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Milk-clotting enzymes produced by culture of *Bacillus subtilis* natto

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ABSTRACT

Factors affecting the production of milk-clotting enzyme (MCE) by *Bacillus subtilis* (natto) Takahashi, a ready available commercial natto starter, were studied. Remarkable milk-clotting activity (MCA), 685.7 SU/ml or 12,000 SU/g, was obtained when the bacteria were cultivated in the medium containing sucrose (50 g/L) and basal salts at pH 6, 37 °C with shaking at 175 rpm for 1 day. The MCA and MCA/PA ratio of the crude enzyme obtained are comparable with those of Pfizer microbial rennin and *Mucor* rennin. The crude enzyme showed excellent pH and thermal stability; it retained 96% of MCA after incubation for 40 min at 40 °C and retained more than 80% of its activity between pH 4 and pH 7 for more than 30 min at 30 °C. The MCE of *B. subtilis* (natto) Takahashi has potential as calf rennet substitutes.

1. Introduction

Milk coagulation is a basic step in cheese manufacturing. For a long time calf rennet, the conventional milk-clotting enzyme obtained from the fourth stomach of suckling calves [1] is the most widely used coagulant in cheese making all over the world to manufacture most of the cheese varieties. The worldwide reduced supply of calf rennet and the ever increase of cheese production and consumption have stimulated the research for milk-clotting enzyme (MCE) from alternative sources to be used as calf rennet substitutes [2–5]. Various animals, plants and microbial proteases have been suggested as milk coagulants [6–13]. However, attention has been focused on the production of milk-clotting enzymes (MCEs) from microbial sources for use as rennin substitutes [8,14–26]. Although there are many microorganisms that produce MCEs [3], only the MCEs produced by strains of *Rhizomucor miehei*, *Rhizomucor pusillus* var. *Lindt*, *Aspergillus oryzae* and *Enthothia parasitica* are widely used [27–29].

Bacillus subtilis (natto) Takahashi, a commercial natto starter, is commonly used to prepare fermented soybean product-natto, which is a traditional Japanese food for more than 1000 years. Recently, we have shown that *B. subtilis* (natto) Takahashi produced simultaneously a mixture of poly(γ-glutamic acid) and levan when it was grown in a basal medium containing sucrose and L-glutamate [30]. Furthermore, it can produce poly(γ-glutamic acid) and levan selectively under chosen conditions in that poly(γ-glutamic acid) was mainly produced in a medium containing L-glutamic acid and without sucrose; in contrast, levan was the only product when the bacteria were cultivated in a medium containing 20% (w/w) sucrose and without L-glutamate. Nonetheless, *B. subtilis* (natto) was never investigated for the production of MCE(s). *B. subtilis* is one of the most investigated microbial groups, because they can produce varieties of biotechnological interesting substances [31]; it is known to secrete several proteases during the fermentation process [32]. The capacity of selected *Bacillus* strains to produce and secrete large quantities of extracellular enzymes has led them to be among the most important industrial enzyme producers. While *B. subtilis* (natto) produces many enzymes, including amylases and cellulases, the most important enzymes in the production of natto are proteases; many of them have been characterized [33]. The proteases are responsible for the main flavor, through hydrolysis of soybean protein. It is conceivable that *B. subtilis* (natto) may produce MCE(s). In fact, we have recently discovered that the fermentation product of *B. subtilis* (natto) Takahashi displayed strong milk-clotting activity (MCA) in the process of making bakery products [34]. To further investigate the possibility of the use of *B. subtilis* (natto) Takahashi for the efficient production of the rennin-like enzymes for cheese making, the present work was undertaken to investigate the pattern of milk-clotting and proteolytic activities of *B. subtilis* (natto) in extracelluar bacterial enzyme preparations under different conditions of culturing.

