View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by IntechOpen

We are IntechOpen, the world's leading publisher of Open Access books Built by scientists, for scientists

4,800
Open access books available

122,000
International authors and editors

135M
Downloads

Our authors are among the

154
Countries delivered to

TOP 1%
most cited scientists

12.2%
Contributors from top 500 universities

CLARIVATE ANALYTICS BOOK CITATION INDEX INDEXED

WEB OF SCIENCE™

Selection of our books indexed in the Book Citation Index in Web of Science™ Core Collection (BKCI)

Interested in publishing with us?
Contact book.department@intechopen.com

Numbers displayed above are based on latest data collected.
For more information visit www.intechopen.com

Chapter 3

New Cheese-Like Food Production from Soy Milk — Utility of Soy Milk Curdling Yeast

Makoto Kanauchi, Sakiko Hatanaka and Makoto Shimoyamada

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/60848

Abstract

Soybeans are a traditional food in eastern Asia, particularly in Japan and China. They were eaten in 100 BC in China. The beans can be processed into *Tofu*, soy milk, fermented seasonings, soy sauce or *Miso* paste, and *Natto* and green beans. Soybeans have rich nutrition, protein lipid, and other functional substances such as isoflavones. However, soybeans are difficult to process for use as food because of tissue and cell wall hardness. Therefore, soybeans are conducted to do some treatments, *e.g.*, boiling, steaming, roasting, crushing/grinding, and some enzyme treating, to eat soy protein easily. Soy storage proteins mainly comprise two proteins as 7S globulin composed with β-conglycinin and 11S globulin containing glycinin composed of 5 subunits. β-Conglycinin, included in 7S globulin, is composed of three subunits.

To modify the physical properties of soy protein, a new type of enzyme for curdling soybean milk enzyme was purified as an extract from yeast. Yeast producing curdling soybean milk enzyme, the SCY003 strain, was isolated from 1345 yeast strains. According to the morphology, physiology, and molecular and characteristics, SCY003 was identified as *Saccharomyces bayanus*. The soy milk curdling enzyme having proteolytic activity was approximately 45 kDa and monomer protein. The optimum pH for the protease activity was pH 7.5; the optimum temperature was 50°C.

The enzyme cleaved the β-conglycinin as α–, α′-, and part of glycinin as A_3 A_4, A_{1b}, and A_2 in soy protein by endoproteolysis. Soybean protein became loosely curdled with the addition of other proteases from microorganisms or plants. Soybean milk curdled after cleaving endoproteolysis enzyme in SCY003 strain.

INTECH
open science | open minds

© 2015 The Author(s). Licensee InTech. This chapter is distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/3.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

The breaking point of curd curdled by enzyme was 58.4% strain. Their breaking stress was 10,900 ($N \cdot m^{-2}$). The brittleness point is 81.2% and 10,200 ($N \cdot m^{-2}$), and the brittleness of this curd produced using the enzyme was 727 ($N \cdot m^{-2}$). Brittleness of the curd produced by the enzyme was less. Their breaking point was greater than that of the curd produced using the glucono-δ-lactone (GDL). Furthermore, the curd had sticky and chewy texture. The curd made by enzyme has resilience more than normal *Tofu*. It is considered that the curd produced by enzyme was not like *Tofu* rheologically.

Keywords: Soybean milk, curdling, *Saccharomyces bayanus*, protease

1. Introduction

1.1. Utility of soybeans

Soybeans have been used as traditional foods from ancient times. They are rich in nutrients such as rich proteins, lipids, and others. Furthermore, soybeans can be eaten after processing in various ways.

Eastern Asian people and particularly Japanese people have eaten soybeans after various stages of processing. Soybean seedlings are eaten in many dishes as bean sprouts. Furthermore, soybeans are eaten as green beans in the pod after boiling, as *Edamame*. Soybean flour made from roasted soybeans is eaten as *Kinako* powder. After boiling soybeans, the beans can be fermented can using molds to produce *Chi* or *Tempe*. Furthermore, soy sauce is made from a molded mixture with boiled beans and roasted wheat, and salted water. Soy paste is made from fermented boiled soybeans and *Koji* with salt. The resultant umami taste is an extract from the bean, facilitated by an enzyme reaction because it is thought that umami components are stored as proteins in hard tissue.

After hard tissues in boiled soybeans are crushed and ground physically, the soluble fraction is extracted as soy milk. Soy milk is processed as *Yuba* from a soybean sheet, and *Tofu* is produced. Regarding the insoluble fraction, spent soy is also eaten as *Okara*. Finally, compressed soybeans produce oil that is widely used as cooking oil. The residue of oil pressing can then be used for soy sauce production or soy protein for food manufacture. Comparing soy products to milk casein, which is eaten as cheese, soy products are not used as widely as food. One reason is that soybean curd such as *Tofu* lacks taste and has less elastic properties and texture compared to cheese. Therefore, to make rich nutrition and produce a food that has good taste and texture, soybean protein is modified by enzymes and is extracted by microorganisms [1].

1.2. History of soybeans

Theories about the origin of soybean use and cultivation remain controversial in their details. By some accounts, soybeans used as food originated in the area of Manchuria in China and

http://dx.doi.org/10.5772/60848

Siberia in Russia. Alternatively, their use as food originated in southern China [1]. Yet another possible history holds that soybeans were bred from wild soybeans as *Glycine soja* in China. In fact, the bean was present in ancient Japan: beans were found in the bottom of an earthen vessel produced in the middle Jomon period (3000–2000 B.C.) in Japan. However, soybeans have not been recorded as eaten in that period.

In China, the first literal record can be found in a Chinese dictionary published in 100 B.C. The dictionary inserted "*Chi*," representing fermenting soybeans with salt. Furthermore, archaic *Miso* paste and soy sauce fermented by soybeans were described in the Chinese text "Qi-min-yao-shu," published in the sixth century.

In Japan also, "*Chi*" fermented soybeans with salt was recorded in the Taiho Code in A.D. 701. It is considered that soybean fermentation practices diffused from China. Tofu was recorded in the tenth century in China. It was recorded in the same period in Japan. In Japan, it was initially eaten as a vegetarian dish for Buddhists because, during the Kamakura period, people gradually became more and more vegetarian in their eating practices. In the Edo Era, a *Tofu* recipe book was published, with 283 recipes explained in it.

1.3. Consumption worldwide and in Japan

Soybeans were produced only in eastern Asia for a long time. In contrast, other cereals such as rice, barley, wheat, and corn have diffused throughout the world. Moreover, it is considered that some other endemic bean or pea or pulse had become cultivated in each area already [1].

For instance, in central Asia, broad beans were cultivated, as were chick peas in India, shell peas in western Asia, and kidney beans and ground peas in North America. Nevertheless, soybeans have been cultivated in the United States as oil seed crops since the 1920s. Furthermore, the crop has begun to be cultivated in Canada and South America. In 2012, approximately 82 million tons of soybeans were harvested, with the United States accounting for 34% of the world production. Brazil harvested approximately 66 million tons that year, accounting for 27% of world production. Argentina harvested approximately 40 million tons, or 17% of world production. Therefore, most soybeans (over 80%) consumed worldwide are now produced and harvested in North and South America [2] (Table 1).

In Japan, soybean production was sufficient to provide for domestic consumption until the Taisho Era [1]. Soybean consumption in Japan has been high, but it decreased after the Taisho Era. In 2013, 30 million tons were consumed, but only 240 thousand tons were harvested domestically. That figure is less than 0.1% of the world production amount. Soybeans used domestically account for 104 thousand tons for feed, 6 thousand tons for seed, and 1.9 million tons for oilseeds, all together accounting for 70% of the 30 million tons consumed. Furthermore, those figures indicate that only 30% of soybeans are used as food. The self-sufficiency ratio of soybeans was 97% in 1947. It decreased gradually to 28% in 1959, 11% in 1965, and 7% in 2013 [3]. The ratios of soybeans used for food are 49% used for *Tofu*, 13% used for *Miso* paste and *Natto*, 4% used for soy milk, and 3.5% used for soy sauce production. As mentioned earlier, some soy sauce production companies have used soy meal after oil pressing to produce soy sauce (Table 2).

	Production 2012 (10 thousand tons)	Share of world production (%)
United States	8205	33.9
Brazil	6585	27.2
Argentina	4010	16.6
India	1467	6.1
China	1305	5.4
Canada	509	2.1
Paraguay	434	1.8
Uruguay	300	1.2
Ukraine	241	1.0
Bolivia	206	0.9
Russia	181	0.7
Indonesia	84	0.3
South Africa	65	0.3
Nigeria	58	0.2
North Korea	35	0.1
Japan	24	0.1
Myanmar	21	0.1
Others	454	1.9
World total	24,184	100

Source: http://www.maff.go.jp/j/seisan/ryutu/daizu/d_data/pdf/014.pdf

Table 1. Soybeans production

	2003	2013
Miso paste	138	123
Soy sauce	38	33
Tofu and fried tofu	494	454
Natto	137	125
Frozen *tofu*	30	20
Soy milk	19	40
Delicatessen of soybean	33	30
Kinako soy powder	17	18
Other	128	93
Total	1034	936

(Unit: thousand tons)

Ref. http://www.maff.go.jp/j/seisan/ryutu/daizu/d_data/

Table 2. Changes in amount of soybeans for applications

http://dx.doi.org/10.5772/60848

2. Nutrition

2.1. Soybean protein

Soybeans contain 35% protein as storage protein, which is used for nutrition during germination. That storage protein is stored in granules, called protein bodies, of about 5–8 μm diameter. Soluble soy protein is extracted from insoluble protein bodies that are burst during soy milk and *Tofu* production. All soy protein is stored in the protein body [1]. Other proteins exist as nonstorage proteins, containing important physiological proteins such as trypsin inhibitors. When an animal ingests a trypsin inhibitor, a digestive enzyme, trypsin activity is inhibited by combination of the trypsin inhibitor specifically with trypsin. Consequently, the pancreas works excessively to secrete and supplement trypsin activity [1]. However, after heating, the inhibitor loses its inhibitory activity and does not bind with trypsin.

