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Note

Fermented Soybean with Thrombosis Preventing Activity Using Mushroom Mycelia as Microbial Source

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Bacillus natto **is the main microorganism used to make natto (fermented soybeans), because this microbe has good ability to produce protease. However, it is known that some genera of mushroom also produce protease, and in this study we made a fermented soybean using a mushroom mycelia in place of *B. natto*. We found that the fermented soybean made using mycelia of *Flammulina velutipes* and *Roseofomes subflexibilis* showed a thrombosis preventing activity: a prolonged thrombin clotting time 8.2 fold that of control.**

Keywords: fermented soybean, mushroom mycelia, anti-thrombin substance, thrombosis

Natto (fermented soybeans) is a traditional fermented food in Japan and *Bacillus natto* is used to make natto because it has strong ability to produce protease (Kamata *et al*., 1989). Other organisms also produce protease including the mushrooms *Flammulina velutipes* (Arima *et al*., 2000), *Grifola frondosa* (Okamura *et al*., 1997a) and *Pleurotus eryngii* (Arima *et al*., 2000), which are popular in Japan. These are also rich in fiber such as glucan, protein and a precursor of vitamin D, in addition to having a preventive effect on thrombosis and cancer (Whistler *et al*., 1976; Hirasawa *et al*., 1997; Okamura *et al*., 1997b; Okamura *et al*., 2000). In this study we produced a fermented soybean with mushroom mycelia because we hypothesized it might have a preventive effect against thrombosis and cancer, as well as other health benefits. In this study, we report on the production of this fermented soybean produced with mushroom mycelia and its characteristics.

Materials and Methods

Cultivation of mushrooms *Agaricus blazei* MWU-C20, *Flammulina velutipes* MWU-C3, *Grifola frondosa* MWU-C9, *Laetiporus sulphureus* MWU-W8, *Pleurotus cornucopiae* var. *citrinopileatus* MWU-C8, *Pleurotus eryngii* MWU-C21 and *Roseofomes subflexibilis* MWU-W117 were used in this experiment because these mushrooms grow well on soybean and have anti-thrombin and fibrinolytic activities. *A. blazei* (dried type), *F. velutipes*, *G. frondosa*, *P. cornucopiae* var. *citrinopileatus* and *P. eryngii* were purchased at a local market in Nishinomiya Japan. *L. sulphureus* and *R. subflexibilis* were isolated from wild mushrooms in Ashibidani, Shiga and Sendai, Miyagi prefecture, Japan, and cultures were obtained by aseptic inoculation of the tissue from the fruit bodies onto a medium containing 2% malt extract (pH 5.6). A small quantity of mushroom cultures grown on an incline were inoculated into 200 ml of the medium in a 500 ml Erlenmeyer flask. Cultivation was carried out at 25°C for 2 weeks under aerobic conditions with a rotary shaker (100 r.p.m.). The weights (fresh weight (dry weight)/200 ml medium) and pH after cultivation of *A. blazei, F. velutipes, G. frondosa, L. sulphureus, P. cornucopiae* var. *citrinopileatus, P. eryngii* and *R. subflexibilis* were 2.2 g (0.15 g), 1.1 g (0.02 g), 1.2 g (0.03 g), 1.8 g (0.03 g), 1.3 g (0.08 g), 1.1 g (0.09 g) and 2.0 g (0.10 g), and 5.3, 5.2, 3.3, 1.7, 5.2, 6.1 and 4.8, respectively.

Mycelia prepared from each mushroom fruit body were collected by centrifugation at 10,000×*g* for 10 min and washed twice with autoclaved ice-cold saline solution.

Assay for proteolytic activity The fresh mushroom mycelia (fresh weight 0.5 g), suspended in 0.5 ml of 10 mM Tris-HCl buffer (pH 8.5) or 0.5 ml of 10 mM McIlvaine buffer (pH 5.0) was subjected to sonication with an ultrasonic oscillator (BRANSON, 20 kHz) for 16 min at below 8°C. The undestroyed cells and debris were removed by centrifugation at 10,000×*g* for 20 min. The supernatant solution obtained was used as the cell-free extract.

