Original paper

Crude Enzymes from a *Hericium* Edible Mushroom Isolated in Japan: Variability in Milk-clotting Activity and the Ability to Coagulate Ultra-high-temperature Pasteurized Milk

Munekazu KISHIMOTO^{1,2*}, Kazuo NAKAMURA³, Keiya KANEMARU³, Takuto TASAKI³, Tomoyuki NAKAMURA⁴, Kaoru SATO⁵ and Morimasa TANIMOTO²

¹The Institute of Enology and Viticulture, University of Yamanashi, 1-13-1 Kitashin, Kofu, Yamanashi 400-0005, Japan

²Department of Local Produce and Food Sciences, Faculty of Life and Environmental Sciences, University of Yamanashi, 4-4-37, Takeda, Kofu, Yamanashi 400-8510, Japan

³Department of Biotechnology, Faculty of Life and Environmental Sciences, University of Yamanashi, 4-4-37, Takeda, Kofu, Yamanashi 400-8510, Japan

⁴Applied Fungi Institute, IBI Corporation, 7841 Anayama, Nirasaki, Yamanashi 407-0263, Japan

⁵Department of Food Science and Technology, Faculty of Applied Life Science, Nippon Veterinary and Life Science University, 1-7-1, Kyonan, Musashino, Tokyo, 180-8602, Japan

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Eleven mycelial strains of the genus *Hericium* were isolated to investigate the variability in milk-clotting activity and ability to coagulate ultra-high-temperature pasteurized milk (UHT-milk) of their crude enzymes. As well, the antifungal activity of cheese prepared using the crude enzymes was assessed. Based on sequence analysis of ITS-5.8S ribosomal DNA, 8 strains were identified as *H. erinaceus*, 2 as *H. abietis*, and 1 as *Hericium* sp. Notable differences were observed among the species and within strains in terms of milk-clotting activity of the crude enzyme preparation. The UHT-milk coagulation ability was detected in 5 strains of *H. erinaceus* and showed no correlation with the potency of milk-clotting activity. Furthermore, all cheese samples prepared from low-temperature pasteurized milk using the crude enzymes from the 8 strains of *H. erinaceus* and ripened for 30 days at 13°C showed growth inhibitory activity toward *Aspergillus niger* NBRC 105649. Thus, the crude enzyme preparation from *H. erinaceus* may be useful for cheese production.

Keywords: *Hericium erinaceus*, milk-clotting activity, ultra-high-temperature pasteurized milk coagulation ability, antifungal activity, cheese

Introduction

In the production of cheese, rennet (chymosin), which is synthesized in the abomasum (a part of the stomach) of young ruminant animals, has traditionally been used for milk coagulation. Nonetheless, an inadequate supply of the enzyme, due to increased cheese production and concerns regarding the slaughter of animals,

has prompted the search for microbes that produce milk-clotting enzymes with properties close to those of rennet. Milk-clotting enzymes derived from mold have been developed as replacements for rennet. Mass culture techniques for *Rhizomucor miehei*, *Rhizomucor pusillus*, and *Cryphonectria parasitica* have been established (Jacob *et al.*, 2011). Milk-clotting enzymes derived

*To whom correspondence should be addressed.

