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Characteristics of a Cheese-Like Food Produced by Fermentation of the Mushroom *Schizophyllum commune*

TOKUMITSU OKAMURA-MATSUI,[1]* KAORI TAKEMURA,[1] MITSUE SERA,[1] TOMOMI TAKENO,[1] HIROKO NODA,[1] SHOKO FUKUDA,[1] AND MASAHIRO OHSUGI[1]

Department of Food Science and Nutrition, School of Human Environmental Sciences, Mukogawa Women's University, 6-46 Ikebiraki-cho, Nishinomiya, Hyogo, 663-8137, Japan[1]

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Lactate bacteria of the *Lactobacillus* and *Streptococcus* genera are normally employed in cheese making because these microbes have potent ability to produce lactate dehydrogenase. A milk-clotting enzyme is also necessary to make cheese. Recently, we discovered that some mushroom genera produce both lactate dehydrogenase and a milk-clotting enzyme. Using the mushroom *Schizophyllum commune* in place of a lactate bacterium, we produced a cheese-like food that contained about 0.58% β-D-glucan, which has been shown to have preventive effects against cancer. The food also exhibited thrombosis prevention activity, prolonging the thrombin clotting time to 49.6-fold that of the control.

[**Key words:** cheese, mushroom, fermentation, β-D-glucan, lactate dehydrogenase, anti-thrombin substance, *Schizophyllum commune*]

Cheese is a popular lactate-fermented food consumed by many people around the world. Lactic acid bacteria particularly *Lactobacillus* and *Streptococcus* genera, have long been used in making cheese since they are potent lactate dehydrogenase producers. A milk-clotting enzyme is also necessary to make cheese. Rennet preparations from the stomachs of young ruminants are the traditional coagulants used (1). Recently, we discovered that some mushrooms, including *Schizophyllum commune* (2), possess lactate dehydrogenase and a milk-clotting enzyme. *S. commune* is also rich in fiber, protein, and vitamins such as thiamin and riboflavin, in addition to having possible preventive effects against cancer and thrombosis (3, 4). We therefore produced a cheese-like food (hereafter referred to as 'cheese') with *S. commune* in anticipation that its consumption could inhibit cancer and thrombosis, as well as having other health benefits. Here, we report on this cheese, and its characteristics.

MATERIALS AND METHODS

Cultivation of *Schizophyllum commune* and preparation of cell-free extract *S. commune* (Fig. 1) was cultivated in a medium contained 2% malt extract (pH 5.6). Mushroom cultures grown on an incline were inoculated into 200 ml of the medium in a 500-ml Erlenmyer flasks. Cultivation was carried out at 25°C for 2 weeks under aerobic conditions on a rotary shaker (100 rpm). Mycelia were collected by centrifugation at 10,000 × *g* for 10 min and washed twice with an ice-cold saline solution. The mycelium pellet, suspended in 10 mM Tris-HCl buffer (pH 7.5), was sonicated with an ultrasonic oscillator (Branson, 20 kHz) for 16 min at below 8°C. The undestroyed mycelia and debris were discarded by centrifugation at 10,000 × *g* for 10 min. The supernatant solution obtained was used as the cell-free extract.

Assay of lactate dehydrogenase The standard reaction mixture contained 200 μmol sodium lactate, 1 μmol NAD^+, 200 μmol Tris-HCl buffer (pH 7.5), and cell-free extract in a final volume of 1.0 ml. The substrate was replaced by water in a blank mixture. The mixture was incubated at 30°C in a cuvette with a 1-cm light path. The reaction was started by the addition of NAD^+ and monitored by measuring the initial change in the absorbance at 340 nm with a Hitachi 150-20 double beam spectrophotometer equipped with a thermostatically controlled cuvette holder and continuous chart recorder. One unit of enzyme was defined as the amount that catalyzed the formation of 1 μmol of NADH per min during the reaction. The specific activity is expressed as units per mg protein. Protein was measured by the method of Lowry *et al.* (5) with crystalline bovine serum albumin as the standard.

Electrophoresis of LDH Gel electrophoresis of the native enzyme was carried out on a 7.5% polyacrylamide gel using the method of Davis (6). Lactate dehydrogenase (LDH) activity staining was done in a solution containing 50 mM Tris-HCl buffer (pH 7.5), 1.25 mM NAD^+, 10 mM sodium lactate, 0.4 mM phenazine methosulfate, and 0.5 mM nitro blue tetrazolium.

Purified LDH was obtained by gel filtration on a TSK gel G3000SW column with an HPLC system and extraction from the

FIG. 1. Fruiting bodies of *S. commune* used in the experiment.

* Corresponding author. e-mail: tokamura@mwu.mukogawa-u.ac.jp phone: +81-(0)798-45-9873 fax: +81-(0)798-45-9873

active staining gel after PAGE.

