Influence of Phytase Treatment on the Gelation Property of Soymilk

Kazunobu TSUMURA, Tsutomu SAITO and Wataru KUGIMIYA

New Ingredients Research Institute, Tsukuba R&D Center, Fuji Oil Co., Ltd., 4-3 Kinunodai, Yawara, Tsukuba-gun, Ibaraki 300-2497, Japan

Received April 26, 2004; Accepted July 30, 2004

The contribution of phytate to the gelation properties of soymilk was studied by a phytase treatment to reduce the phytate in soymilk. The breaking stress of the gel prepared from the phytase-treated soymilk was higher than that from the untreated soymilk when glucono- δ -lactone (GDL) was used as a coagulant. As the phytate content of the soy protein isolate was decreased, so the breaking stress of the GDL-gel was increased as in the case of soymilk. This result indicates that the phytate in soy protein affects its coagulative reaction and hardness of the GDL-gel. An increase in viscosity and change in the zeta potential of soymilk resulted from the decomposition of phytate.

Keywords: soymilk, phytase, phytate, glucono- δ -lactone, gel

Introduction

Soymilk and tofu are popular soybean foods in many Asian and some Western countries. The quality of tofu, a product manufactured by curdling soymilk with glucono- δ -lactone (GDL) or calcium chloride as a coagulant, depends largely on its physical properties. The protein in soymilk plays an important role in the physical properties of tofu. Soybeans contain about 35% protein which is comprised of glycinin and β -conglycinin. It is known that the hardness of tofu curd is influenced by the ratio of glycinin to β -conglycinin (Saio *et al.*, 1969).

Phytate, the hexaphosphate form of myo-inositol, is commonly contained in soymilk at the level of about 2 g per 100 g of protein and in soy protein isolate at 1–2 g per 100 g of protein (Anno *et al.*, 1985; Anderson *et al.*, 1995; Brooks & Morr 1982). Phytate may form a soy protein-phytate complex with soy protein, and thus inhibit the protein digestibility (Ritter *et al.*, 1987) and may interfere with the essential mineral bioavailability (Cheryan 1980; Prattley *et al.*, 1982). In addition, phytate may affect the solubility and related functions of soy protein in a commercial food application (Okubo *et al.*, 1976; Chen & Morr 1985; Lapveteläinen *et al.*, 1991). It would therefore seem worthwhile to obtain reduced-phytate proteins for making high-quality foods.

In the course of our study on reduced-phytate soybean protein (Saito *et al.*, 2001), we observed the formation of a gel when heating soymilk in which the phytate had been hydrolyzed by phytase. Similar gel formation upon treating soybean protein with ion-exchange resin has been reported (Kondo *et al.*, 1988). However, there is little information on the gel-forming properties of reduced-phytate soy protein resulting from a phytase treatment. We report in this paper the influence of a phytase treatment on the gel-forming properties of soymilk.

Materials and Methods

Materials Commercially available soybean (*Glycine max* var. Enrei) was used in this study. Phytase (Phytase Novo-L, 5000 FYT/g) was obtained from Novozymes Japan, Ltd. (Chiba, Japan). All of the chemicals were of the highest purity available and were used without further purification.

Preparation of the soymilk Soybeans (100 g) were soaked in deionized water for 16 h at 4°C. The soaked beans were drained and ground into a homogenate with 600 ml of deionized water by an SM-58 blender (Sanyo Electric Co., Tokyo, Japan), this homogenate then being squeezed through a double layer of nylon gauze. The resulting soymilk was centrifuged at 800 $\times$ g for 10 min to remove the precipitate, before being heated at 95°C for 5 min and then quickly cooled to 40°C. The soymilk contained 11.5% solids with 5.4% protein.

Phytase treatment of the soymilk The soymilk was adjusted to pH 6.4 with 2 N HCl. Phytase was added to this soymilk (250 or 1000 FYT/100 g of protein in the soymilk), and the resulting mixture was incubated for 30 min at 40°C. One enzyme unit (FYT) is defined as the amount of enzyme that released 1 μ mol of inorganic orthophosphate per min under the following conditions: pH 5.5, 37°C, and 5 mM sodium phytate according to the manufacturer's instructions.