Protein digestibility-corrected amino acid score (PDCAAS) values are used to evaluate the protein quality based on the amino acid requirements of humans and their ability to digest it. It was long thought that the amount of amino acid requirements of humans dictate a low score for soybeans because methionine and cysteine residues, sulfur amino acids, in soybean storage proteins have a low composition. The score was only 86 points based on the amino acid requirements of a developing rat. However, in 1985, the score was modified to 100 points, the same as milk and eggs, based on the amino acid requirements of humans. Soy storage proteins are rich in nutrition for human needs [4].

Throughout the world, the recently improving healthy image of soy protein is interesting. In particular, the health benefits of soy foods attract attention in the United States. Health claims are authorized by the Food and Drug Administration (FDA) in the United States: foods containing 6.25 g of soy protein or more can be said by manufacturers to reduce the risk of heart disease if a consumer ingests 25 g/day of soy protein [5]. In Japan, some soybean containing foods are manufactured as *Tokuho*: government-approved foods for specified health benefits, as for hypocholesterolemic activity in this case.

The taxonomy of soybean storage protein has been conducted according to the sedimentation coefficient by an ultracentrifugal fraction as 2S globulin, 7S globulin, 11S globulin, and 15S globulin. Yamauchi [1] reported details of soybean proteins: 2S globulin contained α-conglycinin, 7S globulin composed with β-conglycinin and γ-conglycinin, and 11S globulin-containing glycinin. In addition, the 11S globulin composes hexamer. It is a 350,000 Da protein. Furthermore, their proteins are composed with five subunits as G_1–G_5; their subunit was 10 polypeptides as A_{1a}, A_2, A_{1b}, A_3, A_5A_4, A_4, B_2, B_{1b}, B_4, and B_3. Their polypeptides are combined specifically as $A_{1a}B_{1b}$, $A_{1b}B_2$, A_2B_{1a}, A_3B_4, and $A_5A_4B_3$. In fact, β-conglycinin, called 7S globulin, combines a dimer protein and a monomer protein, which are 150,000–200,000 Da, or an average of 180,000 Da. They are composed of three subunits: an α-subunit of 63,000 Da, an α'-subunit of 67,000 Da, and a β-subunit of 48,000 Da. The protein has a low concentration of sulfur amino acids. In particular, the β-subunit does not contain methionine, cysteine, and tryptophan [6].

2.2. Lipid

Soybeans have 20% lipids. The lipid concentration varies among harvested regions. Soybeans harvested in the United States have more lipids than those in China [1]. A main reason is that soybeans there have long been bred and modified to contain high oil concentrations as oilseed.

	Palmitic acid	Stearic acid	Oleic acid	Linoleic acid	Linolenic acid
Pork fat	26.2	13.5	42.9	9	0.3
Beef fat	38.7	3.8	42.1	2.3	2.4
Milk lipid	31.1	9.2	21.7	1.6	0.4
Soybean oil	10.5	3.2	22.3	54.5	8.3
Sunflower oil	trace	4	27.6	58.3	trace
Cotton oil	27.3	3.1	16.7	50.4	trace
Safflower oil	6	3.4	12.2	77	0.3

Source: Yamauchi and Ookubo (1992).

Table 3. Components of fatty acids in foods (%)

Components of fatty acids in foods shows Table 3. Soy oil comprises a small amount of saturated fatty acids, such as palmitic acid and stearic acid, and large amount of unsaturated fatty acids such as oleic acid, linoleic acid, and linolenic acid. Polyunsaturated fatty acids (PUFAs) containing more than unsaturated bonds are important nutrition as necessary lipids for humans. Soybeans have over 60% PUFA. In particular, one kind of PUFA as linoleic acid contained approximately 54.5%.

Actually, PUFAs in animal lipids have low concentration. Therefore, they are insufficient nutritionally. Saturated fats and unsaturated fats are ideally in the following ratio: saturated–unsaturated (1:2) [1]. Soybean lipids were well known to be much stable against oxidation because they are covered as oil body particle by oleosin and other proteins.

2.3. Isoflavone

Isoflavone is one kind of flavonoid (Fig. 1). Fabaceae sp. contain high concentrations (Fig. 1).

Generally, soybeans have totally 12 isoflavones in 3 aglycones, and they have three types of glycosides as glucoside, acetyl-glycoside and malonyl-glycosides: genistein, daidzein, glystein, genistin, daidzin, glycitin, acetyl-genistin, acetyl-daidzin, acetyl-glycitin, malonyl-genistin, malonyl-daidzin and malonyl-glycitin [7]. After soybean consumption, glycoside isoflavone, which is contained in food as soy milk or *Tofu*, hydrolyzes aglycon and glycoside by bacteria in intestines. Their aglycon are absorbed by the body. Genistein and daidzein have estrogenic effects and hormone-like activity. The isoflavone binding with estrogen receptor

http://dx.doi.org/10.5772/60848

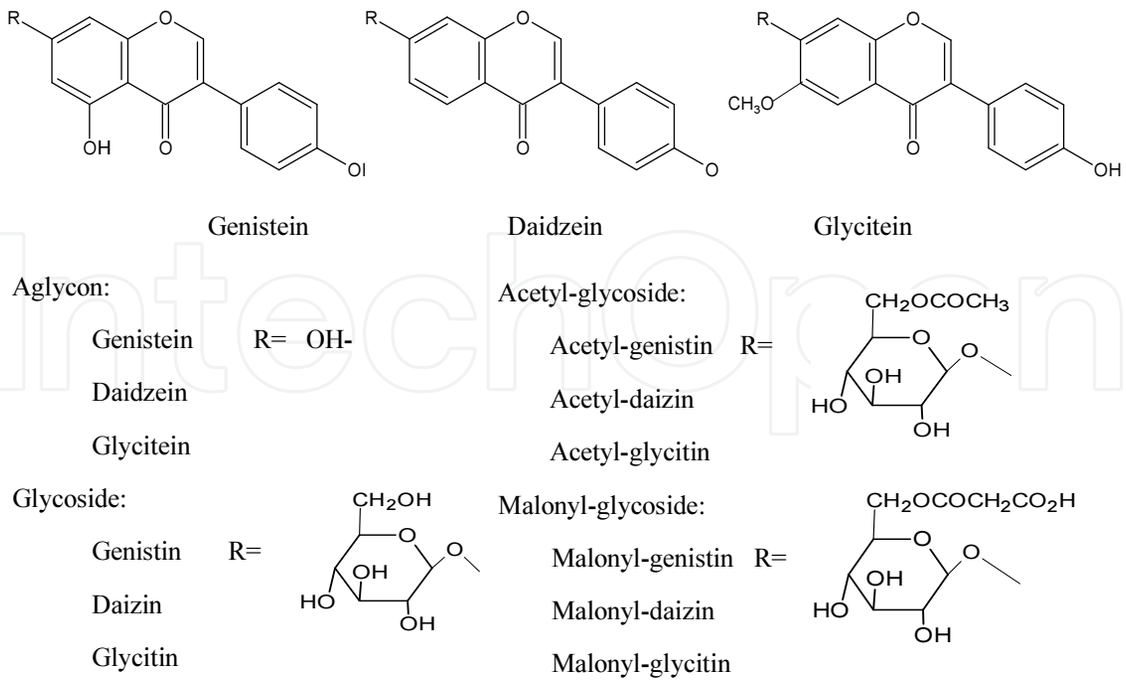


Figure 1. Isoflavone structure.

reacts as an estrogen agonist in the human body. Their substances from plants having estrogenic effects are called "plant estrogen." *Miso* paste has a high level of isoflavone because glucosyl isoflavone is hydrolyzed to their related aglycons.

Many Japanese and throughout eastern Asia intake isoflavone from soybeans. Some researchers have reported negative opinions about plant estrogen [8]. Isoflavones are produced via phenyl–propanoid pathway from phenylalanine in plants. Two intermediate substances, naringenins, are converted to genistein by two specific enzymes in soybeans: isoflavone synthetase and dehydrogenase. Chalcones are converted to daidzein by three specific enzymes in soybean: chalcone reductase, chalcone isomerase, and isoflavone synthetase. Isoflavone and a similar substance, phytoalexin, are used as antibacterial substances against phytopathogenic fungi and bacteria. In addition, they grow well as root nodule bacteria at the root for nitrogen fixation [9, 10].

Other beans and peas, legumes, have isoflavones: chickpeas have biochanin A [11]; alfalfa has formononetin and coumestrol [12]; and ground peas have genistein [13].

Isoflavones in many plants store glucosyl, malonyl-glucosyl, and acetyl-glucosyl conjugate as hydrophilic substances. After invasion of phytopathogenic fungus and bacteria, the glucosyl conjugate isoflavone is transferred to infested wounds, where it hydrolyzes for phytoalexin [14]. Isoflavone has health functions against climacteric disturbances and type 2 diabetes. In Japan, soybean isoflavones are a *Tokuho* (government-approved food for specified health purpose) for the prevention of osteoporosis. The Ministry of Health, Labour and Welfare in Japan alerts consumers to avoid overdosing on isoflavones. The amount of isoflavone intake was 30 mg a day, omitting isoflavone intake from meals and by supplements.

3. Food processing

3.1. Formation of *Tofu* curdling

Soy proteins have properties that produce curd to add specific metal ions. The property is applied for *Tofu* production. Tofu, soybean curd from soybean milk, is consumed throughout Asia. It was eaten in the tenth century in China and Japan. *Tofu* is traditionally consumed after it is produced with a combination of magnesium dichloride ($MgCl_2$) as *Nigari* and calcium dichloride ($CaCl_2$) as *Sumashi-ko*. More recently, glucono-δ-lactone (GDL) has been added to it for commercial production. Tofu resembles cheese or yogurt made from milk curd of cows or other mammals. It is made from soy milk. It curdles by *Nigari* or a coagulant agent [15]. The forming system of *Tofu* curdling is shown in Fig. 2.