The alkaline proteolytic activity (Tris-HCl buffer (pH 8.5)) was assayed using the method described by Arima *et al*. (1967). The substrate employed for the assay of proteolytic activity was 1.2% solution of Hammersten casein (Merck, Germany). The acid proteolytic activity (McIlvaine buffer (pH 5.0)) was assayed using the Anson-hemoglobin method described by Anson (1939). One unit of the enzyme was defined as the amount that catalyzed the formation of 1 μmol of L-tyrosine per min in the measurement by the method of Lowry *et al*. (1951) with crystalline bovine serum albumin (Wako Co. Ltd., Osaka) as the standard (Fig. 1).

Fermented soybean making Fermented soybean was made by following the conventional methods except that mushroom mycelia were used in place of *Bacillus natto*. Briefly, 20 g (about 2 g/soybean) of soybean (Tsurunoko soybean, Hokkaido) was washed thoroughly with water and placed in a flat-bottomed tube. The beans were soaked in water overnight at room temperature, the water was drained off, and the flat-bottomed tube was

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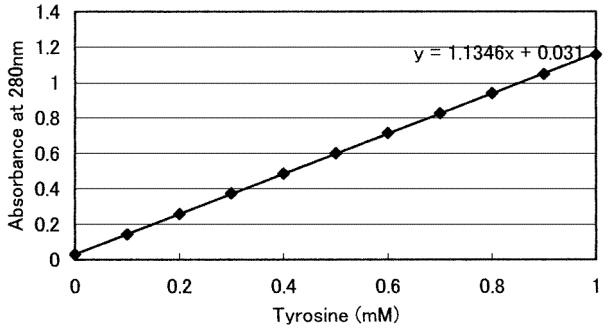

Fig. 1. Standard curve of tyrosine.

sealed with aluminum foil. After autoclaving for 30 min at a pressure of 1 kg/cm^2, the soybean was cooled and inoculated with fresh mycelia (1 g), obtained by the method described in the cultivation of mushrooms, and incubated at 25°C±1°C for 12 days. The same soybean without inoculation was prepared as a control (soybean).

Coagulability test The coagulability test was done using the thrombin time (TT): the time elapsing until the fibrin formation of thrombin, by the method described by Kinoshita and Horie (1993). Briefly, after fermentation, the fermented soybean (1 g), suspended in water (4 ml), was subjected to sonication with an ultrasonic oscillator (BRANSON, 20 kHz) for 3 min at below 8°C. The undestroyed fermented soybean and debris were discarded by centrifugation at 10,000×*g* for 10 min, and the supernatant was applied for determining thrombin activity. Bovine α-thrombin was purchased from Mochida Phamaceutical Co., Ltd.. The thrombin clotting time in a reaction mixture (37°C) containing 50 μl of the supernatant, 50 μl of 12.5 NIH unit/ml thrombin and 200 μl of 0.33% bovine fibrinogen was measured using a KC1A coagulometer (Henrich Amelung).

Fibrinolytic activity test The method of Astrup and Mullertz (1952) using fibrin plates was employed to determine fibrinolytic activities. Briefly, 30 μl of the supernatant, obtained by the method described in the coagulability test was used, an artificial thrombus was prepared in a disk by coagulating 0.4% bovine fibrinogen using thrombin, and the potency required to dissolve the thrombus was determined.

Results and Discussion

As shown in Table 1, the proteolytic activities on pH 8.5 and pH 5.0 of *A. blazei, F. velutipes, G. frondosa, L. sulphureus, P. cornucopiae* var. *citrinopileatus, P. eryngii, R. subflexibilis* and *B. natto* were 3.8, 0.01, 2.3, 6.8, 10.8, 0.06, 0.1 and 5.6 units (μmol/mg/min) and 3.5, 0.1, 1.8, 0.7, 1.5, 2.5, 0.4 and 0.5 units (μmol/mg/min), respectively.

The fermented soybeans produced using mushrooms are shown in Fig. 2; their hardness and flavor was soft and the original flavor, respectively.

The effects of fermented soybean produced using mushroom mycelia on thrombin time (TT) are summarized in Table 1. All six soybeans produced using mycelia of *F. velutipes*, *G. frondosa*, *L. sulphureus*, *P. cornucopiae* var. *citrinopileatus*, *P. eryngii* and *R. subflexibilis* showed anti-coagulative activities on TT, with the TT of fermented soybeans produced by mycelia of *F. velutipes* and *R. subflexibilis* being longer than that of the other mushrooms, and the thrombin clotting times more than 8.2 times longer than that of the control (soybean).