Phytase treatment of soy protein isolate Soy protein isolate (SPI) was prepared as described in the previous paper (Tsumura *et al.*, 1999). The protein and ash contents (dry base) in SPI were 92.0% and 3.5%, respectively. SPI was dissolved with distilled water (5.4 g protein in 100 ml), before being heated at 95°C for 5 min and then quickly cooled to 40°C. SPI solution was adjusted to pH 6.4 with 2 N HCl. Phytase was added to the solution (250–2000 FYT/100 g of protein in the SPI solution), and the resulting mixture was incubated for 30 min at 40°C.

Preparation of GDL-gel and measurement of the breaking stress and pH value of the gel The phytase-treated

soymilk or SPI solution was immediately mixed with a GDL solution, freshly prepared in ice-cold water. The final concentration of protein was adjusted to 5.0% w/v, and the final concentration of GDL was adjusted in the range of 0.05–0.15% w/v. Then, 750 μ l aliquots of the resulting protein solutions were placed in the wells of a 24-well tissue culture plate (16 mm I.D. $\times$ 18 mm height, Becton Dickinson Co., USA). The wells were tightly sealed with heat-resistant film, and the plate was placed in a water bath at 90°C for 30 min, cooled, and then kept at room temperature for 1 h before measurement. Compression testing of the gel samples was carried out with a Texo Graph compression tester (Japan Food R&D Institute, Kyoto, Japan) with a cylindrical plunger 5.6 mm in diameter. The compression rate was 0.1 mm/s, and the peak force was recorded as breaking stress of the gel. Data reported represent mean $\pm$ standard deviation values calculated from three gel parts obtained from two separate gel preparations. The pH value of a gel sample was measured by preparing a homogenate from 1 ml of deionized water poured into each well and suspended with a Polytron PT 10/35 homogenizer (Kinematica, Switzerland). The pH value of the resulting homogenate was measured at room temperature by a pH meter (Horiba Co., Kyoto).

Determination of phytate Phytate was determined according to the method of Tezuka *et al.* (2000). Briefly, two milliliters of soymilk or soy protein solution was mixed with 2.0 ml of 6% trichloroacetic acid. The mixture was incubated for 20 min at 20°C and then centrifuged at $1,800 \times g$ for 15 min. Two milliliters of the resulting supernatant was mixed with 6.0 ml of an iron chloride solution (2.0 g of $\text{FeCl}_3/\text{H}_2\text{O}$ + 16.3 ml of concentrated HCl in 1 l of H_2O). The mixture was incubated for 30 min at 97°C, cooled to room temperature, and then centrifuged at $1800 \times g$ for 15 min. The precipitate was dispersed in 2.0 ml of a washing solution (0.6% HCl + 2.5% Na_2SO_4) and then centrifuged at $3,000 \times g$ for 15 min. The resulting precipitate was further dispersed in 2.0 ml of 0.3 N NaOH and centrifuged at $1800 \times g$ for 15 min, then was dissolved in 3.0 ml of 0.3 N HCl, and the volume was adjusted to 10.0 ml with deionized water. The iron content was colorimetrically measured at 510 nm based upon o-phenanthroline. It was assigned one phytate molecule linked with four iron molecules, and the weight of phytate could be

numerically calculated as 2.98 times the weight of iron.

Analysis The protein content was determined by the Kjeldahl method, employing a nitrogen-to-protein conversion factor of 6.25. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (1980), using 10–20% gradient acrylamide gel (Daiichi Pure Chemicals Co., Tokyo). The gel for electrophoresis was stained by Coomassie brilliant blue R-250. The apparent viscosity of the soymilk was measured at 40°C by a CJV-5000 vibroviscometer (A&D Co. Ltd., Tokyo).

Estimation of the zeta potential of the soymilk A soymilk sample was diluted with a 10 mM phosphate buffer (pH 6.0–7.0) and measured at 20°C by an ELS-800 light-scattering system (Otsuka Electronics Co., Osaka). The computer program supplied with this system was used to calculate the zeta potential of the soymilk.

Scanning electron microscopy A gel sample was cut into 2 mm cubes that were prefixed overnight in a 0.1 M phosphate buffer (pH 6.5) containing 2.5% glutaraldehyde. The cubes were then postfixed for 2 h with 1% OsO_4 in a 0.1 M phosphate buffer (pH 6.5), dehydrated with 50% to 100% ethanol and then with isoamyl acetate, and dried with an HPC-2 critical point drier (Hitachi Co., Tokyo). Each sample was coated with OsO_4 in an E-102 osmium plasma coater (Hitachi Co.). Observations were made by an S-3500N scanning electron microscope (Hitachi Co.) at an accelerating voltage of 5 kV.