Milk-clotting enzyme assay Rennin (Meito rennet) was obtained from Meito Sangyo Co. Ltd. The milk-clotting enzyme was assayed using the method described by Arima *et al.* (7). A 10% solution of skim-milk powder fortified with 0.01 M $CaCl_2$ was used as the substrate. For the assay of enzyme activity, 5 ml substrate solution was added to 0.5 ml suitably diluted enzyme solution at 35°C. The time required for curd particles to form was measured with a stop watch. Under the above assay conditions, the amount of enzyme that clotted the milk solution in 1 min was defined as containing 400 units of activity.

The substrate employed to assay the proteolytic activity was a 1.2% solution of Hammersten casein (pH 6.0 with 0.05 N phosphate buffer). The substrate solution (5 ml) was incubated with 1 ml enzyme solution at 35°C for 10 min and the enzyme reaction was stopped with 5 ml of 0.44 M trichloroacetic acid. After 20-min incubation, the reaction mixture was centrifuged at $10,000 \times g$ for 10 min (4°C), and 2 ml of the supernatant was added to 5 ml of 0.55 M Na_2CO_3 and 1 ml Folin's reagent (×3). This mixture was held at 35°C for 20 min and the optical density at 660 nm was then measured.

Cheese making Cheese was made by the conventional method except that *S. commune* was used in place of a lactate bacterium and rennin. Two grams of *S. commune* mycelia cultivated as described above were added to 900 ml sterilized milk in an Erlenmyer flask. After incubation at 25°C ± 1°C for 6 d, the supernatant was discarded by decantation. The cake was collected, and fermented at 25°C ± 1°C for 2 months. The same milk without inoculation and fresh cheese made using *Lactobacillus bulgaricus* and rennin were used as control 1 (milk) and control 2, respectively.

Measurement of β-D-glucan β-D-Glucan (β-D-1,3-glucan) was estimated by HPLC on a TSK-gel G5000PW column (0.75 × 30 cm) at a flow rate of 1 ml/min using water with RI. Fifty microliters of the supernatant obtained for the coagulability test as described below was used to determine the β-D-glucan content.

Coagulability test The coagulability was tested by measuring the thrombin time (TT), *i.e.* the time elapsing until the fibrin formation of thrombin, using the method described by Kinoshita and Horie (8). After fermentation, 1 g cheese suspended in 2 ml of 10 mM Tris-HCl buffer was sonicated with an ultrasonic oscillator (Branson, 20 kHz) for 3 min at below 8°C. Undestroyed cheese and debris were discarded by centrifugation at $10,000 \times g$ for 10 min and the supernatant was employed to determine the 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 units/ml thrombin, and 200 μl of 0.33% bovine fibrinogen was measured using a KC1A coagulometer (Henrich Amelung).

Fibrinolytic activity test The fibrinolytic activity was determined by the method of Astrup and Mulertz (9) using fibrin plates. Thirty microliters of the supernatant obtained as described above 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

Lactate dehydrogenase and milk-clotting enzyme of *S. commune* Potent LDH activity (4.6 units/ml) in cell-free extracts of *S. commune*. Polyacrylamide gel electrophoresis (PAGE) of the native LDH showed a band on activity staining. Electrophoresis of purified LDH by gel filtration on a TSK gel G3000SW column with an HPLC system and extraction from the active staining gel after PAGE also gave a single band (Fig. 2).

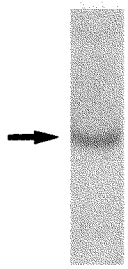

FIG. 2. Active staining after polyacrylamide gel electrophoresis of *S. commune* lactate dehydrogenase (LDH). The purified enzyme was electrophoresed at a current of 2.5 mA using the method of Davis (6). The arrow indicates the position of alcohol dehydrogenase.

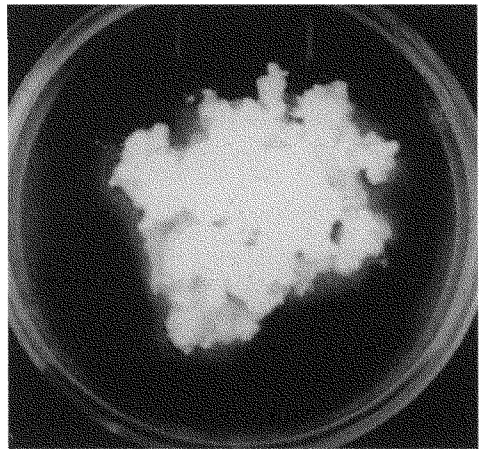

FIG. 3. Cheese made using *S. commune*.

