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Characteristics of Beer-Like Drink Produced by Mushroom Fermentation

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In general, S*accharomyces cerevisiae* is a main microorganism in beer brewing, because this microbe has potent ability to produce alcohol dehydrogenase. Recently, we discovered that some genera of mushroom produce alcohol dehydrogenase, and made a beer-like drink using a mushroom in place of *S. cerevisiae*. The highest alcohol concentration in this drink was achieved with *Tricholoma matsutake* (1069 mM, 4.6%). This beer-like drink contained about 0.17% ß-D-glucan, which is known to have preventive effects against cancer. The drink showed thrombosis preventing activity: prolonged thrombin clotting time 2.3 fold that of control. Thus, the beer-like drink made using mushroom seems to be a healthful alcoholic beverage.

Keywords: beer, mushroom, fermentation, alcohol dehydrogenase, β-D-glucan, anti-thrombin substance

Beer is a popular alcoholic beverage, and it is consumed by many people around the world. In general, *Saccharomyces cerevisiae* has long been used for producing beer since it has potent alcohol dehydrogenase (Ayres *et al*., 1980). Recently, we discovered that some mushrooms also possess alcohol dehydrogenase. Mushrooms such as *Flammulina velutipes* and *Tricholoma matsutake* are eaten by many people in Japan, and they are rich in fiber, protein and vitamins, in addition to having a preventive effect on cancer and thrombosis (Whistler *et al*., 1976; Hirasawa *et al*., 1997). Therefore, we produced a beer-like drink (hereafter referred to as beer) with mushrooms because it was expected that drinking this beer could have a preventive effect against cancer and thrombosis, as well as other health benefits. In this study, we report on this beer produced with mushrooms and its characteristics.

Materials and Methods

Cultivation of mushrooms and preparation of cell-free extract *Flammulina velutipes* and *Tricholoma matsutake* were used in this experiment (Fig. 1). The mushrooms were cultured 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 flask. Cultivation was carried out at 25°C for 2 weeks under aerobic conditions with a rotary shaker. Mycelia were collected by centrifugation at 10,000×*g* for 10 mins and washed twice with ice-cold saline solution. The mycelium pellet, suspended in 10 mM Tris-HCl buffer (pH 7.5), was subjected to sonication 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 mins. The supernatant solution obtained was used as the cell-free extract.

Enzyme assay The standard reaction mixture contained 200 µmol of ethyl alcohol, 1 µmol of NAD^+, 200 µmol of 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. Incubation was done 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 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. Specific activity was expressed as units per mg protein. Protein was measured by the method of Lowry *et al*. (1951) with crystalline bovine serum albumin as the standard.

Electrophoresis Gel electrophoresis of the native enzymes was done on a 7.5% polyacrylamide gel using the method of Davis (1964). The alcohol dehydrogenase (ADH) activity stain was performed in a solution containing 50 mM Tris-HCl buffer (pH 7.5), 1.25 mM NAD^+, 10 mM ethyl alcohol, 0.4 mM phenazine methosulfate, and 0.5 mM nitro blue tetrazolium.

Measurement of molecular mass The molecular mass was estimated by gel filtration on a TSK gel G3000SW column (0.75 ×30 cm) at a flow rate of 700 µl/min with 0.01% ß-mercaptoethanol, and 10% glycerol. A calibration curve was made with the following proteins; glutamate dehydrogenase (290 kDa), lactate dehydrogenase (142 kDa), enolase (67 kDa), adenylate kinase (32 kDa) and cytochrome *c* (12.4 kDa).

Beer brewing Beer brewing was performed by following the conventional methods except that mushrooms were used in place of *S. cerevisiae:* two grams mycelia of mushroom were added to autoclaved malt-hop extract medium (pH 5.8) containing 10% malt extract and 0.1% hop extract in an Erlenmyer flask, and incubated at 20±1°C for 14 days. The same malt-hop extract medium without inoculation was prepared as a control (malt-hop extract).