Results and Discussion

Gel formation of soymilk induced by the phytase treatment When heated soymilk was incubated with phytase at pH 6.4 and then heated at 90°C, gel formation occurred. A firm, self-supporting gel could be obtained from the soymilk without using a coagulant (Fig. 1). Based on this finding, we investigated the effect of the phytase treatment on the gel-forming ability of soymilk in the presence of GDL. Figure 2 shows the effect of various amounts of phytase on the breaking stress of the GDL-gel and the phytate content in the resulting soymilk. GDL was added to soymilk at a final concentration of 0.1%, and the pH value of the gel was about 6.2. As the amount of phytate decreased from 2.05 g to 0.01 g per 100 g of protein in the soymilk, the breaking stress of the GDL-gel increased

Fig. 1. Soymilk samples heated at 90°C for 30 min. A, phytase-treated soymilk (1000 FYT/100 g of protein); B, control soymilk.

Fig. 2. Relationship between the breaking stress of the GDL-gel and phytate content. ○, breaking stress; ●, phytate content.

from zero gf/cm² to 180 gf/cm². The relationships between the breaking stress and pH value for the GDL-gel are shown in Figure 3. As the amount of phytase used was increased, an increase in breaking stress was observed at identical pH value. These results indicate that phytate in the soymilk played a significant role in the gel-forming ability of soymilk in the presence of GDL. Saio (1979) previously reported that the hardness of tofu gel decreased with increasing amount of added phytate in a calcium-coagulated gel system. One explanation is that phytate reacted with both calcium and protein and produced a colloidal precipitate that could hold more moisture in the gel. The present results are consistent with the previous information, although there is a difference between calcium and GDL as the coagulant used.

Effect of the phytase treatment on the characteristics of soymilk Several experiments were done to obtain

Fig. 3. Breaking stress and pH value of the GDL-gel. ○, control soymilk; ▲, 250 FYT/100 g of protein; ●, 1000 FYT/100 g of protein.

further information on the effect of the phytase treatment on the characteristics of soymilk. An increase in the apparent viscosity of soymilk could be clearly observed during the phytase reaction (Fig. 4). This result indicates that the increase in apparent viscosity may depend upon the decomposition of phytate. In general, the viscosity of a protein solution is attributable to increased interaction between the protein molecules (Damodaran, 1997). Therefore, such activity of protein molecules as hydrophobic interaction, hydrogen bond interaction and charge-charge interaction may occur due to the decomposition of phytate.

Fig. 4. Viscosity of soymilk during the phytase treatment. ○, control soymilk; ▲, 250 FYT/100 g of protein; ●, 1000 FYT/100 g of protein.

Phytate may influence the net charge of the protein molecules. The zeta potential as a function of the pH value of soymilk is shown in Fig. 5. A slightly larger negative value is apparent for the zeta potential of the phytase-treated soymilk than the control soymilk. It has been reported that the solubility (Hayakawa & Nakai, 1985) and aggregate size of soy protein (Chan *et al.*, 1982) were correlated well

Fig. 5. Zeta potential of the soymilk. ○, control soymilk; ●, phytase-treated soymilk (1000 FYT/100 g of protein).

with the zeta potential. It seems likely that the net charge of the phytase-treated soymilk was slightly changed from its original state due to decomposition of phytate by phytase.

There have been several reports on the enzyme-induced coagulation of soy protein (Fuke *et al.*, 1985; Inouye *et al.*, 2002). Murata *et al.* (1987) made a series of studies on the feasibility of using proteolytic enzymes to make tofu. As shown in Fig. 6, no proteolysis during the phytase treatment was apparent from the results of the SDS-PAGE analysis. Hence, we exclude any effect from proteolysis on the gelation of soymilk.

Fig. 6. SDS-PAGE profiles of the soymilk. Lane 1, molecular mass markers; lane 2, control soymilk; lane 3, 250 FYT/100 g of protein; lane 4, 1000 FYT/100 g of protein.