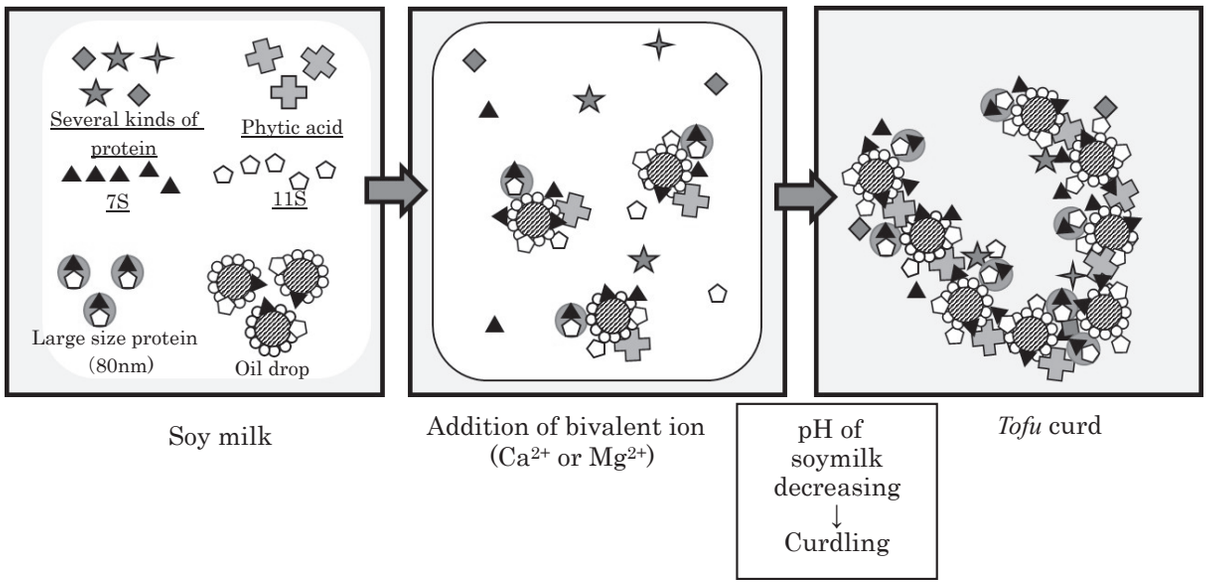


Figure 2. Formation of *Tofu* curdling [15].

Soy milk is an emulsion composing lipid and soy protein, mainly glycinin and β-conglycinin. Actually, 60% of the protein in soy milk protein is composed of these two proteins, and a large size of the protein body was constructed with their proteins and others. Lipid is a triacylglyceride composed of linoleic acid, oleic acid, and phospholipids. Before treating, triacylglyceride is stored in oil bodies in soybeans. During soy milk production, and of course during *Tofu* production, oil drops are suspended, forming an emulsion after crushing of soybeans. The oil drop is formed and held stably by lecithin, phospholipid, and oleosin, all proteins forming oil bodies in soybeans. Furthermore, an outside layer is covered by soy protein. Phytic acid (*myo*-inositol-1,2,3,4,5,6-hexaphosphate) and mineral and oligosaccharide are in the soluble component of soy milk [16]. When some bivalent ions such as calcium ion and magnesium ion are added to soy milk, the ions combine with phytic acid. As a result, decreasing the pH of the soybeans immobilizes the protein in it [17].

http://dx.doi.org/10.5772/60848

This phenomenon induces the charge of protein to dissipate by a combination of phytic acid and bivalent ion. Repulsion among their proteins is decreased. Moreover, the immobilized protein combines with outside layer protein easily around an oil drop [18]. Consequently, the steric network structure of soy protein is formed to gather oil drops with intermediary soy protein. Becoming low pH in soy milk, soluble soy protein becoming dissoluble is taken into the network. The curd produces a hard gel that is water retentive, such that moisture is trapped in the network.

4. Soy milk curdling yeast and characteristics of curdling enzymes

4.1. Soy milk curdling

As discussed above, soybean proteins provide rich nutrition [4, 19]. In fact, soymilk consumption is increasing quickly throughout the world because of its health benefits. Moreover, differently from bovine milk, it contains no cholesterol. Yogurt-like foods and cheese-like foods made from soybeans can be consumed by people who are concerned about health issues or allergies related to bovine milk. Tofu, soybean curd from soybean milk, resembles cheese or yogurt made from milk curd of cows or other mammals. However, the cheese mouthful sense and physical properties are not identical to those of casein protein. Unlike the protein casein in bovine milk, enzymatic curdling of soybean milk produces poor flavor and texture.

It is not yet a viable alternative to dairy foods. For that reason, the commercial use of enzymes such as bromelain, ficin, and papain for the curdling of soybean milk has been unsuccessful [20–22]. The authors have reported that physical properties of soy protein modified by enzyme reactions such as germinated proteolysis in soybeans. Therefore, in this section, along with a report of yeast containing soybean curdling enzyme [23], this investigation was undertaken to screen and identify specific food yeast strains (*Saccharomyces* sp.) that produce a soybean milk curdling enzyme and to purify the enzyme using chromatographic procedures.

4.2. Screening of yeast producing curdling soybean milk enzyme

The yeast strains (1345 strains) stored in the laboratory were screened using soybean milk agar plate medium. The strains were inoculated by streaking on a plate surface. Then they were incubated at 30°C for 7 days. After cultivation, the clear zone diameter was measured using calipers. Yeast strains that produced a clear zone were selected. Results show that 1242 yeast strains among all 1345 yeast strains produced no clear zone on the plate medium. The yeast strains (42 strains) produced less than 1 mm of a clear zone. Also, 57 yeast strains produced 1.0–5.0 mm; 4 yeast strains produced more than 6 mm.

In the second screening of curdling soybean milk enzyme-producing yeast, the screened strains (103 strains) were inoculated to the soybean milk liquid medium. Purchased soybean milk was added to them aseptically.

The soybean milk medium was incubated at 30°C for 24 h. When curdling occurred, the pH of whey was measured using a pH meter (Horiba Ltd.). Results show that three yeasts curdled

at pH greater than 5.90. The media were pH 5.90 (SCY 001), pH 6.05 (SCY 002), and pH 6.38 (SCY003) (Table 4).

pH	Curdling soy milk		
	(++)	(+)	(–)
≥6.50	0	0	7
6.49–5.90	1	2	43
5.89–5.50	0	17	17
5.49–5.00	2	11	0
≤4.99	3	0	0

Initial pH was 6.70. ++, coagulating very well; +, coagulating; –, noncoagulating.

Table 4. Curdling soybean milk condition by screened yeast

The curd activity of strain SCY003 was the highest among the strains. Therefore, the SCY003 strain was finally screened. Isolated yeasts were classified taxonomically and were identified according to methods described in earlier studies [24].

The morphology was observed by microscope. Their 1.5- to 6.5-μm-long cells were short and ovaloid. The yeast, which buds by multibudding reproduction, does not form pseudomycelia or pellicles on the liquid medium. It forms ascospores. It was identified as *Saccharomyces* sp.

For researching physiological characteristics of the strain, the yeast was inoculated into a yeast nitrogen base medium (Difco Laboratories) adding 0.5% of each carbon source: sugar or organic acid as glucose, galactose, sucrose, maltose, raffinose, trehalose, lactose, melibiose, cellobiose, melezitose, starch, D-xylose, L-arabinose, D-ribose, L-rhamnose, erythritol, D-mannitol, salicin, inositol, dulcitol, ethanol, D-sorbitol, disodium succinate, and trisodium citrate. The yeast was inoculated into yeast carbon base medium (Difco Laboratories) adding sodium nitrate solution.

The glucose, galactose, sucrose, maltose, and raffinose in the medium were fermented as carbon sources using strain SCY003. The yeasts grew in a vitamin-free medium. Furthermore, the strain did not grow in 0.01% cycloheximide. According to the morphological, physiological, and molecular characteristics, it was identified as *Saccharomyces bayanus*.

For researching molecular biological characteristics of the strain, primers were used for amplification and sequencing of 18S-rRNA-encoding genes. The PCR products were sequenced using a kit (ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction; Applied Biosystems). Analyses of DNA sequence reactions were performed using a sequencer (3130; Applied Biosystems). The 18S rRNA coding DNA was sequenced. Homology was assessed using the Basic Local Alignment Search Tool (BLAST; http://www.ncbi.nlm.nih.gov/BLAST/).

http://dx.doi.org/10.5772/60848

As a result, the yeast showed homology of 99% with *S. bayanus* (accession no. AY046227). It was identified as *S. bayanus* through homology research and phenotypic testing.

4.3. Purification of the protease as a soybean milk curdling enzyme

Enzyme extraction, intercellular, in *S. bayanus* SCY003, soybean milk curdling test/activity was conducted using the method described [25-27] for modified soybean milk from bovine milk-curdling activity. The mixture was centrifuged at 400×*g* for 10 min. The supernatant was removed gently using a Pasteur pipette. The weight of the precipitate was measured using a chemical balance.

Generally, commercial soy milk has dispersion stability attributable to the presence of oleosomes or forming aggregate formation of soy proteins on it [28, 29]. Therefore, no precipitate is produced from commercial soybean milk by low centrifugal gravity as 400×*g*. However, precipitation ratios increase with the enzyme reaction period.

The precipitation ratio was related with the reaction period, and with the enzyme solution volume. They are mutually correlated: $R^2 = 0.9978$. Therefore, one curdling unit expressed the ratio of curdling (%) from 1 mL of soybean milk at 40°C for 1 h. The precipitation ratio from soybean milk was assayed efficiently to propose a new curdling method. The precipitation ratio was assayed (Fig. 3).

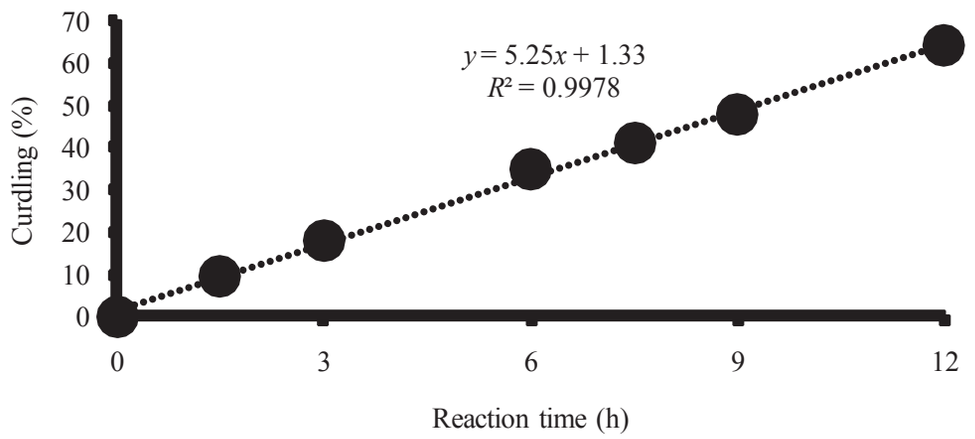


Figure 3. Curdling soybean milk enzyme activity.