Figure 3 shows the fibrinolytic activities of fermented soybean produced using mushroom mycelia on a fibrin plate. Table 1 shows the fibrinolytic activities of the extract solution of the soybean produced using mushroom: the fermented soybean produced using *R. subflexibilis* and *P. cornucopiae* var. *citrinopileatus* showed high fibrinolytic activities on the fibrin plate, while the control did not.

Based on these findings we theorized that fermented soybean produced using mycelia of these mushrooms may have a preventive effect on thrombosis (Okamura *et al*., 2000), and that it may also contain β-D-glucan, because mycelia of mushrooms contain β-D-glucan and this is known to have a preventive effect against cancer (Whistler *et al*., 1976).

In general, *B. natto* is used to make natto (fermented soybeans), because this microbe has a potent ability to produce protease. On the other hand, the activities on pH 8.5 and pH 5.0 of *A. blazei*, *F. velutipes*, *G. frondosa*, *L. sulphureus*, *P. cornucopiae* var. *citrinopileatus*, *P. eryngii* and *R. subflexibilis* are almost 0.01 to 11 units (μmol/mg/min) and 0.1 to 3.5 units (μmol/mg/min), respectively. The activities on pH 8.5 and pH 5.0 of *B. natto* are 5.6 units (μmol/mg/min) and 0.5 units (μmol/mg/min), respectively. The proteolytic activities of *P. cornucopiae* var. *citrinopileatus* and *L. sulphureus* were higher than that of *B. natto* on pH 8.5. Further, those of most mushrooms were higher than that of *B. natto* on pH 5.0.

Terashita *et al*. (1984) reported that the carboxy proteinase from *Lentinus edodes* fruit-bodies was most active between pH

Table 1. Proteolytic activities of mushroom mycelia, and thrombin time, fibrinolytic activities and final pH of the fermented soybeans.

Mushroom mycelia used	Proteolytic activity (unit[a])		Thrombin time (s)	Fibrinolytic activity (mm^2)	Final pH
	pH 5.0	pH 8.5			
Agaricus blazei	3.5	3.8	73.6±0.3	35±1	8.5
Flammulina velutipes	0.1	0.01	600[c]	132±2	8.8
Grifola frondosa	1.8	2.3	100.1±0.4	42±1	6.1
Laetiporus sulphureus	0.7	6.8	173.7±0.5	36±1	8.0
Pleurotus cornucopiae var. *citrinopileatus*	1.5	10.8	160.6±0.4	288±3	7.8
Pleurotus eryngii	2.5	0.06	179.6±0.5	63±1	5.9
Roseofomes subflexibilis	0.4	0.1	600[c]	500±3	8.4
Bacillus natto	0.5	5.6	—	—	8.3
Control[b]	0	0	73.6±0.4	16±1	6.4

[a] μmol/mg (protein)/min
[b] soybean
[c] more than 600 s

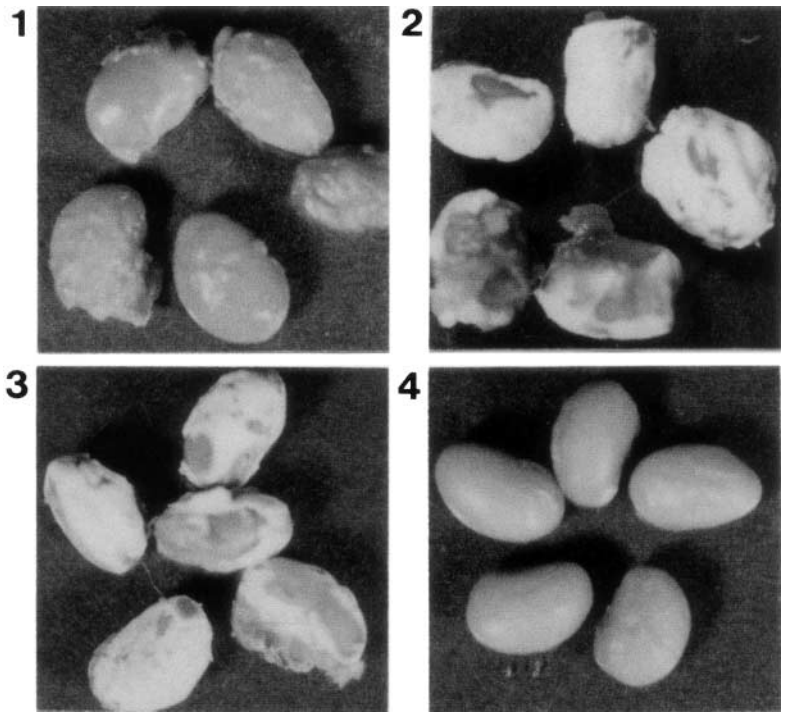

Fig. 2. Fermented soybean produced using mushrooms mycelia.
1, *A. blazei*; 2, *F. velutipes*; 3, *R. subflexibilis*; 4, Control (soybean).

