus, with larger diameter of interspaces, the unstable gel network of S₆ sogurt was in accordance with the lower water holding capacity determined in our previous research (20).

Protein hydrolase system

The activities of acidic, neutral and alkaline protease in germinated seeds are shown in Table 2. Proteases showed activities towards casein over a wide pH range. Active acid and neutral protease activities were detected right after imbibitions, at around 29.20 and 8.57 $\mu\text{g}/(\text{day}\cdot\text{mg})$, respectively. After minor decrease or increase,

Table 2. Protease activities of crude enzyme extracts of soybean seedlings

pH value	E ₀	E ₃	E ₆
	$\mu\text{g}/(\text{day}\cdot\text{mg})$	$\mu\text{g}/(\text{day}\cdot\text{mg})$	$\mu\text{g}/(\text{day}\cdot\text{mg})$
pH=3.0	29.20±0.00	16.76±0.44	13.79±2.57
pH=7.0	8.57±0.63	14.79±0.29	22.41±0.64
pH=10.0	0.41±0.13	0.92±0.18	3.46±0.17

Values are mean±standard deviations, $N=2$; E₀, E₃, E₆ indicate the crude enzyme extracts of soybeans with different hypocotyl lengths

the activities maintained relatively high level as hypocotyls grew to 6 cm. Most of the acidic proteases might belong to the papain-like family of cysteine protease as they showed optimum activities at acid pH (34). The activities of alkaline proteases were fairly low over the entire germinating period.

As shown in Table 3, endopeptidase in soybean seedlings showed high activity against the substrate NBZ-Gly-Pro-Arg-pNA, ranging from 15.37 to 28.49 $\mu\text{mol}/(\text{day}\cdot\text{mg})$. The Arg-X bond is an important split site of legumain-like endopeptidase during the proteolysis of phaseolin in beans (34). It could be speculated that endopeptidase which showed activity towards the Arg-pNA site in this work might be a legumain-like cysteine protease as well. By assaying the N-terminal sequences of hydrolyzates, Qi *et al.* (13) reported that there are several acidic amino acid residues (such as Asp and Glu) around the split site of protease C1. Nonetheless, the substrates NBZ-Asp-pNA, NBZ-Glu-pNA and NBZ-

Table 3. Endopeptidase activities of crude enzyme extracts of soybean seedlings

Substrate	E ₀	E ₃	E ₆
	$\mu\text{mol}/(\text{day}\cdot\text{mg})$	$\mu\text{mol}/(\text{day}\cdot\text{mg})$	$\mu\text{mol}/(\text{day}\cdot\text{mg})$
NBZ-Gly-Pro-Arg-pNA	28.50±0.47	15.37±2.19	28.39±0.43
NBZ-Glu-pNA	N.D.	N.D.	N.D.
NBZ-Phe-pNA	N.D.	N.D.	N.D.
NBZ-Asp-pNA	N.D.	N.D.	N.D.

Values are mean±standard deviations, $N=2$; E₀, E₃, E₆ indicate the crude enzyme extracts of soybeans with different hypocotyl lengths; NBZ=N-benzoyl, pNA=*p*-nitroaniline; N.D.=not detected

-Phe-pNA remained totally unattacked by endopeptidases in this work (Table 3), which demonstrates that the split sites of protease C1 are located in the X-Glu and X-Asp, rather than in the Glu-X and Asp-X sites. As some of the specific peptide bonds were cleaved, the polypeptide chains were further hydrolyzed by nonrestrictive proteolysis. Thus, after the restrictive degradation of α' , α subunits of 7S and acidic chains of 11S by endogenous endopeptidases in soybean seeds, the microstructures of soy protein aggregated gel were more ordered and open (Fig. 2).