E-mail: mkishimoto@yamanashi.ac.jp

from mold are advantageous for replacement of the costly calf rennet. Nevertheless, because of concomitant proteolytic activity, the cheese thus produced often tastes bitter (Sousa *et al.*, 2001). *Basidiomycetes* may also be a useful source of milk-clotting enzymes without this problem. *Irpex lacteus* (Kobayashi *et al.*, 1985, Kikuchi *et al.*, 1988, Kobayashi and Kasamo, 1992, Chemeris *et al.*, 2016), *Phellinus chrysoloma*, *Kuehneromyces mutabilis*, *Ganoderma applanatum* (Nerud *et al.*, 1989), *Schizophyllum commune* (Okamura-Matsui *et al.*, 2001), *Coprinus lagopides* (Shamtsyan *et al.*, 2014), *Termitomyces clypeatus* (Majumder *et al.*, 2015), *Piptoporus soloniensis* (El-Baky *et al.*, 2011), *Laetiporus sulphureus* (Kobayashi and Kim, 2003), and *Pleurotus ostreatus* (Lebedeva and Proskuryakov, 2009, Yin *et al.*, 2014) have been found to produce milk-clotting enzymes. Except for *P. ostreatus*, these *Basidiomycetes* are not commonly used in foods in Japan; moreover, some are suspected to cause infection in humans (Amitani *et al.*, 1996, Buzina *et al.*, 2005). Thus, Nakamura *et al.* (2014) explored milk-clotting-enzyme-producing strains among mushrooms consumed routinely and found high milk-clotting activity and low protease activity in three strains of *Hericium erinaceum* MAFF 435060, MAFF 430234 and NBRC 100328, indicating its potential use in cheese production. Furthermore, Sato *et al.* (2016) found that the milk-clotting enzyme in *H. erinaceum* MAFF 435060 has the ability to coagulate milk pasteurized at a low temperature (66°C, 30 min) as well as milk pasteurized at ultra-high temperatures (130°C, 2 s). Additionally, during the course of research, we found that microbial spoilage during ripening did not readily occur in cheeses prepared using the crude enzymes from *H. erinaceum* MAFF 435060 (unpublished results).

Although the classification of the genus *Hericium* remains unclear, phylogenetic classification based on ribosomal DNA (rDNA) internal transcribed spacer (ITS) sequences revealed *Hericium abietis*, *H. alpestre*, *H. americanum*, *H. coralloides*, and *H. erinaceus*, among others, as the most promising species (Hallenberg *et al.*, 2013). These findings have generated interest in the variability of milk-clotting activity as well as the UHT-milk coagulation ability of the crude enzymes among species and strains of the genus *Hericium*.

The purpose of this study was to examine the variability in milk-clotting activity and UHT-milk coagulation ability among species and strains of the genus *Hericium* newly isolated in Japan. Moreover, we evaluated the antifungal activity of the cheese produced using a crude enzyme preparation.

Materials and Methods

Isolation and identification of mycelial strains Fruiting bodies presumed to originate from the genus *Hericium* and growing on dead wood were harvested in 2015 in 4 prefectures: Yamanashi, Nagano, Wakayama, and Kochi. At the time of harvest, the spine length and spine arrangement typical of the genus *Hericium* were

observed. To isolate the mycelium, cells from the central part of the fruiting body were cultured on MYS medium (pH 5.6) consisting of 1.0% malt extract (Kyokutoseiyaku, Tokyo, Japan), 0.4% yeast extract (Kyokutoseiyaku), 1.0% sucrose, and 1.5% agar or on potato dextrose agar (Nissui, Tokyo, Japan) at 25°C. The generated mycelia were purified and stored at −80°C. After culturing on MYS medium, the purified mycelia were subjected to ITS-5.8S rDNA base sequence analysis, BLAST analysis, and identification at Techno Suruga Lab Co., Ltd. (Shizuoka, Japan).

Cultivation of mycelia and preparation of crude enzymes As described by Nakamura *et al.* (2014), the isolated strains were precultured on MYS plates at 25°C until the entire plate was covered with mycelia. Five discs were collected from each precultured plate using a sterilized cork-borer (7 mm in diameter). To 5 g of wheat bran in a 100-mL Erlenmeyer flask, 5.9 mL of water was added, followed by sterilization at 110°C for 20 min. The discs were inoculated into the sterilized wheat bran medium (water content of approximately 60%, w/w). After 14 days of cultivation at 25°C, 50 mL of 0.05 M McIlvaine buffer (pH 6.0) was added to the medium, and the suspension was kept overnight at 4°C. The suspension was filtered to remove the wheat bran and the mushroom mycelia using a metal mesh cloth (32-mesh). The filtrate was centrifuged (10,000 × g, 15 min, 7°C) and filtered to remove solid particles using No. 2 filter paper (Advantec, Tokyo, Japan). The filtrate served as the crude enzyme preparation. Similarly, *H. erinaceum* MAFF 435060, selected as a suitable strain for cheese making by Nakamura *et al.* (2014), was cultured, and the extracted crude enzyme was then used to prepare cheese.

