Food Chemistry 111 (2008) 921–924

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Effects of alginate microencapsulation on the fibrinolytic activity of fermented soybean paste (Cheonggukjang) extract

J.A. Ko [a,*], S.Y. Koo [a,b], H.J. Park [a,c,*]

[a] College of Life Sciences and Biotechnology, Korea University, 1,5-Ga, Anam-Dong, Sungbuk-Gu, Seoul 136-701, Republic of Korea
[b] Natural Products Research Center, Korea Institute of Science and Technology, Gangneung Institute, Gangneung, Gangwon-do 210-340, Republic of Korea
[c] Department of Packaging Science, Clemson University, Clemson, SC 29634-0370, USA

ARTICLE INFO

Article history:
Received 31 December 2007
Received in revised form 19 March 2008
Accepted 1 May 2008

Keywords:
Cheonggukjang
Fibrinolytic activity
Alginate microparticle

ABSTRACT

Alginate microparticles were prepared to evaluate the effect on the fibrinolytic activity of Korean fermented soybean paste, Cheonggukjang (CGJ). The mean diameter of microparticles was 110.37 μm and the microparticles had generally spherical and some wrinkled surfaces. The fibrinolytic activities of encapsulated and non-encapsulated CGJ extract were measured at various ranges of pH and temperature. When non-encapsulated CGJ extract was exposed to simulated gastric juice of pH 2.0, the fibrinolytic activity was rapidly reduced. However, fibrinolytic activity of encapsulated CGJ extract (14.27 units) was significantly higher than that of non-encapsulated CGJ extract (4.15 units). The fibrinolytic activity of non-encapsulated CGJ extract decreased rapidly with increased temperature, but stability of fibrinolytic activity of encapsulated CGJ was improved under high temperature conditions. These results indicate that the microencapsulation technique is an effective tool to protect the fibrinolytic activity of CGJ extract from ingestion and heating effects.

1. Introduction

Fibrin is an insoluble protein component that is formed by the specific cleavage of fibrinogen during recovery from a wound (Voet & Voet, 1990). Normally, the fibrin formed in the blood is dissolved by fibrinolytic enzymes, such as plasmin, which is activated from plasminogen by a tissue-type plasminogen activator, within a few days after injury has healed (Marks, Marks, & Smith, 1996). Redundant accumulation of fibrin in the blood vessels can interfere with blood circulation and lead to myocardial infarction, stroke and other serious cardiovascular diseases (Kim & Choi, 2000; Kim et al., 1996; Mine, Wong, & Jiang, 2005). Synthetic compounds, such as coumarin and warfarin, have been used for treatment of thrombosis (Electricwala, Sawyer, Jones, & Atkinson, 1991). Although the intravenous administration of urokinase obtained from human urine has been widely used for thrombosis therapy, this enzyme has a low specificity to fibrin and is expensive (Kim et al., 1996; Sumi, Sasaki, Toki, & Robbins, 1980).

Fibrinolytic effect can be found in a variety of foods, such as the Japanese Natto (Sumi, Hamada, Tsushima, Mihara, & Muraki, 1987) and Korean Cheonggukjang (Kim et al., 1996). A traditional Japanese fermented food, Natto, has been reported to enhance fibrinolytic activity in plasma and the production of tissue-type plasminogen activator (Sumi, Hamada, Nakanishi, & Hiratani, 1990). The oral administration of fibrinolytic enzyme obtained from Natto has been shown to enhance the solubilisation of fibrin in the blood (Sumi et al., 1990).

Cheonggukjang (CGJ) is a traditional and popular fermented soybean food in Korea and similar to Japanese Natto. A procedure of Cheonggukjang production is shown in Fig. 1. In the past, soaked and boiled soybeans have been spread on rice straw and fermented for 2–3 days. *Bacillus subtilis*, present in the straw, propagates and creates sticky mucilage on the soybean. In the modified CKJ, soybeans are inoculated with *B. subtilis* and fermented. Many researchers have studied the functional components of CGJ (Kwon et al., 2006; Park, Jung, & Kwon, 2003) and their fibrinolytic activity (Kim, Kim, & Oh, 1995; Kim et al., 1996; Ko, Yan, Zhu, & Qi, 2004; Yoo, Seo, Lee, & Kang, 1998). However, a major barrier to the effect of fibrinolytic activity of CGJ is that fibrinolytic enzymes are unstable under the acidic conditions of the stomach and heating conditions during the cooking process (Kim et al., 1996).