Measurement of ethyl alcohol concentration The alcohol (ethyl alcohol) concentration was estimated by HPLC on a TSK-gel Oapak-A column (0.78×30 cm) at a flow rate of 1 ml/min

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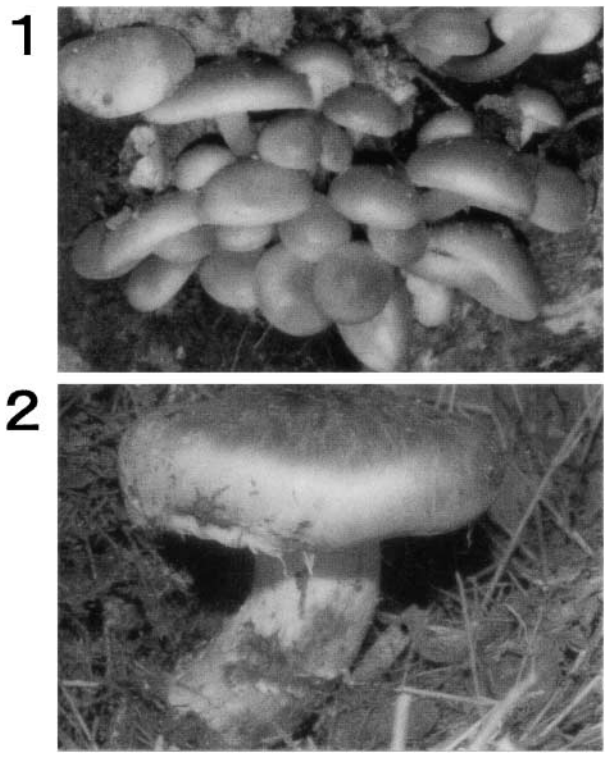

Fig, 1. Fruit-bodies of mushrooms used in this experiment. 1, *Flammulina velutipes*; 2, *Tricholoma matsutake*.

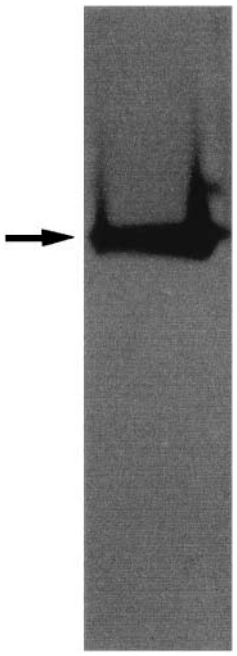

Fig. 2. Active staining after polyacrylamide gel electrophoresis of alcohol dehydrogenase (ADH) of *T. matsutake*. The purified enzyme was electrophoresed at a current of 2.5 mA using the method of Davis (1964). The arrow indicates the position of alcohol dehydrogenase.

using water with RI.

Measurement of ß-D-glucan The ß-D-glucan (ß-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.

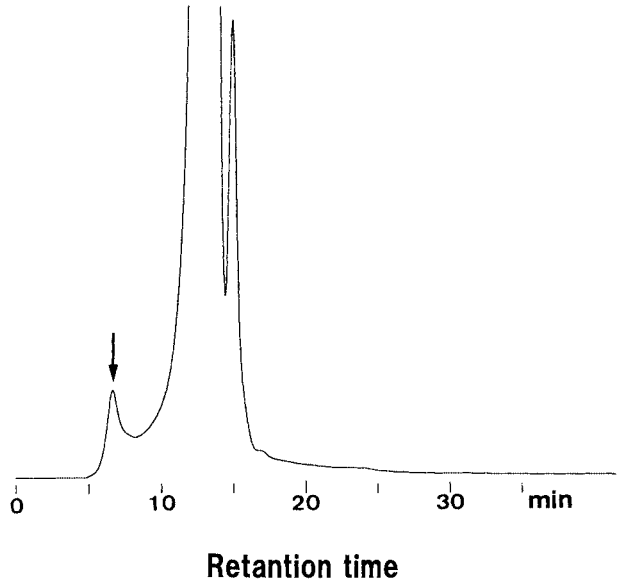

Fig.3. β-D-glucan of beer produced using *T. matsutake*. The arrow indicates the position of β-D-glucan.

Table 1. Effects of beer produced by mushroom on thrombin time.

Mushroom Used	Thrombin time (s)
Flammulina velutipes	303.4±0.5
Tricholoma matsutake	600[b)]
Control[a)]	268.5±0.4

[a)]malt-hop extract medium, [b)]more than 600 s.