Results in Table 4 demonstrate different activities of aminopeptidase, dipeptidyl aminopeptidase and carbo-

Table 4. Exopeptidase activities of crude enzyme extracts of soybean seedlings

Substrate	E ₀	E ₃	E ₆
	$\mu\text{mol}/(\text{day}\cdot\text{mg})$	$\mu\text{mol}/(\text{day}\cdot\text{mg})$	$\mu\text{mol}/(\text{day}\cdot\text{mg})$
Leu-pNA	23.96±0.39	11.41±0.22	15.98±0.91
Val-pNA	1.97±0.02	0.54±0.00	0.59±0.01
Ala-pNA	69.22±1.88	33.69±0.33	46.01±0.32
Glu-pNA	0.48±0.04	0.21±0.01	N.D.
Gly-Phe-pNA	4.81±0.00	4.34±0.00	5.38±0.06
NCBZ-Ala-Tyr	0.01±0.00	0.02±0.00	0.07±0.00
NCBZ-Ala-Phe	0.01±0.00	0.04±0.01	0.09±0.00
NCBZ-Ala-Leu	0.03±0.00	0.15±0.02	0.09±0.02
NCBZ-Glu-Phe	0.01±0.00	0.04±0.01	0.08±0.00
NCBZ-Trp-Leu	0.06±0.00	0.12±0.02	0.18±0.01

Values are mean±standard deviations, $N=2$; E₀, E₃, E₆ indicate the crude enzyme extracts of soybeans with different hypocotyl lengths; pNA=*p*-nitroaniline, NCBZ=N-carbobenzoxy; N.D.=not detected

xypeptidase in sprouted seeds. Aminopeptidase exhibited the highest activities against substrate Ala-pNA at 69.22 $\mu\text{mol}/(\text{day}\cdot\text{mg})$ right after soybean imbibitions, followed by Leu-pNA at about 23.96 $\mu\text{mol}/(\text{day}\cdot\text{mg})$. Couton *et al.* (16) reported an aminopeptidase that shows activities against substrates with N-terminal hydrophobic amino acid residues, which might explain the potent cleavage abilities towards substrates with amino acid residues Ala and Leu in this study. Aminopeptidases that catalyze the efficient hydrolysis of substrates with specific N-terminal acidic amino acid residues have been studied as well (17). Yet, low activity towards Glu-pNA was detected at around 0.48 units at the onset of sprouting. The activity was then dropped until it was not detected in E₆. Soy enzyme extracts also exhibited dipeptidyl aminopeptidase activity towards substrate Gly-Phe-pNA, which fluctuated within the range of 4.34–5.38 units over the entire period. Furthermore, the carboxypeptidase activity in sprouted seeds was extremely low, especially in substrates with hydrophobic C-terminal amino acid residues like -Phe, -Tyr and -Leu (Table 4). Nonetheless, other carboxypeptidases exhibiting activities against substrates of NCBZ-Phe-Ala and NCBZ-Ala-Gly were assayed by Wilson *et al.* (8), indicating substantial amount of carboxypeptidases in soybean seedlings.

Changes of free amino acids

The changes of free amino acids are shown in Table 5. High content of total free amino acids was obtained in S₀ specimen at around 470 $\mu\text{g}/\text{mL}$, which was in accordance with the potent exopeptidase activities right after soaking (Table 4). As expected, total level of FAAs dramatically rose as the germination continued, which was finally increased up to well over 1100 $\mu\text{g}/\text{mL}$ in S₆ specimen. Most of the increased amino acids are attributed