To overcome this disadvantage, a microencapsulation technique was applied. Microencapsulation has been successfully used to improve the survival of microorganisms in dairy products, protect sensitive food components, ensure against nutritional loss and incorporate unusual or time-release mechanisms into the formulation (Desai & Park, 2005; Lee, Cha, & Park, 2004). The alginate microparticles have been widely used for the immobilization of

* Corresponding authors. Address: College of Life Sciences and Biotechnology, Korea University, 1,5-Ga, Anam-Dong, Sungbuk-Gu, Seoul 136-701, Republic of Korea. Tel.: +82 2 3290 3450; fax: +82 2 953 5892 (H.J. Park).
E-mail addresses: elfinja@korea.ac.kr (J.A. Ko), hjpark@korea.ac.kr (H.J. Park).

0308-8146/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2008.05.005

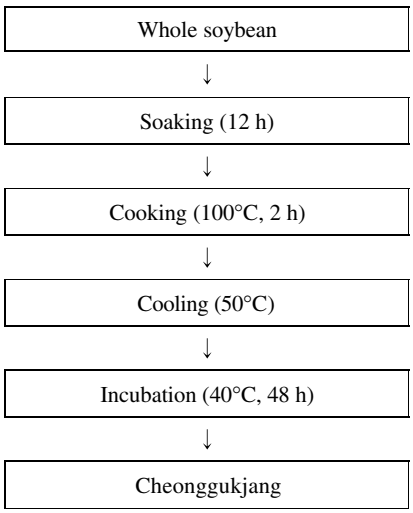

Fig. 1. The procedure of Cheonggukjang production.

target materials owing to their ease of handling, nontoxic nature and low cost (Albarghouthi et al., 2000; Hari, Chandy, & Sharma, 1996; Park & Chang, 2000). In this study, alginate microparticles loaded with CGJ extract were prepared by an air-atomizing device. The air atomization technique is not harmful to live organisms and produces small particles (Kim, Hwang, Park, & Park, 2002; Kwok, Groves, & Burgess, 1991).

The objective of this study was to investigate the effect of alginate microparticles loaded with CGJ on the fibrinolytic activity in simulated gastric juices, as well as their temperature stability (40–90 °C).

2. Materials and methods

2.1. Materials

Fifteen Cheonggukjang samples were purchased in various regions in Korea. Human fibrinogen, thrombin, plasmin, fibrin, Folin–Ciocalteu's phenol reagent, sodium alginate of medium viscosity and phosphate-buffered saline tablets (pH 7.4 at 25 °C) were purchased from Sigma Chemicals (St. Louis, USA). Oxgall was purchased from Difco (Detroit, MI), glycerol was purchased from Junsei (Tokyo, Japan), and xanthan gum (Keltrol F) was obtained from NutraSweet (Chicago, IL).

2.2. Fibrinolytic activity

CGJ (1 g) was mixed with 9 ml of sterilized distilled water for 3 h. After centrifugation (12,000 rpm, 50 min) of the mixture, the supernatant fluid was analysed for its fibrinolytic activity by a modified fibrin solution method of Ehrlich et al. (1987).

The fibrin solution (0.6% (w/v)) was prepared in 1 N NaOH and adjusted to pH 8.0. This solution was dialysed against water at room temperature to remove base, for 24 h. CGJ extract (0.1 ml) was mixed with 0.4 ml of fibrin solution, and then incubated at 40 °C for 20 min. The reaction mixture was added to 0.45 ml of 0.44 M trichloroacetic acid (TCA) and the solution was allowed to stand for 30 min at room temperature. After centrifugation (12,000 rpm, 10 min), 0.1 ml of supernatant was reacted with 1 ml of 0.55 M Na_2CO_3 and 0.2 ml of Folin–Ciocalteu's phenol reagent for 30 min at room temperature. The absorbance was measured at 660 nm. One unit of fibrinolytic activity was defined as the amount of enzyme required to produce 1 μg of tyrosine per min.