After crude extraction of the enzyme, the enzyme protein was purified using chromatography. After crushing cells and extracting the enzyme, the enzyme was precipitated to between 30% and 40% saturation of ammonium sulfate. After redissolving the precipitate, the solution was dialyzed overnight at 4°C, and they carried out ion-exchange chromatography using a column (25 mm × 300 mm) of DEAE gel. The protein was eluted using a 0- to 1-M NaCl linear gradient. Proteolytic activity and curdling were assayed each fraction. Proteolytic activity was measured in duplicate using a commercial kit fluorescein isothiocyanate-labeled casein (FTC). Fluores-

cence was measured using a 485-nm excitation wave and a 535-nm emission wave. The proteolytic activity was decided for one unit expressed equal amount of trypsin (1 ng·mL^{-1}) doing proteolysis of FTC solution.

After ion chromatography, fractions containing the highest level of activity were pooled and reprecipitated using 80% saturation of ammonium sulfate. After redissolving the precipitate, gel filtration chromatography (10 mm × 350 mm, P-100 gel; Bio-Rad Laboratories Inc., CA, USA) was carried out. Then the molecular weight of the enzyme was analyzed using also the chromatography as various molecular weight standards (myosin, 200 kDa; serum albumin, 66.2 kDa; ovalbumin, 45.0 kDa; trypsin inhibitor, 21.5 kDa).

The results are portrayed in Figs. 4a and 4b. One main curdling activity peak was identified using ion-exchange chromatography. The peak (fraction no. 49) agreed with protein and activity. Furthermore, curdling activity agreed with the same fractions presenting protease activity (fraction no. 49).

As a result, the larger peak of proteolysis activity was found around fraction number 25, and a small peak was found at fraction number 49. The fraction of the proteolysis enzyme around number 25 was not representative of curdling activity. It is considered that the former fractions are attributable to intense proteolysis enzymes and that the latter fractions are attributable to soybean milk-curdling enzymes.

After reprecipitation, the sample was analyzed using gel filtration chromatography. A peak was found at fraction numbers 11–14. Their fractions agreed with soybean milk curdling activity, proteolytic activity, and protein. This result on their chromatograms demonstrates that protease and soybean milk curdling enzyme have some mutual relation of activity.

After purification, the enzyme protein band was approximately 45 kDa (Fig. 5a), which agrees with data of other proteases. The protease molecular weight was measured using gel filtration chromatography (Fig. 5b). The molecular mass is about 45 kDa. The protease was inferred.

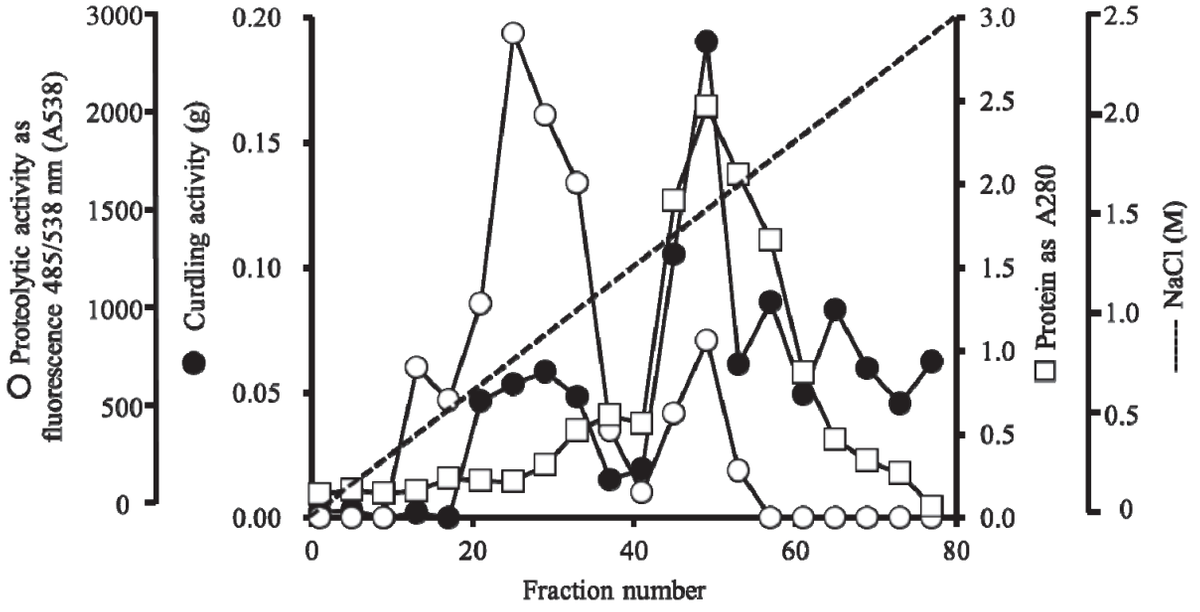
The soy milk curdling enzyme has proteolytic activity. Results suggest that the soy milk curdling enzyme was a proteolysis enzyme. Many researchers have reported protease produced by yeasts as *Candida albicans* [30, 31, 32], *Candida humicola* [33], and *Saccharomyces cerevisiae* [34]. Extracellular proteases produced by yeasts as *Candida* spp. are 42–45 kDa [35, 32]; those by bacteria are 21 kDa [36].

By contrast, few reports describe intracellular protease producing *Saccharomyces cerevisiae*, although many intracellular proteases in the vacuole or other organelles are known to be related to proteinase A, which is 42 k Da [34]. The molecular weight of curdling soy protein enzyme protease agreed with protease produced by other yeast as Ascomycota. However, the *Mucor* sp. enzyme, which curdles bovine milk, produced a 49-kDa protease [37], which is larger than those produced by these yeasts.

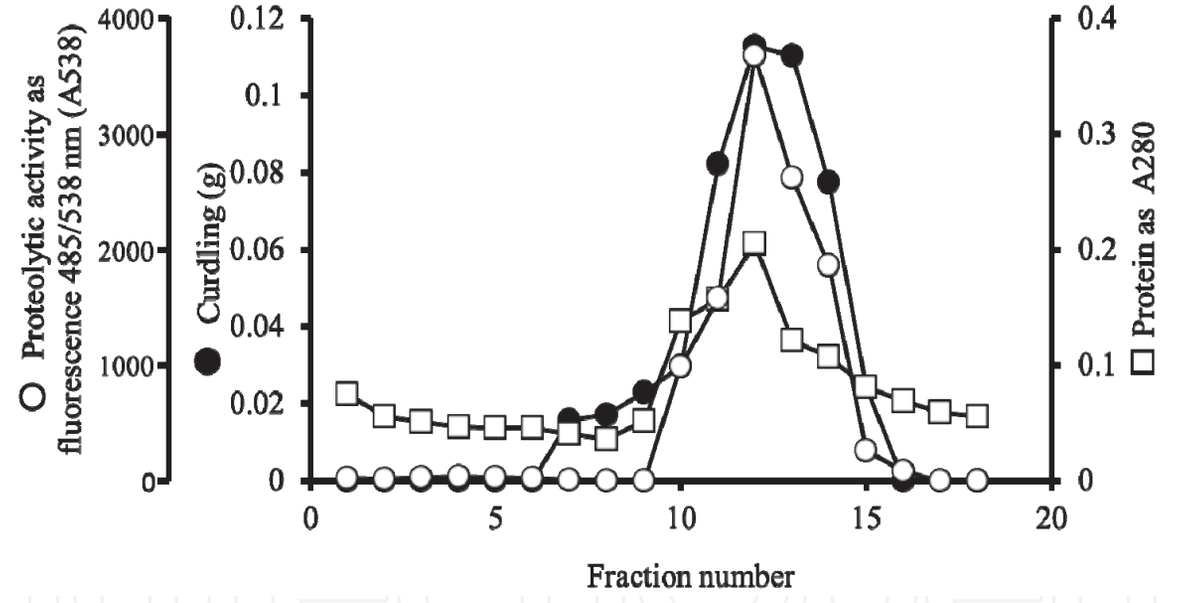
4.4. Characteristics of the protease as a soybean milk curdling enzyme

Optimum pH, temperature, and stability of the enzyme are presented in Figs. 6a and 6b. Optimum pH was assayed at pH 4.0–8.0 using 50 mM phosphate-citric buffer or phosphate-

http://dx.doi.org/10.5772/60848



(a)



(b)

Figure 4. (a). Ion-exchange chromatography of soybean milk curdling. **enzyme.** (b). Gel filtration chromatography of soybean milk curdling. **enzyme**.

NaOH. For optimum pH of the enzyme assaying, 0.1 mL of a fluorescein isothiocyanate-labeled casein (FTC) solutions, which dissolved in each phosphate buffer (50 mM, pH 4.0–8.0), and 20 μL of enzyme solution were reacted at 40°C for 60 min. For optimum temperature of the enzyme assaying, FTC solution at pH 7.5 (0.1 mL) and 20 μL of enzyme solution were reacted at 15°C–70°C for 60 min to find optimum temperature. After reaction, fluorescence was measured using a 485-nm excitation wave and a 535-nm emission wave.