Table 2. Fibrinolytic activities of the beer produced by mushroom.

Mushroom Used	Fibrinolytic activity (mm^2)
Flammulina velutipes	0
Tricholoma matsutake	36±0.5
Control[a)]	0

[a)]malt-hop extract medium.

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). After fermentation, the beer was centrifuged at 10,000×*g* for 10 mins, 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 by a KC1A coagulometer (Henrich Amelung).

Fibrinolytic activity test In order to determine the fibrinolytic activities, the method of Astrup and Mulertz (1952) using fibrin plates was employed. 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.

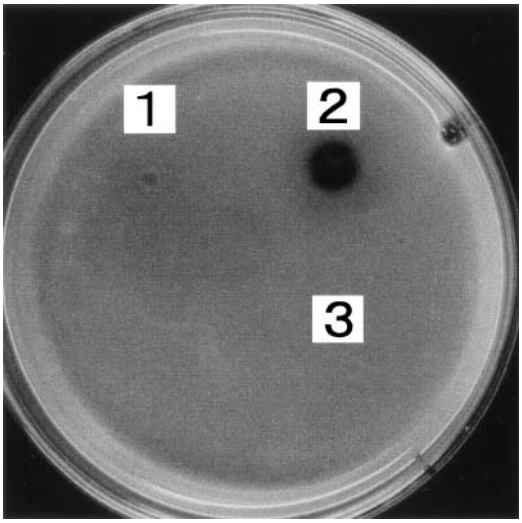

Fig. 4. Fibrinolytic activity of beer produced using mushrooms. 1, *F. velutipes*; 2, *T. matsutake*; 3, control (malt-hop extract medium). Showing a dissolved zone on the fibrin plate.

Results and Discussion

Mushroom alcohol dehydrogenase The ADH activity in cell-free extracts of the two types of mushroom were examined. Potent activity was found in the extracts of *F. velutipes* (15.6 units/ml), whereas low activity was found in the extracts of *T. matsutake* (2.5 units/ml).

Polyacrylamide gel electrophoresis (PAGE) of the native ADH from *T. matsutake*, followed by activity staining, is shown in Fig. 2. The electrophoresis of purified ADH, that was obtained by gel filtration on a TSK gel G3000SW column with HPLC system and extraction from active staining gel after PAGE, showed a single band on activity staining. Additionally, the ADH from *F. velutipes* showed one band on activity staining.

The molecular masses of the enzymes were estimated to be about 90 kDa (*F. velutipes*) and 30 kDa (*T. matsutake*) by gel filtration on a TSK gel G3000SW column.

Beer brewing Two genera of mushrooms possessing ADH activity were used in the following beer brewing. The highest alcohol concentration in the beer was achieved using *T. matsutake*, and was 1069 mM (4.6%), while the *F. velutipes* beer (651 mM, 3.0%) had low alcohol content. The flavor of beer produced by *T. matsutake* was the same as that of the fruit-body.

As shown in Fig. 3, beer produced by *T. matsutake* contained about 0.17% ß-D-glucan, which has been reported to have preventative activity against cancer (Whistler *et al*., 1976). Considering these facts, drinking beer produced using *T. matsutake* is expected to be effective in preventing of cancer.

The effects of beer produced by mushrooms on thrombin time (TT) are summarized in Table 1. The two beers produced using *T. matsutake* and *F. velutipes* showed anti-coagulative activities on TT. The TT of beer produced by *T. matsutake* was longer than that of *F. velutipes*. The thrombin clotting times of the beer produced by *T. matsutake* was determined to be more than 2.3 times longer than that of the control (malt-hop medium).

Table 2 shows the fibrinolytic activities of the beer produced by mushroom. The beer produced by *T. matsutake* showed fibrinolytic activities on fibrin plate, though that of *F. velutipes* and control did not (Fig. 4).

Thus, beer produced with these mushrooms may have a preventive effect on thrombosis (Okamura *et al*., 1997).

In general, conversion of carbohydrate into ethyl alcohol requires the action of alcohol dehydrogenase produced by the yeast *S. cerevisiae* during beer production, and therefore *S. cerevisiae* has been used to make beer. In this research, beer was produced using only mushrooms because they also have alcohol dehydrogenase activity.

This is the first report on beer produced using mushrooms.

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