2.3. Preparation of alginate microparticles

The preparation of alginate microparticles followed the modified method of Lee et al. (2004). An alginate mixture was prepared in distilled water containing 1% (w/v) sodium alginate, 5% (v/v) glycerol, 0.15% (v/v) xanthan gum, and 0.1% (v/v) Tween 80 and homogenised at 4 °C for 1 day. The extract solution (250 ml) of CGJ was mixed with the alginate mixture solution (500 ml) and sprayed into 0.5 M $CaCl_2$ solution to form beads using an air-atomizing device (Spray Systems Co., Incheon, Korea) while stirring the solution in the stainless steel pan (30 cm × 30 cm × 8 cm) with a magnetic bar. The air-atomizing device was operated at an air pressure and a liquid pressure of 1.0 kg f/cm^2. The microparticles were allowed to stand for about 30 min for gelation to occur and then washed twice with distilled water. The capsules were harvested by filter paper (Cat. No. 1004 110, Whatman, Maidstone, UK) and freeze–dried.

2.4. Physical examination of alginate microparticles

2.4.1. Size analysis

The size of the microparticles was determined by using a particle size analyser (CILAS-1064, France). In order to use uniform size of <200 μm, size segregation was carried out using a 200 μm sieve.

2.4.2. Morphology

The morphology of the microparticles was observed under a scanning electron microscope (SEM). The encapsulated samples were sprinkled onto a double-sided tape, and coated by an ION COATER (IB-5, Eiko Co., Japan) for 6 min. Observations were made using the scanning electron microscope (FE-SEM, S-4700, HITACHI, Japan) at an accelerating voltage of 5.0 kV.

2.5. Effect of pH on the fibrinolytic activity of CGJ extract

The fibrinolytic activity of CGJ extract was studied under different pH conditions, including KCl–HCl buffer (artificial gastric juice, pH 2.0), glycine–HCl buffer (pH 4.0), phosphate-buffered saline tablet (artificial intestinal juice, pH 7.4), and glycine–NaOH (pH 10.0). CGJ extract (0.1 ml) and 1 M buffer (0.1 ml) were mixed with 0.6% fibrin solution (0.3 ml), and then incubated at 40 °C for 20 min. Alginate microparticles (1 g) loaded with CGJ extract were added to 30 ml buffer solution and stirred for 5 h. The mixture was centrifuged at 12,000 rpm for 30 min and the supernatant was obtained. The fibrinolytic activity was measured using the modified fibrin solution method (Ehrlich et al., 1987).

2.6. Effect of temperature on the fibrinolytic activity of CGJ extract

CGJ extract solution or microparticles loaded with CGJ extract were incubated at various temperatures (40, 50, 60, 70, 80 and 90 °C) for 60 min. Distilled water (3 ml) was added to 0.1 g of microparticles and it was centrifuged at 12,000 rpm for 30 min to obtain the supernatant. The supernatant (0.1 ml) was mixed with 0.6% fibrin solution (0.4 ml) and then the fibrinolytic activity was measured by the modified fibrin solution method (Ehrlich et al., 1987). CGJ extract (0.1 ml) was prepared, using the same method as for the non-encapsule sample.

2.7. Statistical analysis

The mean values and the standard deviation were calculated from the data obtained with triplicate trials. The data were analysed by Duncan's multiple comparison method, using the statistical analysis system (SAS Institute Inc., Cary, NC, USA). Significance was determined at $p < 0.05$ level for all analyses.