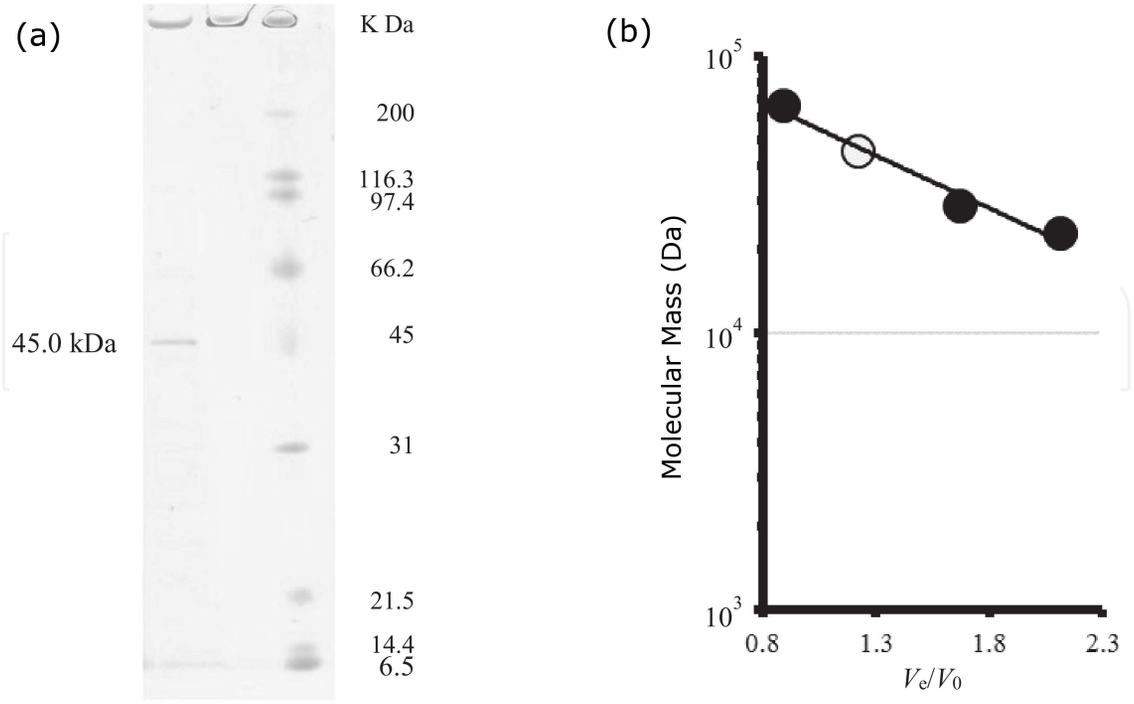


Figure 5. (a). Photograph of SDS-PAGE of soybean curding enzyme. (b). Measurement of molecular weight.

The optimum pH for the protease activity as curdling was pH 7.5; the optimum temperature was 50°C. The optimum pH of a bovine milk curdling protease, *Mucor pusillus*, is pH 5.0. The optimum pH values of many commercially available proteases are pH 5.9–6.7. However, soymilk curdling activity decreases concomitantly with increasing alkalinity.

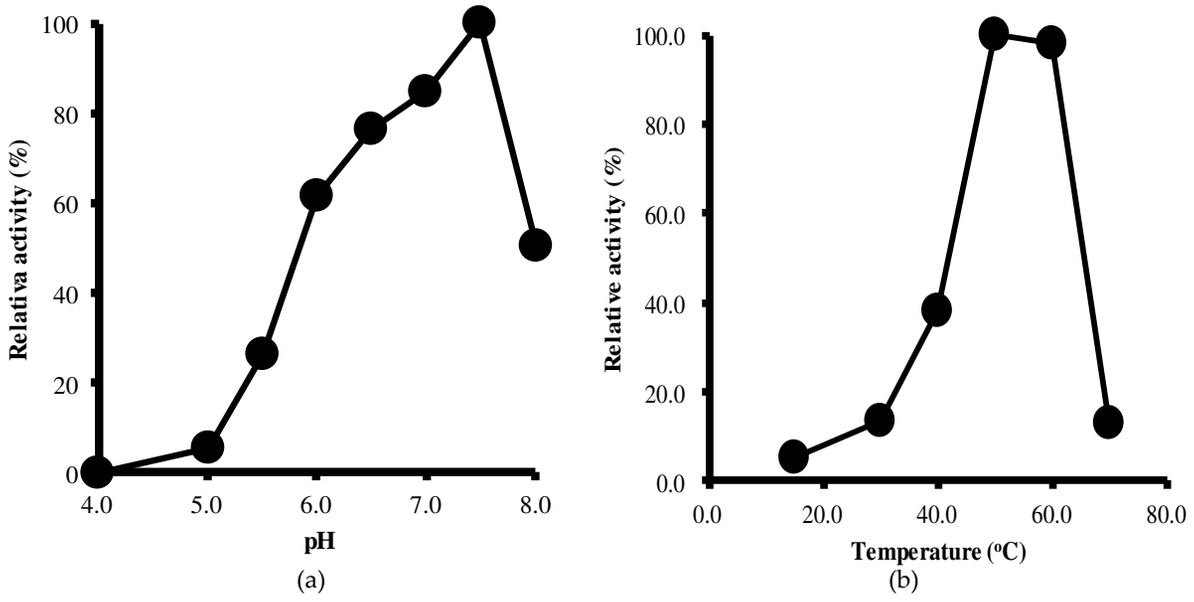


Figure 6. (a). Optimum pH of soybean curdling enzyme. (b). Optimum temperature of soybean curdling enzyme.

http://dx.doi.org/10.5772/60848

Park *et al.* [38] reported that the optimum pH of soybean milk curdling protease produced by *Bacillus* was pH 6.0. Regarding this enzyme, the optimum pH of the protease was pH 7.5. The optimum temperature of the protease was 50°C. The enzyme also curdled soybean milk at pH 7.5 and 50°C. Commercial soybean milks sold in Japan are pH 7.0–7.2. The pH range agrees with their optimum pH range.

Effects of metal ions and inhibitors on protease are presented in Table 5. In fact, zinc, copper, and mercury all inhibit protease activity. The amino acid of the active site contains cysteine residue because of inhibition by mercury [39]. Furthermore, EGTA (10 mM) inhibited protease activity (62.0% of relative activity).

Metal ion and inhibitor	Concentrations	Relative activity (%)
Na^{+}	1 mM	101.5
K^{+}	1 mM	105.9
Mn^{2+}	1 mM	101.9
Mg^{2+}	1 mM	46.2
Fe^{2+}	1 mM	95.4
Zn^{2+}	1 mM	23.6
Co^{2+}	1 mM	47.9
Cu^{2+}	1 mM	17.9
Ca^{2+}	1 mM	100.9
Iodo acetate	1 mM	74.1
Hg^{2+}	1 mM	34.1
EGTA	1 mM	101.6
EGTA	10 mM	62.0
EDTA	1 mM	96.1
EDTA	10 mM	89.8
NEM	1 mM	98.5
Azid	1 mM	100.4
NBS	1 mM	97.6
Cont.	–	100.0

Table 5. Effects of metal ion and inhibitor of the protease

The activity was not activated by metal ions, but it was inactivated by mercury. Soybean milk-curdling enzyme [38] was inhibited by zinc ions and mercury ions. These results agree with our data related to zinc and mercury. Its survival activity was 18% by mercury. The protease

was not activated by metal ions, which indicates that the protease is not a metalloprotease: a metal-dependent enzyme. The amino acid of active site contains cysteine residue.

The mechanisms of curdling soybean milk protease were investigated. At first, The curdled soybean milk samples with added protease and without protease were treated with sample buffer solution.

Soybean milk was poured into a glass vessel (inner diameter 32 mm, height 45 mm). After 0.1 mL of enzyme solution adding to the soybean milk, or without enzyme 0.1 mL D.W., the mixtures were incubated at 40°C, and they were sampled sequentially; between 4- and 24-h. samples (0.01 mL) were added to 0.01 mL of sample buffer and then heated at 100°C for 3 min. Then samples (10 μl) were added in each well. The samples were electrophoresed on a 12.5% uniform gel at 20 mA.

They were subsequently examined using SDS–PAGE (Fig. 7) of curdled soybeans. The left side lane shows the standard of protein size. The next lane (0 h.) shows soybean milk protein without reaction of protease. The other lanes show soybean milk protein decomposed for 4, 8, 12, 16, and 24 h. Lane 0 h shows the α'- and α-subunits of β-conglycinin (approximately 84–73 kDa), the A_3 acidic subunit (approximately 40 kDa), other acidic subunits as A_4, A_{1a}, A_{1b}, and A_2 (approximately 30–42 kDa) of glycinin, the β subunit (approximately 50 kDa), and basic subunits as B_3, B_{1a}, B_{1b}, and B_4 (approximately 20 kDa) [40, 41].

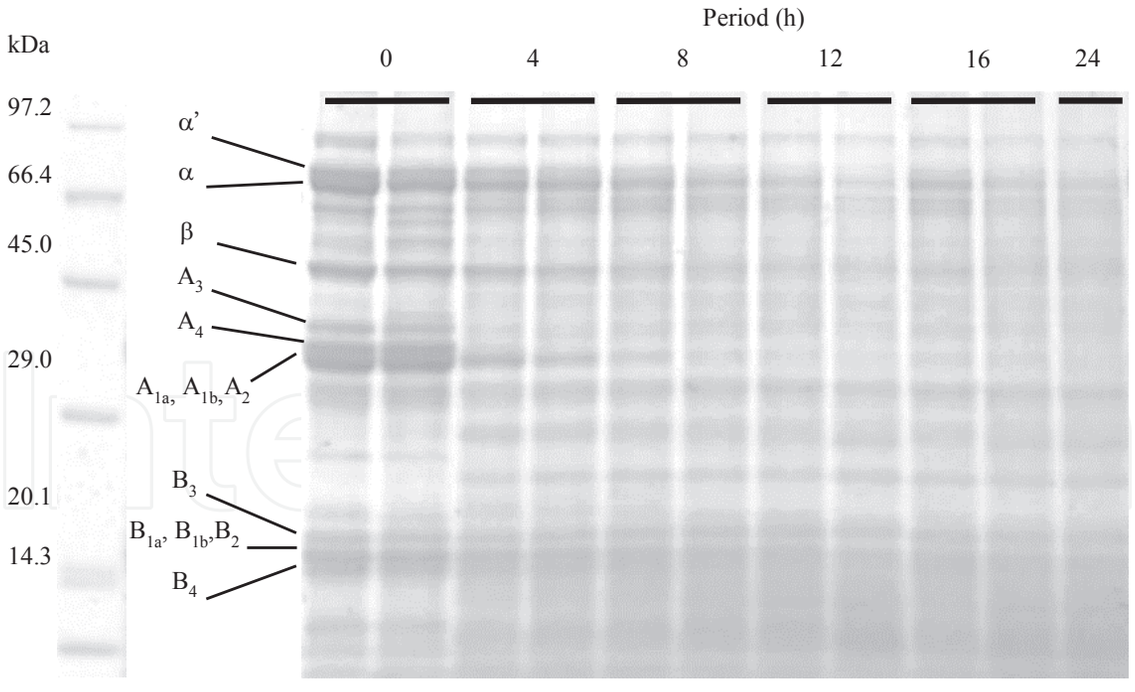


Figure 7. Digestion of soy protein during curding by soybean milk curdling enzyme.