As shown in Table 1, *S. commune* has clotting activity, though the ratio of the clotting to proteolytic activity is lower than that of rennin.

Cheese making and functional appreciation *S. commune*, which was shown to possess both LDH and milk-clotting activities, was used for cheese making. Figure 3 shows the cheese made, which had a flavor similar to that of the *S. commune* fruiting body.

As shown in Fig. 4, cheese produced using *S. commune* contained about 0.58% (weight of β-D-glucan/weight of cheese fermented for 2 months) β-D-glucan, which has been reported to have preventative activity against cancer (for example, in Sarcoma 180/ICR mice.) (3). Considering these findings, consuming cheese produced using *S. commune* could be effective in the prevention of cancer.

The effect of the cheese produced using *S. commune* on the thrombin time (TT) is shown in Table 2. The cheese exhibited anti-coagulative activity, with a thrombin clotting time 49.6 times longer than that of the controls (milk and normal fresh cheese).

Figure 5 shows the fibrinolytic activity of the cheese on

TABLE 1. Ratio of clotting activity to proteolytic activity

	Clotting activity[a] (units/ml)	Proteolytic activity[b] (OD 660 nm)	Ratio (a/b)
Rennin	404	0.04	10100
S. commune	17.4	0.10	174

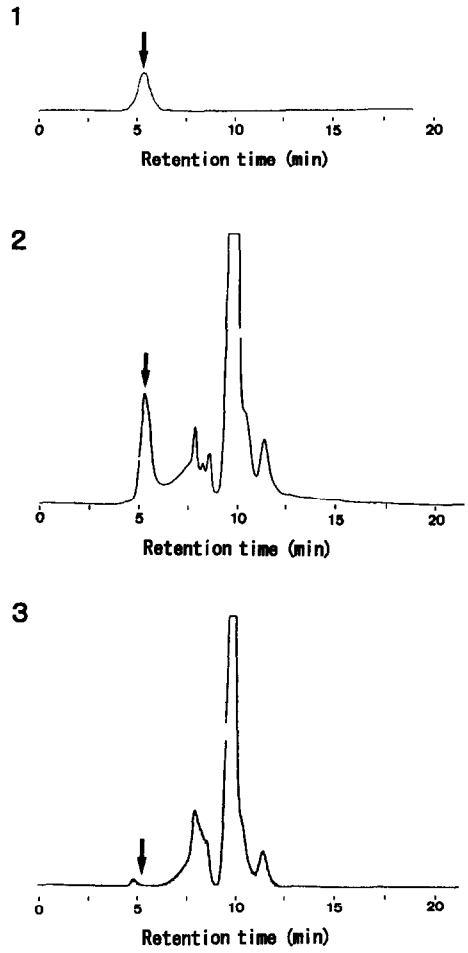

FIG. 4. β-D-Glucan of cheese produced using *S. commune*. (1) 0.25% authentic β-D-glucan (M.W. 2,000,000); (2) β-D-glucan of cheese produced using *S. commune*; (3) after β-D-1,3-glucanase treatment. The arrows indicate the position of β-D-glucan.

a fibrin plate, which the measured fibrinolytic activity of the extract solution is given in Table 3. The cheese clearly showed fibrinolytic activity on the fibrin plate, whereas the control did not. Thus, the cheese produced with *S. commune* may have a preventive effect against thrombosis (10).

In general, the conversion of carbohydrate into lactate requires the action of lactate dehydrogenase produced by a lactate bacterium during cheese production, and for this reason lactate bacteria we normally employed to make cheese. We were able to produce cheese using only *S. commune* mushroom because it has both lactate dehydrogenase and milk-clotting enzyme activities. Utilization of different fermentative microorganisms should permit the development of new fermented foods that possess attractive functional properties.

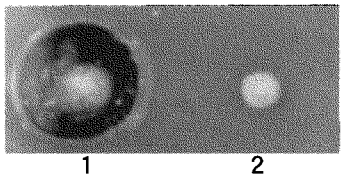

FIG. 5. Fibrinolytic activity of cheese produced using *S. commune*. 1, *S. commune*; 2, control (milk). The dissolved zone on the fibrin plate is shown.

TABLE 2. Effect of cheese produced using *S. commune* on thrombin time

	Thrombin time (s)
Cheese made with *S. commune*	600[c]
Control 1[a]	12±0.5
Control 2[b]	12±0.5

[a] Milk.
[b] Fresh cheese made with *L. bulgaricus*.
[c] More than 600 s.

TABLE 3. Fibrinolytic activity of cheese produced using *S. commune*

	Fibrinolytic activity (mm^2)
Cheese made with *S. commune*	356±0.5
Control 1[a]	0
Control 2[b]	0

[a] Milk.
[b] Fresh cheese made with *L. bulgaricus*.

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