3. Results and discussion

3.1. Selection of excellent fibrinolytic crude enzyme extract from various CGJs

Fig. 2 shows the relative fibrinolytic activity of crude enzyme solution from various CGJs. The relative activities of CGJ extracts varied between 34% and 92% due to varieties of soybean, fermenting bacterial strains, the ratio of materials and various processes of the region in which it is manufactured (Park et al., 2003). The C2 sample had significantly higher fibrinolytic activity among 15 kinds of CGJ, so it was used in this study.

3.2. Physical examination of alginate microparticles loaded with CGJ

Alginate microparticles loaded with CGJ were prepared by an air atomizer. The particle sizes of those ranged mainly between 90 and 120 μm and the mean diameter was 110.37 μm. Alginate microparticles had generally spherical shape and some wrinkled surface (Fig. 3). Kwok et al. (1991) reported that the wrinkled surface was formed due to the loss of water content during the freeze–drying process.

3.3. Effect of pH on the fibrinolytic activity of non-encapsulated and encapsulated CGJ extracts

Fig. 4 shows the fibrinolytic activity of non-encapsulated and encapsulated CGJ extracts at various pH values. The fibrinolytic activity of non-encapsulated CGJ extract and encapsulated CGJ extract were 19.5 and 18.5 units at pH 7.4, respectively, and 4.15 and 14.3 units at pH 2, respectively. Fibrinolytic activities of both non-encapsulated CGJ extract and encapsulated CGJ extract decreased with decreasing pH. This shows that the fibrinolytic activity of CGJ was sensitive to the acidic condition and that ingestion of Cheonggukjang would result in reduced fibrinolytic activity. It was in agreement with the results of Kim et al. (1996) who reported that *Bacillus* sp. strain CK11-4, which produced a strongly fibrinolytic enzyme, was screened from CGJ and optimum pH range of fibrinolytic enzyme was pH 7–12. Also Yoo et al. (1998) reported that fibrinolytic enzyme isolated from *B. subtilis* K-54 was stable within a pH range 8–12 and Lee et al. (2001) reported that the stable pH range of fibrinolytic enzyme isolated from *Bacillus* sp. KDO-13 was 6–10.

Most proteins cannot be delivered orally because of problems related to degradation in the acidic environment of the gastrointestinal tract (Dai, Wang, & Zhao, 2005). To overcome these disadvantages, the encapsulation method has been used as an alternative technology to the entrapment of cells and enzymes. Proteins encapsulated in the gel matrix can be protected from harsh environmental conditions, such as pH, temperature, organic solvent and poison (Park & Chang, 2000).

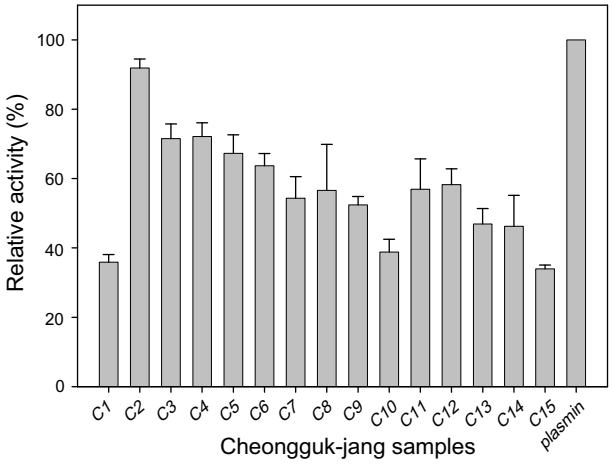

Fig. 2. The relative fibrinolytic activity of crude enzyme solutions from various Cheonggukjang samples. Standard plasmin is equal to 100% relative activity.