2.5–2.8, and stable over a range of pH 3.1–5.7. However, final pH of the fermented soybean was not low value such as 2.5–2.8. Also, Terashita *et al*. (1981) reported that the optimal pH of intracellular proteinase in the mycelium was pH 2.7 and 7.0, and when *Streptomyces*-pepsin inhibitor (S-PI) was added to the culture medium, the activities were strikingly changed; the carboxyl proteinase activity was remarkably decreased, while the metal proteinase activity was increased to 1.5 times that of the control (Terashita *et al*., 1981).

Therefore, the proteinase activity may be different from fruit body and mycelia, and may change depending on the cultural conditions. In this experiment, the mycelia of mushrooms were grown on the soybeans resulting in fermenting them.

Table 1 shows the final pH of the fermented soybeans. That of fermented soybeans produced by *G. frondosa* and *P. eryngii* was 6.1 and 5.9, respectively, while that of the soybeans produced by the other mushrooms and *B. natto* was pH 7.8 to pH 8.8. Different fermentation by protease and the other enzymes between each mushroom and *B. natto* may produce various amino acids and organic acids, thus leading to different pH of the final fermented soybean product.

Kim J.H. and Kim Y.S. (1999) reported that a fibrinolytic metalloprotease has been purified from the fruiting bodies of the edible honey mushroom (*Armillariella mellea*), and hydrolyzes fibrinogen as well as fibrin, but shows no proteolytic activity for other blood proteins such as thrombin, human albumin, bovine albumin, human IgG, hemoglobin, or urokinase. They also reported that the optimum pH of the fibrinolytic activity of metalloprotease from *Armillariella mellea* was pH 7 when N-Suc-Ala-Ala-Pro-Phe pNA was used as a substrate, indicating that it is a neutral protease. It may be a fibrinolytic metalloprotease the same as *Armillariella mellea*, and therefore the fermented soybean showed fibrinolytic activities.

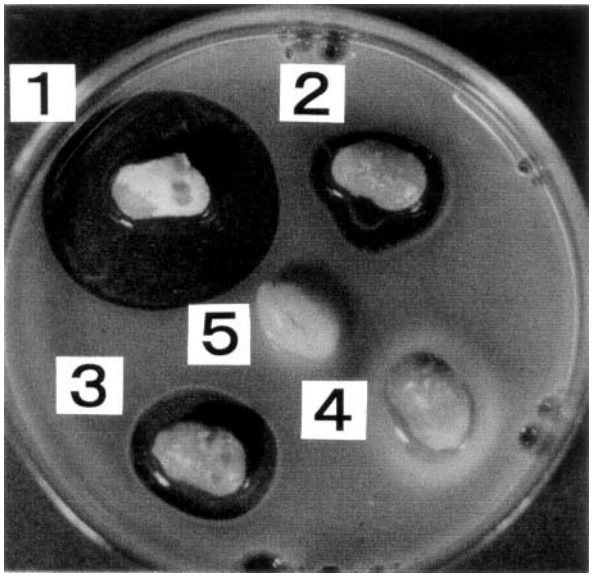

Fig. 3. Fibrinolytic activity of fermented soybean produced using mushroom mycelia. 1, *R. subflexibilis*; 2, *F. velutipes*; 3, *P. cornucopiae* var. *citrinopileatus*; 4, *G. frondosa*; 5, Control (soybean). A dissolved zone is shown on a fibrin plate.

Sumi *et al*. (1987) reported the presence of a strong fibrinolytic enzyme (nattokinase) in natto, and nattokinase may be an equally good protease for oral fibrinolytic therapy because of its confirmed safety for long-term intake, stability and the strong fibrinolytic activity. The fermented soybeans produced using mushroom mycelia, however, may have a preventive effect on not only thrombosis but also cancer.

Therefore, the utilization of different fermentative microorganisms should ensure the development of new fermented foods with attractive functional and beneficial properties.

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