The two bands shown as α' and α disappeared gradually after the reaction, showing the protein band from curd making the protease. In the glycinin subunits, the band of the A_3 acidic subunit disappeared completely after 4 h. Furthermore, A_4, A_{1a}, A_{1b}, and A_2 disappeared to a partial

http://dx.doi.org/10.5772/60848

degree same as A_3 acidic subunit. Peptides smaller than 20 kDa were detected on the gel. The β-conglycinin as α, α', and part of glycinin as A_3 A_4, A_{1b}, and A_2 were decomposed. Soybean protein became loosely curdled with the addition of other proteases from microorganisms or plants. The protein was decomposed. The low-molecular-weight peptides increased on the polyacrylamide gel. Generally, 11S glycinin was related to the formation of a stiffer gel. Furthermore, Ono *et al.* [42] reported hydrophobic bonding and hydrogen bonding related to curdling *Tofu*. Utsumi *et al.* [43] reported that the basic subunit and β-subunit formed macro complexes by heating. The complexes were regarded as forming cores for *Tofu* coagulation. The complexes were reportedly wrapped in α-, α'-, and acidic subunits [42].

According to our data, after the curdling soy milk by enzyme, α- and α'-subunits cleaved by the protease easily, whereas basic and β-subunit remained. It is considered that surface proteins as α- and α'-subunits were decomposed easily. Some decomposed subunits such as α, α', A_3, acidic, and basic subunits are regarded as related to the curdling soybean milk.

The enzyme of mechanisms for proteolysis was searched that the enzyme had the peptidase activity as exotype proteolysis activity and protease as endotype proteolysis activity. The synthesis peptide substrates, Z-glutamyl-tyrosine, and casein, FTC, were reacted by the enzymes. Peptidase (carboxypeptidase) activity was determined by the increase in ninhydrin after hydrolysis of benzyloxycarbonyl-glutamyl-tyrosine (pH 8.0) at 40°C.

The results show that the enzyme had 0.14 $U \cdot mg^{-1}$ protein as peptidase activity and 0.55 $U \cdot mg^{-1}$ protein protease as endotype proteolysis activity (data not shown). Results also show that the soybean milk-curdling enzyme as a proteolysis enzyme had endotype proteolysis activity.

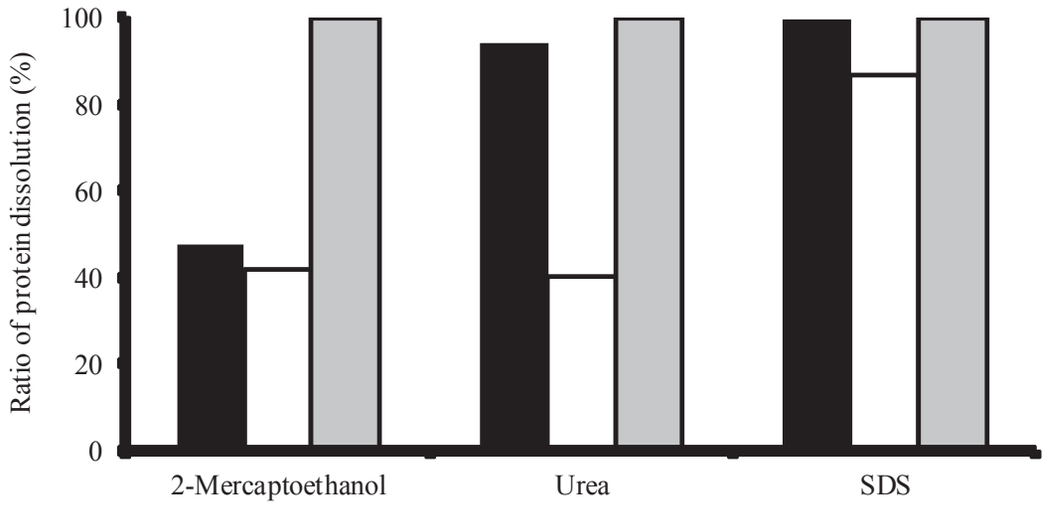
Jones *et al.* [34] reported proteolysis enzymes of three types in yeast classes: cytosolic protease, vacuolar proteases, and proteases located within the secretory pathway. They belong to aspartic type, serine, or metallo-type proteolysis enzyme cleaved substrates with endotype or exotype. Generally, metallo-type enzyme requires metal ions such as zinc. The optimum condition of aspartic protease is an acidic condition. The enzyme did not require ion metal. Its optimum pH was 7.5, which is weakly alkaline. It is therefore considered that the enzyme is a serine protease of one kind. These results agreed with serine protease from *Bacillus* sp. curdling soy protein [44]. For future studies, we will ascertain the amino acid sequence in a substrate cleaved by enzyme using synthesis substrate.

4.5. Characteristics of curd curdling by enzyme

As mechanisms that are closely involved in curdling soybeans, curdled soybean milk by enzyme and glucono-δ-lactone (GDL, 3.0% solution) as control samples were dissolved in chemical solutions. The enzyme solution (0.1 mL) was added to the soybean milk. The mixture was incubated at 40°C for 4 h., or 0.1 mL of glucono-δ-lactone (GDL, 3.0% solution) was added to the soybean milk (1.0 g) and incubated at 80°C for 1 h as control sample. To each of the two curdled soybean milk samples, 9 mL of chemical solution as 2% SDS solution, 4 M urea solution, and 10 mM 2-mercaptoethanol were added. They were held for 1 h at room temper-

ature and were then centrifuged at 4000×*g* for 10 min. Protein in the supernatant was assayed using the Lowry method.

Results show the relative ratio of protein (%); that is, 100% relative ratio represents the amount of protein dissolving no-curdling soy milk in each solution (Fig. 8).



▪, SCE; □, GDL; ▪, no-curdled soy milk.

Figure 8. Curdled soy bean milk dissolved in chemical solutions. SCE shows soymilk curdled by enzymes; GDL shows soymilk curdled by glucono δ-lactone.

After dissolving the solutions, curd produced by the enzyme dissolved 47.3% of relative ratio from curd to the 2-mercaptoethanol solutions. That curdled by GDL dissolved 41.6% of relative ratio from the curd to the urea solutions. With urea solution, the curd making the enzyme dissolved 93.8% of relative ratio and GDL dissolved 40.3% of relative ratio. The curd produced by the enzyme can dissolve with urea. Both the curd produced by the enzyme and GDL dissolved with SDS solution. Actually, 2-mercaptoethanol solution cleaves the disulfide bond in protein. The urea solution cleaves the hydrogen bond, and the SDS solution cleaves the hydrophobic bond. That inference agrees with results reported by Yasuda *et al.* [44] that serine protease from *Bacillus* sp. curdled soybean milk and produced a protein bond through mutual hydrophobic bonding.

Next, the effects of the proteolysis enzyme against two protein in soybean, glycinin and β-conglycinin, were researched. Glycinin and β-conglycinin were extracted from commercial soy protein according a process described by Nagano *et al.* [44]. The soy protein (100 g) suspended 1500 mL of distilled water at pH 7.5 adjusted 0.1 M NaOH. From their extraction, the glycinin was carried out to do isoelectric precipitation at pH to 6.4. Moreover, β-conglycinin was also precipitated at pH 4.8. The two fractions were freeze-dried. Each fraction as glycinin fraction and β-conglycinin (50 mg) was resolved to 1 mL of 50 mM phosphate buffer (pH 7.5). Fur-

http://dx.doi.org/10.5772/60848

thermore, enzyme solution (0.1 mL) was added. The mixture were incubated at 40°C. After reaction, soybean milk curdling activity was assayed according the preceding method. Curdling activity was assayed against two substrates: glycinin and β-conglycinin (Fig. 9). Glycinin was curdled strongly: soybean curdling activity was 86.9 ($U \cdot mL^{-1} \cdot min^{-1}$). However, β-conglycinin was curdled weakly: 38.0 ($U \cdot mL^{-1} \cdot min^{-1}$).

The data agree with reports in the relevant literature [45, 46, 47]. Bromelain decomposes 11S globulin to curdling. The entire band of acidic subunits and most basic subunits disappeared [46]. Glycinin-rich soybean milk was curdled strongly [47]. However, the enzyme made glycinin curdle without metal ion or GDL. Generally, glycinin is known to contain more sulfur amino acid than β-conglycinin does. According to Fig. 7, soymilk was curdled by a hydrogen bond or hydrophobic bond. Furthermore, some alkaline protease as subtilisin and chymotrypsin cleaves hydrophobic amino acid residue.

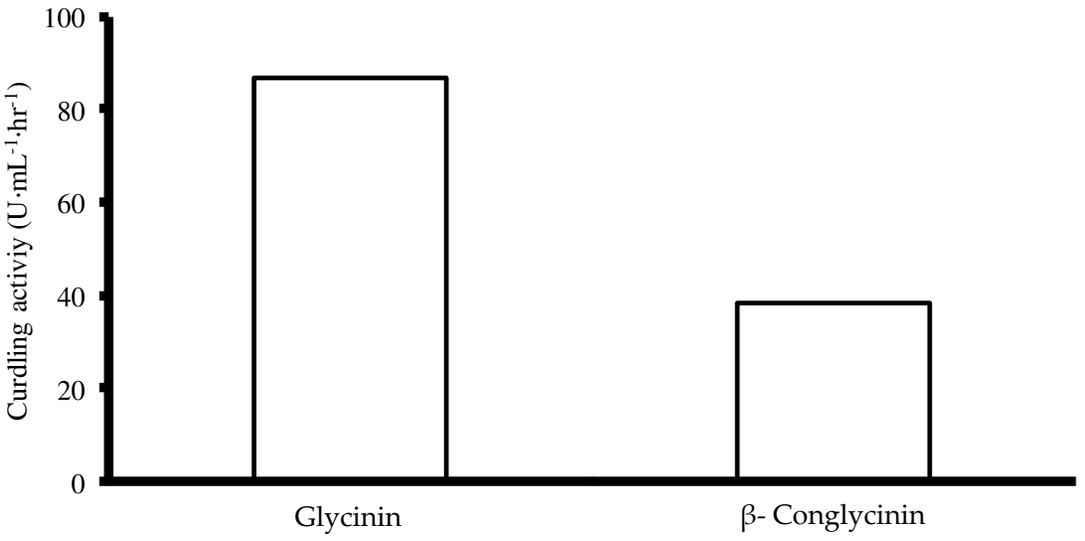


Figure 9. Curdling activity against two soy protein as glycinin and β-conglycinin.