Measurement of milk-clotting activity and UHT-milk coagulation ability Milk-clotting activity of the crude enzymes extracted from the wheat bran culture of the isolated strain was determined as described by Arima *et al.* (1970). As a substrate solution, 5 mL of 10% (w/v) skim milk (Morinaga Co., Ltd., Tokyo, Japan) containing 0.01 M calcium chloride was incubated at 35°C. To this solution, we added 0.5 mL of a crude enzyme preparation, after which the time required for coagulated particles to form the curd was measured. Milk-clotting activity was expressed in Soxhlet units. Moreover, to determine the coagulation ability toward UHT-milk (130°C, 2 s), 5 mL of commercial UHT-milk (Meiji Co., Ltd., Tokyo, Japan) was incubated at 35°C, then 0.5 mL of a crude enzyme preparation was added, and the status of coagulation was checked after 40 min.

Preparation of cheese curd and measurement of antifungal activity Cheese curd was made from 100 mL of low-temperature pasteurized milk (Takanashi Milk Products Co., Ltd., Kanagawa, Japan) as described by Sato *et al.* (2016). The milk was incubated at 35°C for 10 min before addition of 10 mL of a crude enzyme preparation from the isolated mycelial strains of *H. erinaceus*—or 1 mL of 2 mg/mL commercial rennet (CHY-MAX Powder-Extra, Christian Hansen Japan Co., Ltd., Tokyo, Japan) in place of the crude enzymes as the control—and mixed gently. The milk was

Table 1. Characteristics of the harvested fruit bodies and results of BLAST searching based on analysis of the ITS-5.8S rDNA sequence of mycelial strains isolated in Japan.

Mycelial strain	Sampling place	Arrangements of spines	Length of spines	Presumed genus ^{a)}	BLAST match sequence			
					Reference strain No. ^{b)}	Accession No.	Similarity(%)	
WH01	Yamanashi	Spines in bundles	1-4 cm	<i>Hericium</i>	<i>H. erinaceus</i> KUMC1008	AY534593	100	
WH02	Wakayama	Spines in bundles	1-4 cm	<i>Hericium</i>	<i>H. erinaceus</i> KUMC1008	AY534593	100	
WH03	Yamanashi	Spines in bundles	1-4 cm	<i>Hericium</i>	<i>H. erinaceus</i> KUMC1008	AY534593	100	
WH04	Yamanashi	Spines in bundles	1-4 cm	<i>Hericium</i>	<i>H. erinaceus</i> KUMC1008	AY534593	100	
WH06	Yamanashi	Spines in bundles	1-4 cm	<i>Hericium</i>	<i>H. erinaceus</i> KUMC1008	AY534593	100	
WH07	Yamanashi	Spines in bundles	1-4 cm	<i>Hericium</i>	<i>H. erinaceus</i> KUMC1008	AY534593	100	
WH08	Yamanashi	Spines in bundles	1-2 cm	<i>Hericium</i>	<i>H. erinaceus</i> KUMC1008	AY534593	100	
WH10	Yamanashi	Spines single, combilike	<1 cm	<i>Hericium</i>	<i>H. coralloides</i> ATCC52480	AY534584	95.9	
WH13	Yamanashi	Spines in bundles	1-4 cm	<i>Hericium</i>	<i>H. erinaceus</i> KUMC1008	AY534593	99.8	
WH20	Nagano	Spines in bundles	<1 cm	<i>Hericium</i>	<i>H. abietis</i> CBS 125851	JN201334	99.5	
WH21	Kochi	Spines in bundles	<1 cm	<i>Hericium</i>	<i>H. abietis</i> CBS 125851	JN201334	99.5	

a) Genus presumed from morphological features of the fruiting body.

b) Highest-ranking strain in BLAST search with sequence of the strain.

then continuously incubated at 35°C until all milk was clotted completely. The clotted substance was diced into small cubes and then warmed to 50°C to accelerate the discharge of whey. The curd was poured into the mold and kept at 4°C for 3 h with a weight. After the whey was discharged, the hardened curd was immersed in 25% salted water for 1 h and placed in a tightly closed container to ripen at 13°C. The ripened cheese was stored at -20°C if necessary.