Table 5. Changes of free amino acids after fermentation of probiotics

Amino acid	$\gamma(\text{RSM})$	$\gamma(\text{soymilk})$			$\gamma(\text{RSMY})$	$\gamma(\text{sogurt})$		
	$\mu\text{g}/\text{mL}$	S ₀	S ₃	S ₆	$\mu\text{g}/\text{mL}$	S ₀	S ₃	S ₆
Asp	2.90	9.80	29.20	48.80	6.10	1.60	7.00	14.50
Glu	13.10	41.60	79.20	78.20	22.30	20.20	36.20	40.50
Ser	5.20	10.50	25.90	172.00	9.20	8.90	13.60	25.00
Gly	2.20	8.60	8.50	25.70	5.50	8.90	6.30	9.50
His	9.60	30.60	38.70	89.50	24.90	24.40	25.10	35.70
Arg	21.30	169.10	185.80	166.00	18.90	14.80	91.50	95.80
Thr	5.30	20.50	32.80	159.20	13.10	15.60	20.10	36.80
Ala	7.50	29.80	378.50	50.50	2.00	22.10	25.60	35.10
Pro	17.90	23.20	29.00	38.90	99.50	51.70	55.50	60.50
Tyr	29.60	22.10	24.90	37.50	10.90	12.20	12.40	16.70
Val	5.20	12.50	20.50	27.20	8.00	5.40	4.80	9.50
Met	0.60	42.30	41.70	76.30	3.40	21.90	18.90	15.90
Cys	0.50	0.20	2.10	2.60	0.50	0.80	0.90	1.00
Ile	6.60	0.50	10.10	26.70	3.50	1.50	1.30	3.30
Leu	9.90	9.10	15.70	34.50	5.40	3.00	3.40	7.60
Trp	0.60	28.80	30.40	66.00	2.60	13.20	14.40	20.70
Phe	7.50	5.60	13.10	39.00	1.30	0.60	0.70	3.60
Lys	8.90	6.10	20.80	28.10	11.60	12.60	10.30	15.00
Total	154.40	470.90	987.00	1166.60	248.60	239.30	348.10	446.60

RSM=reconstituted skimmed milk; S₀, S₃ and S₆ indicate soymilk from soybeans with different hypocotyl lengths; RSMY=reconstituted skimmed milk yogurt

to the release of free amino acids from the carboxylic or amino terminal of polypeptides by active exopeptidases during seedling growth. Arginine was the most abundant amino acid in all soymilk specimens. There might be an aminopeptidase that catalyzes the cleavage of amino-terminal arginine, which is exposed after the cleavage of N-terminal Arg-X bond of soy protein polypeptides by endogenous endopeptidase (shown in Table 3). Moreover, since the amino acid residue Arg is released from the N-terminal domain, the bitterness of polypeptides could be largely reduced (35,36), which is beneficial for the flavour of sogurts.

As shown in Table 5, total content of free amino acids in soymilk samples dramatically dropped after the fermentation, from 1166.60 to 446.60 in S_6 specimen, and 987.00 to 348.10 in S_3 sample. However, the content in S_3 and S_6 sogurts was still higher than that in RSMY (at around 250), which means that far more free amino acids were obtained in sogurts prepared from sprouted seeds. Specifically, some of the free amino acids like Arg, Glu, Ala, His, Thr and Ser decreased considerably after the inoculation of probiotics. According to the report of Bown and Shelp (37), glutamic acid can be converted into γ -aminobutyric acid (GABA) by probiotics, which has a lowering effect on the blood pressure in hypertensive patients. Other amino acids like Thr and His can be transformed into volatile aroma compounds as well, such as benzaldehyde, dimethyl disulphide and 2-methyl propanol (38), all of which are beneficial for the flavour improvement of sogurts.

Most of the free amino acids in RSM obviously increased after the fermentation of probiotics, especially Pro and Met, both of which increased approx. 4.5 times of the initial values. What is demonstrated here is that the combined probiotics can produce active proteases for degradation of casein in reconstituted skimmed milk during proliferation. Further research is needed for the selection of proper probiotics, which are capable of catalyzing the efficient proteolysis of soy protein in soymilk medium. Thus, the sogurt production could be further improved.

Conclusion

By comparison with sogurt from ungerminated soybeans, sogurts obtained from germinated seeds showed less viscoelastic and shear-thinning rheological properties. The interspace diameter increased from 1–2 μm in S_0 sogurt to 5–6 μm in S_6 sogurt; therefore, a more open three-dimensional soy protein network was formed. During the growth of soybean seedling, subunits of soybean storage proteins were partly hydrolyzed by endopeptidases, and then the molecular structures of soy protein were more open, the cross-linking of protein micelles in sogurt therefore decreased. Consequently, the textural, rheological and microstructural properties of sogurts were remarkably improved. Furthermore, since a great amount of free amino acids was released by carboxypeptidases or aminopeptidases, more volatile aroma and health-beneficial compounds could be converted during combined probiotic fermentation. It can be concluded that, after proper proteolysis during germination, the rheo-

logical properties, microstructure as well as the sensory characteristic of sogurts can be dramatically improved.

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