The enzyme made curd from soymilk mainly by an enzyme reaction against glycinin. Choi *et al.* [48] reported that the α-subunit in β-conglycinin contains a hydrophobic sequence.

Soybean milk (10 mL) with 0.01 mL of anti-foam (KM-72F) was poured into a glass cup (32 mm inner diameter, 45 mm height). Then 1.5 mL of enzyme solution was added to the soybean milk. The mixture was incubated at 40°C for 4 h. As a control sample, glucono-δ-lactone (GDL, 0.3%) was added to soybean milk (10 mL) with 0.01 mL of anti-foam (KM-72F) added. Then the mixture was incubated at 80°C for 1 h. The curdled soybean milk samples were held at room temperature for 30 min. The rheological characteristics of enzyme curdling soybean milk were measured directly using a creep meter with a 16-mm-diameter plunger compressing 1 mm s^{-1} with 80.0%. As a control sample, soybean milk was curdled by GDL, 0.3% at 80°C for 1 h.

The rupture strength of the curdled soybean milk in the cups was measured directly using a creep meter (RE-3305; Yamaden Co. Ltd., Tokyo, Japan) with a 16-mm-diameter plunger compressing 1 mm s^{-1} with 80.0%.

The stress–strain curves of curdled soybean milk are presented in Fig. 10. The vertical axis shows stress ($N \cdot m^{-2}$), which represents internal forces of the sample curd pushing back against the strain. The horizontal axis shows the strain of the curd. The sample curd strained by the plunger is broken by a force that exceeds a certain force: the breaking point. The breaking load represents the hardness or softness of the curd sample. The breaking strain represents the resilience of the sample curd: a large value signifies a high-resilience sample. Pressure by the plunger was loaded more. Then the curd was broken more heavily. After strain loading, the stress value decreased partly. The brittleness shows a different breaking point with the local minimal value. A large brittleness load shows brittle sample curd. The soybean milk was poured into a glass vessel and then curdled using the respective methods. After curdling, rheological analysis of the sample was conducted using a creep meter without taking out.

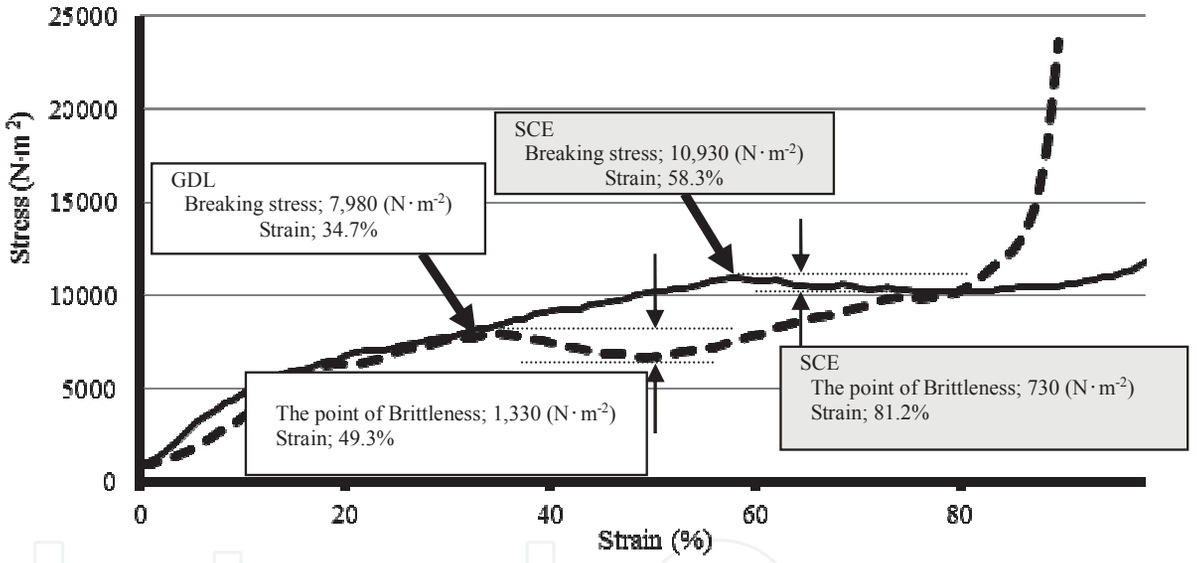


Figure 10. Stress–strain curves of curdled soybean milk.

The breaking point of curd curdled using GDL, which is used by many Japanese *Tofu* production companies to produce commercial *Tofu*, was 34.7% strain. The breaking stress was 7980 ($N \cdot m^{-2}$). The point of brittleness was 49.3% and 6650 ($N \cdot m^{-2}$). The brittleness of this curd produced using GDL was 1330 ($N \cdot m^{-2}$). In fact, their *Tofu* curdled by GDL same as the Japanese *Tofu* is soft and brittle.

By contrast, the breaking point of curd curdled by soy curdling enzymes (SCE) was 58.4% strain. Their breaking stress was 10,930 ($N \cdot m^{-2}$). The brittleness point is 81.2% and 10,200 ($N \cdot m^{-2}$). The brittleness of this curd produced using the enzyme was 730 ($N \cdot m^{-2}$). The curd that used SCE had 1.4 times greater breaking point, shown hardness, than that of the curd produced using the GDL same as the Japanese *Tofu*. Moreover, the curd that used SCE had 1/2

http://dx.doi.org/10.5772/60848

times smaller brittleness than that of GDL. Great breaking point shows hard curd and elasticity. It showed that the curd produced by SCE had hard, but soft, springy and sticky curd.

Heretofore, Yasuda *et al.* [45] reported soy milk curdled by bacteria protease. As a result, the curd produced using the bacteria protease is too soft to measure rheological characteristics, but they also reported that curd produced using the bacteria protease with calcium ion had 2–3×10^5 ($N\cdot m^{-2}$) of the breaking stress. Their curd was as hard as 20–30 times of the curd produced using this yeast enzyme. The curd produced using the bacteria protease with calcium was broken during fermentation and aging for long time. However, the curd produced using the bacteria protease had not springy and sticky texture.

It is considered that the curd produced using yeast enzymes was not like *Tofu* or the curd produced using the bacteria protease of its main characteristics or rheology.

Guo and Ono [47] and Toyokawa *et al.* [49] reported that the breaking stress of normal *Tofu* showed a relationship to soy milk conditions, such as their concentrations of glycinin, proteins, or temperature. Future investigations will examine if the condition of soy milk has a relationship with the breaking stress for the enzyme curdling.

According to this report, the new protease from *S. bayanus* SCY 003 produced a new texture of soy food that is applicable for new healthy foods, anti-milk allergy foods, and others.

5. Conclusion

Soybeans are a traditional food in eastern Asia, particularly in Japan and China. They were eaten in 100 BC in China. The beans can be processed into *Tofu*, soy milk, fermented seasonings, soy sauce or *Miso* paste, and *Natto* and green beans. Soybeans have rich nutrition, protein lipid, and other functional substances such as isoflavones. However, soybeans are difficult to process for use as food because of tissue and cell wall hardness. Therefore, soybeans are conducted to do some treatments, *e.g.*, boiling, steaming, roasting, crushing/grinding, and some enzyme treating, to eat soy protein easily.

Soy storage proteins mainly comprise two proteins as 7S globulin composed of β-conglycinin and γ-conglycinin and 11S globulin containing glycinin composed of 5 subunits. β-Conglycinin, included in 7S globulin, is composed of three subunits.

To modify the physical properties of soy protein, a new type of enzyme for curdling soybean milk enzyme was purified as an extract from yeast. Yeast producing curdling soybean milk enzyme, the SCY003 strain, was isolated from 1345 yeast strains. According to the morphology, physiology, and molecular and characteristics, SCY003 was identified as *S. bayanus*. The soy milk curdling enzyme having proteolytic activity was approximately 45 kDa and a monomer protein. The optimum pH for the protease activity was pH 7.5; the optimum temperature was 50°C.

The enzyme cleaved the β-conglycinin as α and α', and part of glycinin as A_3 A_4, A_{1b}, and A_2 in soy protein by endoproteolysis. Soybean protein became loosely curdled with the addition

of other proteases from microorganisms or plants. Soybean milk curdled after cleaving endoproteolysis enzyme in SCY003 strain. The rheological characteristics of enzyme curdling soybean milk, the breaking point, was 58.4% strain; their breaking stress was 10,900 ($N \cdot m^{-2}$); the brittleness point is 81.2% and 10,200 ($N \cdot m^{-2}$). The brittleness of the curd produced using the enzyme was 727 ($N \cdot m^{-2}$). The curd had a sticky and chewy texture and did not resemble *Tofu* rheologically.

In this way, some properties of soy protein were modified by enzymes, such as decomposing specific subunits in soybeans and making soy milk curdle, which are expected to be applicable for new healthy foods, anti-milk allergy foods, and others.

Acknowledgements

We thank A-STEP, Adaptable and Seamless Technology, Transfer Program through Target Driven R&D by Japan Science and Technology Agency (JST) (AS231Z00291E) for financial support.

Author details

Makoto Kanauchi[1], Sakiko Hatanaka[2] and Makoto Shimoyamada[3]

1 Department of Food Management, Miyagi University, Japan

2 Industrial Technology Institute, Miyagi Prefectural Government, Japan

3 School of Food and Nutritional Sciences, University of Shizuoka, Japan

References

[1] Yamauchi F, Okubo K. editors. Daizu no Kagaku: Science of Soy Bean. Asakura Publishing, Tokyo: 1992 (in Japanese).