Effect of the phytase treatment on the soy protein isolate Soymilk contains not only proteins but other components which affect gel formation such as fats (Yamano, 1990), saccharides (Saio, 1985) and phytate. We then examined the effect of the phytase treatment on the gel forming ability of the soy protein isolate (SPI). Figure 7 shows the effect of various amounts of phytase on the breaking stress of the GDL-gel and on the phytate content in the resulting SPI. GDL was added to SPI solution at a

Fig. 7. Relationship between the breaking stress of the GDL-gel and the phytate content of the soy protein isolate. ○, breaking stress; ●, phytate content.

final concentration of 0.1%, and the pH value of the gel was about 6.2. As the phytate content decreased from 2.10 g to 0.10 g per 100 g of protein in SPI, an increase in the breaking stress of the gels was observed as in the case of soy milk. Phytase was thus able to effectively degrade phytate in SPI and influence the gel-forming ability.

Gel structure Microstructural observation can provide valuable information for understanding the gel formation induced by a phytase treatment. We therefore observed the gel structure by scanning electron microscopy. The gel prepared with the control soymilk containing 0.35% GDL exhibited a fine and uniform network structure (Fig. 8B), consistent with the observation in the previous report (deMan *et al.*, 1986). The network structure of the gel prepared with the phytase-treated soymilk containing 0.1% GDL was almost the same as that of the control soymilk gel (Fig. 8A), although the phytase-treated soymilk needed less GDL to form a gel. These results indicate that the phytase treatment can help a protein to coagulate and form a firm network structure with less coagulant than that required without the phytase treatment (*i.e.* at a higher pH value).

Fig. 8. GDL-gel structure observed by scanning electron microscope. A, phytase-treated soymilk (1000 FYT/100 g of protein); B, control soymilk.

Gelation mechanism Heat treatment of soymilk before adding phytase was essential in the present study for denaturing the proteins so that they could coagulate into a gel after heating. When raw soymilk was incubated with phytase, protein (mainly the glycinin fraction) was precipitated as previously reported (Saito *et al.*, 2001) and no gelation occurred after further heating. Kohyama *et al.* (1995) proposed that the gelation of tofu was a two-step process: protein denaturation by heating and then hydrophobic coagulation promoted by protons from GDL or calcium ions. Since heat-denatured soy protein is hydrophobic and negatively charged, the protons induced by GDL or calcium ions neutralize the net charge of the negatively charged protein molecules. As a result, hydrophobic interaction of the neutralized protein molecules may become more predominant and lead to the gel formation.

The phytase treatment enabled the heated soy protein to form a gel with less coagulant than without treatment (*i.e.* at a higher pH value). This suggests that the soy protein-phytate complex may have changed such surface properties as the net charge and hydration of soy protein and have affected its gel-forming ability. Therefore, it seems likely that the gel-forming ability of soy protein recovered to its original state due to the decomposition of phytate so that the soy protein could coagulate and form a gel at a higher pH value.

Phytase leads to the hydrolysis of phytate, and consequently to a breakdown of the phytate-calcium complexes; hence, free calcium ions may become available for coagulation. It is well known that calcium ions contribute to the formation of bridges between protein molecules, thus generating a stronger gel network (Boye *et al.*, 1997). The effect on the gel-forming ability of calcium ions released from the phytate-calcium complex under the influence of phytase should be examined in further studies.

In conclusion, a phytase treatment can be used to change the gel-forming ability of soy protein that can be applied to prepare new food products such as phytate-reduced tofu with high nutritional quality.

Acknowledgments The authors thank Dr. X. Q. Liu for valuable discussions and Ms. H. Ashida for technical assistance.