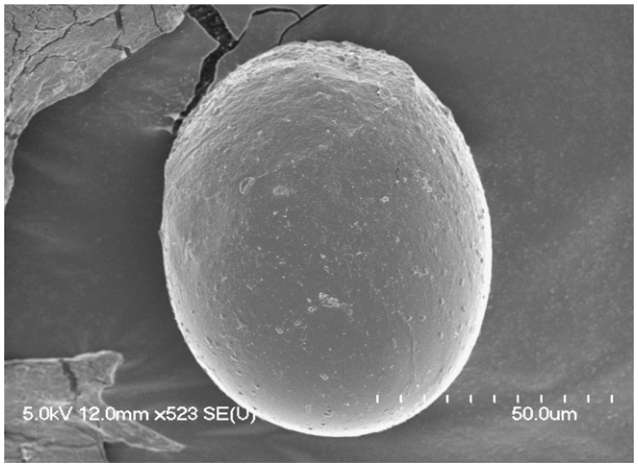

Fig. 3. SEM picture of CGJ loaded alginate microparticle.

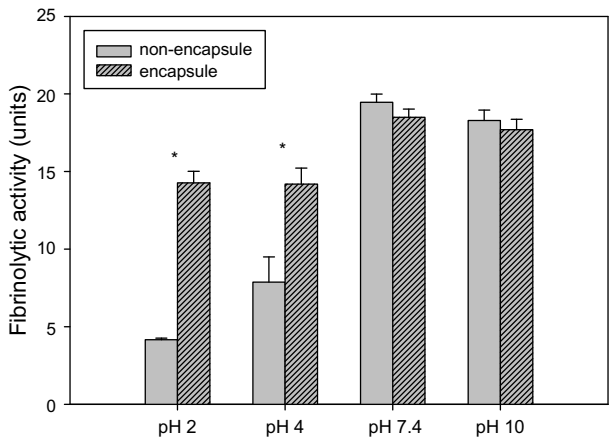

Fig. 4. The effect of pH on the fibrinolytic activity of non-encapsulated CGJ extract and encapsulated CGJ extract. *, significantly different between non-encapsulated and encapsulated at $p < 0.05$ by Duncan's test.

As shown in Fig. 4, there were no significant differences in fibrinolytic activity between non-encapsulated and encapsulated CGJ extracts at pH 7.4 and pH 10. However, the fibrinolytic activity of encapsulated CGJ extract (14.3 units) was significantly higher than that of non-encapsulated CGJ extract (4.16 units) at pH 2 ($p < 0.05$). This result suggested that the microencapsulation could protect fibrinolytic activity of Cheonggukjang extract from the gastric environment.

3.4. Effect of temperature on the fibrinolytic activity of non-encapsulated and encapsulated CGJ extract

The effect of temperature on the fibrinolytic activity of non-encapsulated and encapsulated CGJ extract was evaluated over the range 40–90 °C at pH 7.4 (Fig. 5). Fibrinolytic activities of non-encapsulated and encapsulated CGJ extracts were 21.8 units and 19.5 units at 40 °C, respectively. As the temperature increased, the fibrinolytic activity decreased. The fibrinolytic activity of non-encapsulated CGJ extract decreased rapidly from 21.8 units to 5.98 units with the increased temperature from 40 °C to 90 °C. These results were similar to those of Kim et al. (1996). They

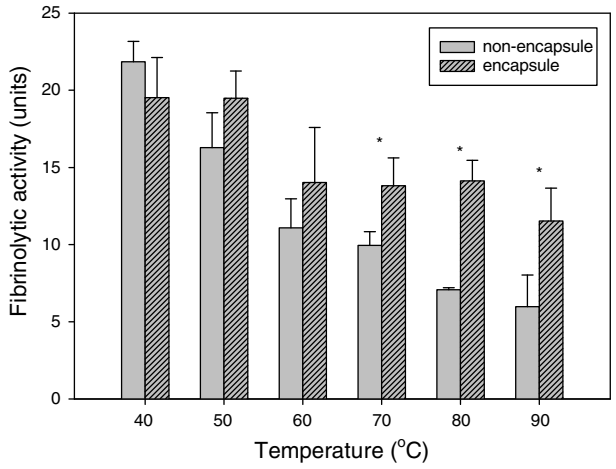

Fig. 5. The effect of temperature on the fibrinolytic activity of non-encapsulated CGJ extract and encapsulated CGJ extract. *, significantly different between non-encapsulated and encapsulated at $p < 0.05$ by Duncan's test.

showed that fibrinolytic activity of CGJ was very stable at 40 °C and decreased with increased temperature. Kim et al. (1997) reported that the optimum temperature of fibrinolytic enzyme from *Bacillus* sp. was 40 °C.