To evaluate the antifungal activity, an MYS plate was mixed with the test mold—*Aspergillus niger* NBRC 105649 spores—to a concentration of approximately 10⁵ spores per plate. A sample hole was made in the plate using a 7-mm Ø dry-heat-sterilized cork-borer, then the hole was packed with 0.1 g of ripened cheese, and the plate was incubated at 25°C for 3 days. Next, the diameter of the circle of inhibition of growth was measured. The diameter of the cork-borer was subtracted from this value to determine the growth-inhibitory ability.

Results and Discussion

Isolation and identification of mycelia from fruiting bodies

Fruiting bodies growing on a dead tree were harvested and a total of 11 mycelial strains were newly isolated. The characteristics of the harvested fruiting bodies and results of BLAST searches based on analysis of the ITS-5.8S rDNA sequence of the strains are presented in Table 1. All the harvested fruiting bodies were presumed to belong to the genus *Hericium* according to their morphological features. The BLAST results based on ITS-5.8S rDNA sequence analysis of the strains indicated that all the isolates belonged to the genus *Hericium*. The shared homology among 8 strains—WH01, WH02, WH03, WH04, WH06, WH07, WH08, and WH13—with *H. erinaceus* KUMC1008 was ≥99.8%; thus, these strains were identified as *H. erinaceus*. Two strains, WH20 and WH21, shared 99.5% homology with *H. abietis* CBS 125851, and thus the strains were identified as *H. abietis*. The homology between WH10 and *H. coralloides* ATCC52480 was 95.9%, which was a somewhat low value, and this strain was assumed to be

closely related to *H. coralloides*. Therefore, the species could not be identified and was predicted to be a *Hericium* sp. The classification of the genus *Hericium* from Japan was investigated by Obatake *et al.* (2008) and reported as follows: Two species, *H. erinaceus* (*Yamabushitake* in Japanese) and *H. abietis* (*Sangoharitakemodoki* in Japanese), were identified, and *Sangoharitake* (in Japanese) had a fruiting body resembling that of Western-produced *H. coralloides* in morphological features and was closely related based on rDNA sequence analysis; however, crossing between single-nucleus mycelia was not confirmed. *H. erinaceus* is distributed in Japan and North America (Boddy *et al.*, 2011), and has been used in folk medicine and in medicinal cuisine (Wang *et al.*, 2014). This species is currently cultivated artificially in Japan.

Milk-clotting activity and UHT-milk coagulation ability of crude enzyme preparations The milk-clotting activity of each crude enzyme preparation extracted from the wheat bran culture of isolated strains is shown in Table 2. The milk-clotting activities of crude enzyme preparations from WH01, WH02, WH03, WH04, WH06, WH07, WH08, and WH13, identified as *H. erinaceus*, were 85.8–333.8 U/mL. When the WH01 strain showing the highest activity and the WH13 strain showing the lowest activity were compared, an approximately 4-fold difference was observed. Milk-clotting activity was not detected in strains WH20 and WH21, which were identified as *H. abietis*. In addition, weak milk-clotting activity was detected in strain WH10 (*Hericium* sp.), equivalent to that of WH13, which showed the lowest activity among the *H. erinaceus* strains. These results revealed that the strength of milk-clotting activity varies considerably among species and strains. Chemeris *et al.* (2016) reported that milk-clotting enzymatic activity in a liquid culture of *I. lacteus* differs among strains.