References

- Anderson, R.L. and Wolf, W.J. (1995). Compositional changes in trypsin inhibitors, phytic acid, saponins and isoflavones related to soybean processing. *J. Nutr.*, **125**, 581s–588s.
- Anno, T., Nakanishi, K., Matsuno, R. and Kamikubo, T. (1985). Enzymatic elimination of phytate in soybean milk. *Nippon Shokuhin Kogyo Gakkaishi*, **32**, 174–180.
- Boye, J.I., Ma, C.-Y. and Harwalk, V.R. (1997). Thermal denaturation and coagulation of proteins. In “Food Proteins and Their Applications,” ed by S. Damodaran and A. Paraf. Marcel Dekker, New York, pp. 25–56.
- Brooks, J.R. and Morr, C.V. (1982). Phytate removal from soy protein isolates using ion exchange processing treatments. *J. Food Sci.*, **47**, 1280–1282.
- Chan, M.Y.Y., Bell, D.J. and Dunnill, P. (1982). The relationship between the zeta potential and the size of soyaprotein acid precipitate particles. *Biotechnol. Bioeng.*, **24**, 1897–1890.
- Chen, B.H. -Y. and Morr, C.V. (1985). Solubility and foaming properties of phytate-reduced soy protein isolate. *J. Food Sci.*, **50**, 1139–1142.
- Cheryan, M. (1980). Phytic acid interactions in food systems. *CRD Crit. Rev. Food Sci. Nutr.*, **13**, 197–335.
- Damodaran, S. (1997). Food proteins: an overview. In “Food Proteins and Their Applications,” ed by S. Damodaran and A. Paraf. Marcel Dekker, New York, pp. 1–24.
- deMan, J.M., deMan, L. and Gupta, S. (1986). Texture and microstructure of soybean curd (tofu) as affected by different coagulants. *Food Microstruct.*, **5**, 83–89.
- Fuke, Y., Sekiguchi, M. and Matsuoka, H. (1985). Nature of stem bromelain treatments on the aggregation and gelation of soybean proteins. *J. Food Sci.*, **50**, 1283–1288.
- Hayakawa, S. and Nakai, S. (1985). Relationships of hydrophobicity and net charge to the solubility of milk and soy proteins. *J. Food Sci.*, **50**, 486–491.
- Inouye, K., Nagai, K. and Takita, T. (2002). Coagulation of soy protein isolates induced by subtilisin carlsberg. *J. Agric. Food Chem.*, **50**, 1237–1242.
- Kohyama, K., Sano, Y. and Doi, E. (1995). Rheological characteristics and gelation mechanism of tofu (soybean curd). *J. Agric. Food Chem.*, **43**, 1808–1812.
- Kondo, T., Kusakabe, M., and Otomo, N. (1988). Preparation of shape-retaining food composed soybean protein. JP 63-063348 A
- Laemmli, U.K. (1980). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685.
- Lapveteläinen, A., Kerrola, K. and Linko, R. (1991). Effect of phytic acid hydrolysis on the functionality of soy protein isolate. *Lebensm. -Wiss. u. Technol.*, **24**, 71–75.
- Murata, K., Kusakabe, I., Kobayashi, H., Kiuchi, H., and Murakami, K. (1987). Selection of commercial enzymes suitable for making soymilk-curd. *Agric. Biol. Chem.*, **51**, 2929–2933.
- Okubo, K., Myers, D.V. and Iacobucci, G.A. (1976). Binding of phytic acid to glycinin. *Cereal Chem.*, **53**, 513–524.
- Prattley, C.A., Stanley, D.W., Smith, T.K. and Van de Voort, F.R. (1982). Protein-phytate interactions in soybeans. III. The effect of protein-phytate complexes on zinc bioavailability. *J. Food Biochem.*, **6**, 273–282.
- Ritter, M.A., Morr, C.V. and Thomas, R.L. (1987). In vitro digestibility of phytate-reduced and phenolics-reduced soy protein isolates. *J. Food Sci.*, **52**, 325–327, 341.
- Saio, K., Kamiya, M. and Watanabe, T. (1969). Food processing characteristics of soybean 11s and 7s proteins. Part I. Effect of difference of protein components among soybean varieties on formation of tofu-gel. *Agric. Biol. Chem.*, **33**, 1301–1308.
- Saio, K. (1979). Tofu – relationships between texture and fine structure. *Cereal Foods World*, **24**, 342–345, 350–354.
- Saio, K. (1985). Tofu processing characteristics of Japanese domestic soybeans. *Rept. Natl. Food Res. Inst.*, **47**, 128–149 (in Japanese).
- Saito, T., Kohno, M., Tsumura, K., Kugimiya, W. and Kito, M. (2001). Novel method using phytase for separating soybean β -conglycinin and glycinin. *Biosci. Biotechnol. Biochem.*, **65**, 884–887.
- Tezuka, M., Taira, H., Igarashi, Y., Yagasaki, K. and Ono, T. (2000). Properties of tofus and soy milks prepared from soybeans having different subunits of glycinin. *J. Agric. Food Chem.*, **48**, 1111–1117.
- Tsumura, K., Kugimiya, W., Bando, N., Hiemori, M. and Ogawa, T. (1999). Preparation of hypoallergenic soybean protein with processing functionality by selective enzymatic hydrolysis. *Food Sci. Technol. Res.*, **5**, 171–175.
- Yamano, Y. (1990). In “Shokuhin no Bussei” ed. by S. Matsumoto and Y. Yamano. Shokuhin Shizai Kenkyukai, Tokyo, pp. 203–222 (in Japanese).