[2] The yield amount of soy bean; http://www.maff.go.jp/j/seisan/ryutu/daizu/d_data/pdf/014.pdf

[3] Trend in demand of soy bean; http://www.maff.go.jp/j/seisan/ryutu/daizu/d_data/pdf/011.pdf

[4] Endres JG. In: Soy Protein Products: Characteristics, Nutritional Aspects, and Utilization. Champaign, Illinois: AOCS Publishing; pp.10–18 (2001).

[5] Food and Drug Administration (FDA) in U.S. Soy Protein and Coronary Heart Disease. In: Federal Register. 1999:57700–57733.

[6] CMC Publishing. β-Conglycinin. Bio industry, 27 (5): 68–69 (2010) (in Japanese).

[7] Yamori Y, Ohta S, Watanabe S. Soybean Isoflavones. Tokyo: Saiwai Shobo; 2001 (in Japanese).

[8] Fritz H, Seely D, Flower G, Skidmore B, Fernandes R, Vadeboncoeur S, Kennedy D, Cooley K, Wong R, Sagar S, Sabri E, Fergusson D. Soy, red clover, and isoflavones and breast cancer: a systematic review. PLoS One 2013;8(11): e81968.

[9] Simons R, Vincken JP, Roidos N, Bovee TF, van Iersel M, Verbruggen MA, Gruppen H. Increasing soy isoflavonoid content and diversity by simultaneous malting and challenging by a fungus to modulate estrogenicity. J Agric Food Chem 2011;59(12): 6748–58.

[10] Zimmermann MC, Tilghman SL, Boué SM, Salvo VA, Elliott S, Williams KY, Skripnikova EV, Ashe H, Payton-Stewart F, Vanhoy-Rhodes L, Fonseca JP, Corbitt C, Collins-Burow BM, Howell MH, Lacey M, Shih BY, Carter-Wientjes C, Cleveland TE, McLachlan JA, Wiese TE, Beckman BS, Burow ME. Glyceollin I, a novel antiestrogenic phytoalexin isolated from activated soy. J Pharmacol Exp Ther 2010;332(1): 35–45.

[11] Kraft B, Barz W. Degradation of the isoflavone biochanin A and its glucoside conjugates by *Ascochyta rabiei*. Appl Environ Microbiol 1985;50(1): 45–8.

[12] Tikhonovich IA. Nitrogen Fixation: Fundamentals and Applications: Fundamentals and Applications. Proceedings of the 10th International Congress on Nitrogen Fixation, St. Petersburg, Russia; 1995.

[13] Takashima M, Nara K, Niki E, Yoshida Y, Hagihara Y, Stowe M, Horie M. Evaluation of biological activities of a groundnut (*Apios americana* Medik) extract containing a novel isoflavone. Food Chem 2013;138(1): 298–305.

[14] Lin LZ, He XG, Lindenmaier M, Yang J, Cleary M, Qiu SX, Cordell GA. LC-ESI-MS study of the flavonoid glycoside malonates of red clover (*Trifolium pratense*). J Agric Food Chem 2000;2(48): 354–65. doi:10.1021/jf991002

[15] Shimoyamada M. Processing characteristics of soy protein—curdling property of soy milk. J Cookery Sci Japan 2007;40(1): 37–40 (in Japanese).

[16] Ono T, Takeda M, Guo ST. Interaction of protein particles with lipids in soybean milk. Biosci Biotechnol Biochem 1996;60: 1165–9.

[17] Ono T, Katho S, Mochizuki K. Influences of calcium and pH on protein solubility in soybean milk. Biosci Biotechnol Biochem 1993;57: 24–8.

[18] Guo ST, Ono T, Mikami M. Incorporation of soy milk lipid into protein coagulum by addition of calcium chloride. J Agric Food Chem 1999;47: 901–5.

[19] Larkin T, Price WE, Astheimer L. The key importance of soy isoflavone bioavailability to understanding health benefits. Crit Rev Food Sci Nutr 2008;48(69): 538–52.

[20] Park YW, Kusakabe I, Kobayashi H, Murakami K. Production and properties of a soymilk-clotting enzyme system from a microorganism. Agric Biol Chem 1985;49(11): 3215–9.

[21] Park YW, Kobayashi H, Kusakabe I, Yoshida S, Murakami K. Action of soymilk-clotting enzyme from *Bacillus* sp. K-295G-7 on the acidic subunit of soybean 11S globulin. Agric Biol Chem 1989;53(8): 2289–90.

[22] Qua DV, Shimizu U, Taga N. Purification and some properties of halophilic protease produced using a moderately halophilic marine *Pseudomonas* sp. Can J Microbiol 1981;27: 505–10.

[23] Hatanaka S, Maegawa M, Kanauchi M, Kasahara S, Shimoyamada M, Ishida M. Characteristics and purification of soybean milk curdling enzyme-producing yeast *Saccharomyces bayanus* SCY003. Food Sci Technol Res 2014;20(5): 927–38.

[24] Kurtzman CP, Fell JW. In: The Yeast, A Taxonomic Study. 4th ed.. Amsterdam, The Netherlands: Elsevier Science Publishers B.V., pp.360–361 and 891–913, 1998.

[25] Arima K, Yu J, Iwasaki S, Tamura G. Milk-clotting enzyme from microorganisms. V. Purification and crystallization of Mucor rennin from *Mucor pusillus* var. Lindt. J Appl Microbiol 1968;16: 1727–33.

[26] Khan MR, Blain JA, Patterson, JDE. Extracellular protease of *Mucor pusillus*. Appl Environ Microbiol 1979;37(4): 719–24.

[27] Nouani A, Belhamiche N, Slamani R, Belbraout S, Fazouane F, Bellal MM. Extracellular protease from *Mucor pusillus*: purification and characterization. Int J Dairy Technol 2009;6(1): 112–7.

[28] Waschatko G, Junghans A, Vilgis TA. Soy milk oleosome behaviour at the air–water interface. Faraday Discuss 2012;158: 157–69.

[29] Shimoyamada M, Tsushima N, Tsuzuki K, Asao H, Yamauchi R. Effect of heat treatment on dispersion stability of soymilk and heat denaturation of soymilk protein. Food Sci Technol Res 2008;14: 32–8.

[30] Remold H, Fasold H, Staib F. Purification and characterization of a proteolytic enzyme from *Candida albicans*. Biochim Biophys Acta 1968; 167: 399–406.

[31] Ruchel R. Properties of a purified proteinase from the yeast *Candida albicans*. Biochim Biophys Acta 1968;659: 99–113.

[32] Negi M, Tsuboi R, Matsui T, Ogawa H. Isolation and characterization of proteinase from *Candida albicans*: substrate specificity. J Invest Dermatol 1984;83: 32–6.

[33] Ray MK, Uma DK, Seshu KG. Shivajo, S. Extracellular protease from the Antarctic yeast *Candida humicola*. Appl Environ Microbiol 1992;88(6): 1918–23.

[34] Jones EW. Three proteolytic systems in the yeast *Saccharomyces cerevisiae*. J Biol Chem 1991;266(13): 7963–6.

[35] Ruchel R. Properties of a purified proteinase from the yeast *Candida albicans*. Biochim Biophys Acta 1968;659: 99–113.

[36] Cowan DA, Daniel RM. Purification and some properties of an extracellular protease (caldolysin) from an extreme thermophile. Biochim Biophys Acta 1982;705: 293–305.

[37] Nouani A, Belhamiche N, Slamani R, Belbraout S, Fazouane F, Bellal MM. Extracellular protease from *Mucor pusillus*: purification and characterization. Int J Dairy Technol 2009;6(1): 112–7.

[38] Park YW, Kobayashi H, Kusakabe I, Murakami K. Purification and characterization of soymilk-clotting enzymes from *Bacillus* sp. K-295G-7. Agric Biol Chem 1987;51(9): 2343–9.

[39] Springham DG, Moses V, Cape RE. In: Biotechnology, the Science and the Business. New York: Harwood Academic Publishers; 1999.

[40] Thanh VH, Shibasaki K. Heterogeneity of b-conglycinin from soybean seeds. Biochim Biophys Acta 1976a;439: 326–38.

[41] Thanh VH, Shibasaki K. Major proteins of soybean seeds: Subunit structure of b-conglycinin. J Agric Food Chem 1978;26: 692–5.

[42] Ono T, Wada T, Imai A. The structure of Tofu for preventing the change of lipid. Daizu Tanpakushitsu Kenkyu 2004;7: 42–7 (in Japanese).

[43] Utsumi S, Damodaran S, Kinsella JE. Heat-induced interactions between soybean proteins: preferential association of 11S basic subunits and β subunits of 7S. J Agric Food Chem 1984; 32: 1406–12.

[44] Nagano T, Hirotsuka M, Mori H, Kohyama K, Nishinari K. Dynamic viscoelastic study of the gelation of 7S globulin from soybeans. J Agric Food Chem 1992;40: 941–4.

[45] Yasuda M, Kuba M, Tachibana S, Aoyama M. Analysis for mechanism of soybean-milk-coagulation by bacterial protease and utilization of the enzyme to the food processing. Daizu Tanpakushitsu Kenkyu 2002;5: 36–40 (in Japanese)

[46] Mohri M, Matsushita S. Improvement of water absorption of soybean protein by treatment with bromelain. J Agric Food Chem 1984;32(3): 486–90.

[47] Guo S, Ono T. The role of composition and content of protein particles in soymilk on Tofu curding by glucono-δ-lactone or calcium sulfate. Food Chem Toxicol 2005;70(4): 258–62.

[48] Choi SK, Adachi M, Utsumi S. Improved bile acid-binding ability of soybean glycinin A1a polypeptide by the introduction of a bile acid binding peptide (VAWWMY). Biosci Biotechnol Biochem 2005;68: 1980–3.

[49] Toyokawa T, Uehara M, Mochizuki T, Tamamura T, Higa K. Comparisons of physiological and chemical characteristics with Okinawa Tofu and Japanese Tofu. Report of Okinawa Industrial Technology Center. Okinawa, Japan: Okinawa Industrial Technology Center; 2008;11: 7–11 (in Japanese).

IntechOpen

IntechOpen