CGJ is usually used after cooking, that is, boiling so the fibrinolytic activity of CGJ would be reduced during the cooking process. Therefore, the microencapsulation technique was applied to improve stability of fibrinolytic activity of CGJ in the high temperature environment. Alginate can form stable gels over a 0–100 °C temperature range (Gombotz & Wee, 1998). Fig. 5 shows that the fibrinolytic activity of encapsulated CGJ extract in alginate microparticles was maintained significantly higher than that of non-encapsulated CGJ at high temperature, although that of CGJ loaded in alginate microparticles decreased due to the rigidity of the alginate gel decreasing with an increase in temperature. This result showed that stability of fibrinolytic activity of CGJ loaded in alginate microparticles was significantly improved over that of non-encapsulated CGJ at high temperature.

4. Conclusion

CGJ is a traditional Korean fermented soybean paste and many researchers have been interested in functions of CGJ, such as fibrinolytic activity. Alginate microparticles were used to protect the fibrinolytic activity of CGJ extract from harsh environmental conditions, such as artificial gastric conditions and the heating process. The fibrinolytic activity of encapsulated CGJ extract was significantly higher than that of non-encapsulated CGJ extract in simulated gastric juice of pH 2.0 or at high temperature. Therefore, microencapsulation of CGJ extract with alginate can offer an effective way of maintaining fibrinolytic activity during oral administration and the cooking process.

Acknowledgements

This study was supported by a Grant of the Korea Health 21 R&D Project, Ministry of Health and Welfare, Republic of Korea (A050376).

References

Albarghouthi, M., Abu Fara, D., Saleem, M., El-Thaher, T., Matalka, K., & Badwan, A. (2000). Immobilization of antibodies on alginate–chitosan beads. *International Journal of Pharmaceutics, 206*, 23–34.

Dai, C., Wang, B., & Zhao, H. (2005). Microencapsulation peptide and protein drugs delivery system. *Colloids and Surfaces B: Biointerfaces, 41*, 117–120.

Desai, K. G. H., & Park, H. J. (2005). Recent developments in microencapsulation of food ingredients. *Drying Technology, 23*, 1361–1394.

Ehrlich, H. J., Bang, N. U., Little, S. P., Jaskunas, S. R., Weigel, B. J., & Mattler, L. E., et al. (1987). Biological properties of a kringless tissue plasminogen activator mutant. *Fibrinolysis, 1*, 75–77.

Electricwala, A., Sawyer, R. T., Jones, C. P., & Atkinson, T. (1991). Isolation of thrombin inhibitor from the leech *Hirudinaria manillensis*. *Blood Coagulation and Fibrinolysis, 2*, 83–91.

Gombotz, W. R., & Wee, S. F. (1998). Protein release from alginate matrices. *Advanced Drug Delivery Reviews, 31*, 267–285.

Hari, P. R., Chandy, T., & Sharma, C. P. (1996). Chitosan/calcium–alginate beads for oral delivery of insulin. *Journal of Applied Polymer Science, 59*, 1795–1801.

Kim, B. K., Hwang, S. J., Park, J. B., & Park, H. J. (2002). Preparation and characterization of drug loaded polymephacrylate microspheres by an emulsion solvent evaporation method. *Journal of Microencapsuation., 19*, 811–822.

Kim, H. K., Kim, G. T., Kim, D. K., Choi, W. A., Park, S. H., & Jeong, Y. K., et al. (1997). Purification and characterization of a novel fibrinolytic enzyme from *Bacillus sp*. KA38 originated from fermented fish. *Journal of Fermentation and Bioengineering, 84*, 307–312.

Kim, S. H., & Choi, N. S. (2000). Purification and characterization of subtilisin DJ-4 secreted by *Bacillus sp*. strain DJ-4 screened from Doen-jang. *Bioscience Biotechnology and Biochemistry, 64*, 1725–1772.