An example of coagulation of UHT-milk by a crude enzyme preparation is shown in Fig. 1. As shown in Fig. 1(B), clearly separate curd and whey were produced by crude enzymes from 5 strains—WH02, WH03, WH04, WH07, and WH08—and their UHT-milk coagulation ability was confirmed. In contrast, the

Table 2. Milk-clotting activity and UHT-milk coagulation ability of crude enzymes from the isolated mycelial strains.

Mycelial strain	Milk clotting activity (U/mL)	UHT-milk coagulation ability
WH01	333.8±103.0	Absent
WH02	310.6±48.3	Present
WH03	260.2±49.8	Present
WH04	251.6±49.7	Present
WH06	218.2±20.2	Absent
WH07	174.9±9.2	Present
WH08	169.0±36.5	Present
WH10	70.6±39.4	Absent
WH13	85.8±25.2	Absent
WH20	ND	NT
WH21	ND	NT

Values are the mean ± standard deviation (n=4). Mean of the enzyme activity in four independent cultures extracts. Absent, milk without coagulation; Present, milk with strong coagulation. ND, not detected; NT, not tested.

Fig. 1. An example of UHT-milk coagulation by a crude enzyme preparation. (A) Milk without coagulation; (B) Milk with effective coagulation.

coagulation ability toward UHT-milk was not detected in strains WH01, WH06, WH10, and WH13 (Table 2). The status of UHT-milk coagulation ability differed among the strains of *H. erinaceus*, and showed no correlation with the potency of milk-clotting activity. According to these results, we estimated that the nature of the milk-clotting enzymes in *H. erinaceus* is strain-dependent. Sato *et al.* (2016) found that the milk-clotting enzyme in *H. erinaceus* MAFF 435060 has coagulation ability not only toward milk pasteurized at a low temperature but also toward UHT-milk, and demonstrated that the milk-clotting effect is not due to degradation of κ -casein. It is known that rennet does not have coagulation ability toward UHT-milk (McMahon *et al.*, 1993). The UHT-milk coagulation ability identified in crude enzyme preparations from WH02, WH03, WH04, WH07, and WH08 is expected to bring about technological innovation to the currently challenging process of cheese production from UHT-milk as a source.

Fig. 2. Inhibition of *A. niger* growth by cheese prepared using rennet (A) or crude enzymes from *H. erinaceus* MAFF435060 (B). Cheese samples were tested before ripening (a) and after ripening at 13°C for 15 days (b), 30 days (c), and 45 days (d). **Fig. 3.** Growth inhibition abilities toward *A. niger* of the cheese prepared using the crude enzymes from 8 strains of *H. erinaceus* and rennet.

Error bars indicate standard deviation (n = 3). Means reflect the growth inhibition shown by 3 independent cheese samples.

Antifungal activity of cheese prepared using crude enzymes from *H. erinaceus* We found that microbial spoilage during ripening did not readily occur in cheeses prepared with crude enzymes from *H. erinaceus* MAFF 435060. The changes in growth inhibition of *A. niger* NBRC 105649 during the ripening of cheese prepared using the crude enzymes from *H. erinaceus* MAFF 435060 were compared with those of cheese prepared using rennet (Fig. 2). When rennet was used, *A. niger* growth was not inhibited during the ripening period. In contrast, for cheeses prepared using the crude enzymes from *H. erinaceus*, the potency of growth inhibition gradually increased with the number of ripening days. We also compared the growth inhibition ability toward *A. niger* of the cheese prepared using the crude enzymes (from the 8 strains of *H. erinaceus*, which all created successful curd, and ripened for 30 days at 13°C) with the inhibition exerted by the cheese prepared using rennet (Fig. 3). Potent growth inhibition was observed for all 8 strains, indicating that cheese prepared using crude enzymes from *H. erinaceus* has antifungal activity. For cheese prepared using rennet, particularly fresh-type cheese, the storage period is short, and microbial spoilage often occurs during storage (Ledenbach *et al.*, 2009). The use of crude enzymes from *H.*

erinaceus for cheese-making may improve the shelf-life of cheese; thus, further studies on the mechanism of antifungal activity are needed.

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