Kim, W. K., Choi, K. H., Kim, Y. T., Park, H. H., Choi, J. Y., Lee, Y. S., Oh, H. I., Kwon, I. B., & Lee, S. Y. (1996). Purification and characterization of a fibrinolytic enzyme produced from *Bacillus sp*. Strain CK 11-4 screened from Chungguk-jang. *Applied and Environmental Microbiology, 62*, 2482–2488.

Kim, Y. T., Kim, W. K., & Oh, H. I. (1995). Screening and identification of the fibrinolytic bacterial strain from Cheonggukjang. *Korean Journal of Applied Microbiology and Biotechnology, 23*(1), 1–5.

Ko, J. H., Yan, J. P., Zhu, L., & Qi, Y. P. (2004). Identification of two novel fibrinolytic enzymes from *Bacillus subtilis* QK02. *Comparative Biochemistry and Physiology Part C, 137*, 65–74.

Kwok, K. K., Groves, M. J., & Burgess, D. J. (1991). Production of 5–15 μm diameter alginate–polylysine microcapsules by an air atomization technique. *Pharmaceutical Research, 8*, 341–344.

Kwon, D. Y., Jang, J. S., Lee, J. E., Kim, Y. S., Shin, D. H., & Park, S. M. (2006). The isoflavonoid aglycone-rich fractions of Chungkookjang, fermented unsalted soybeans, enhance insulin signaling and peroxisome proliferator-activated receptor-γ activity in vitro. *BioFactors, 26*, 245–258.

Lee, J. S., Cha, D. S., & Park, H. J. (2004). Survival of freeze-dried *Lactobacillus bulgaricus* KFRI 673 in chitosan-coated calcium alginate microparticles. *Journal of Agricultural and Food Chemistry, 52*, 7300–7305.

Lee, S. K., Bae, D. H., Kwon, T. J., Lee, S. B., Lee, H. H., & Park, J. H. , et al. (2001). Purification and characterization of a fibrinolytic enzyme form *Bacillus sp*. KDO-13 isolated from soybean paste. *Journal of Microbiology and Biotechnology, 11*, 845–852.

Marks, D., Marks, A., & Smith, C. (1996). *Basic medical biochemistry*. Baltimore, USA: Williams and Wilkins. p. 107.

Mine, Y., Wong, A. H. K., & Jiang, B. (2005). Fibrinolytic enzymes in Asian traditional fermented foods. *Food Research International, 38*, 243–250.

Park, J. K., & Chang, H. N. (2000). Microencapsulation of microbial cells. *Biotechnology Advances, 18*, 303–319.

Park, K. Y., Jung, K. O., & Kwon, E. Y. (2003). Development of a functional Chungkookjang (soybean paste fermented for 2–4 days) with anti-AGS human gastric cancer cell properties. *Nutraceuticals and Food, 8*, 54–60.

Sumi, H., Hamada, H., Nakanishi, H. K., & Hiratani, H. (1990). Enhancement of the fibrinolytic activity in plasma by oral administration of Nattokinase. *Acta Haematologica, 84*, 139–143.

Sumi, H., Hamada, H., Tsushima, K. H., Mihara, H. , & Muraki, H. (1987). A novel fibrinolytic enzyme (Nattokinase) in the vegetable cheese Natto: A typical and popular soybean food in the Japanese diet. *Experientia, 43*, 1110–1111.

Sumi, H., Sasaki, K., Toki, N., & Robbins, K. C. (1980). Oral administration of urokinase. *Thrombosis Research, 20*, 711–714.

Voet, D., & Voet, J. G. (1990). *Biochemistry*. New York: John Wiley and Sons. pp. 1087–1095.

Yoo, C. K., Seo, W. S., Lee, C. S., & Kang, S. M. (1998). Purification and characterization of fibrinolytic enzyme excreted by *Bacillus subtilis* K-54 isolated from Chung Guk Jang. *Korean Journal of Applied Microbiology and Biotechnology, 26*, 507–514.