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2. Materials and methods

2.1. Bacteria strains and reagents

B. subtilis (natto) Takahashi was obtained from Takahashi Yuzo research facility Japan. Other bacteria tested in this study were *B. subtilis* (natto) ATCC 7058, *B. subtilis* (natto) ATCC 7059, *B. subtilis* (natto) IFO 13169, *B. subtilis* (natto) IFO 3335, which were obtained from the Culture Collection and Research Center (CCRC) Taiwan. Reagents for cultivation such as nutrient agar (NA) and nutrient broth (NB) were purchased from DIFCO Laboratories Michigan, USA. $MgSO_4{\cdot}7H_2O$, $NaH_2PO_4{\cdot}2H_2O$, $Na_2HPO_4{\cdot}12H_2O$, rennin from *Mucor meiher* (type II) and Pfizer microbial rennin were obtained from Sigma Chemical (St. Louis, MO) and Pfizer Inc. (Milwaukee, WI), respectively. Dry skim milk powder was from New Zealand milk brands Ltd. All other reagents used were of the highest grade available unless indicated otherwise.

2.2. Sample preparation

2.2.1. Preparation of inoculums

B. subtilis (natto) Takahashi or other tested *B. subtilis* (natto) bacteria were first cultured on NA (Difco Laboratories) containing agar (15 g/L), beef extract (3 g/L), peptone (5 g/L) at 37 °C, pH 7.0 overnight. The colonies appeared on the plate were picked up (1 cm square) and inoculated into 5 ml of NB composed of beef extract (3 g/L), peptone(5 g/L) in a 30-ml test tube, and incubated at 37 °C, pH 7.0 for 20 h with shaking at 175 rpm. After incubation, 1 ml of bacteria were inoculated into 100 ml medium composed of beef extract (3 g/L), peptone(5 g/L), soybean (50 g/L), sucrose 2 g/L) and NaCl (5 g/L) in a 250-ml Erlenmyer flask, followed by incubation at 37 °C, pH 7.0 for 20 h with shaking at 175 rpm. The culture broth was then used as inoculums for the later experiments.

2.2.2. Solid state fermentation (SSF)

After removing impurities, the soybean samples (50 g) were washed, soaked in de-ionized water (15 Ω) at 4 °C for approximately 24 h until the weight increase to about twice of the original dry weight. After being drained, the soaked beans were mixed with 5 g NaCl salt and 1 g sucrose in a 250-ml flask, then placed in autoclave at 121 °C for 15 min. 2 ml bacterial inoculums prepared above or 2 ml of natto bacteria liquid (prepared by suspending 0.1 g of natto bacteria in 10–20 ml of sterilized water) were used to inoculate. The mixture was stirred with a sterilized spatula so that the natto bacteria will be evenly distributed, followed by static inoculation at 37 °C for 24 h. To extract the enzyme for assaying, 50 ml sterilized de-ionized water (15 Ω) was used for extraction. The solid material and bacteria were removed by filtering through cotton cloth, followed by centrifugation at 5600×*g*, 4 °C for 20 min. After removing bacteria, the liquid extract of crude enzyme was used to assay for milk-clotting activity.

2.2.3. Liquid state fermentation (LSF)

Soybean samples (50 g) were washed, soaked and drained as described above, which were then combined with 100 ml of L-medium composed of sucrose (50 g/L), $MgSO_4{\cdot}7H_2O$ (0.5 g/L), $NaH_2PO_4{\cdot}2H_2O$ (3 g/L), $Na_2HPO_4{\cdot}12H_2O$ (3 g/L) and NaCl salt (10 g/L) in a 250-ml flask; the whole mixture was steamed and sterilized in autoclave at 121 °C for 15 min. The soybean contained medium (S-medium) was inoculated with 5 ml (5%, v/v) of bacteria inoculums prepared above and incubated at 37 °C, pH 7.0 with shaking at 175 rpm for 24 h. At the end of incubation, the culture medium was filtered through the cotton cloth to remove soybean, which was followed by centrifugation at 5600×*g*, 4 °C for 20 min and filtering through a syringe filter (0.45 μm) to remove the bacteria. The liquid broth of the crude enzyme was used to assay for milk-clotting activity. To investigate the factors affecting MCE production, the cultivation was carried out in the medium and conditions indicated above except that the factors were varied by one-factor-at-a-time fashion.

2.2.4. Preparation of levan and poly(γ-glutamic acid)

The preparation of levan by *B. subtilis* (natto) Takahashi was carried out by following the protocol described previously [30,35]. The natto bacteria inoculums prepared above were inoculated (5%, v/v) into 100 ml of a medium composed of sucrose (200 g/L), $MgSO_4{\cdot}7H_2O$ (0.5 g/L), $NaH_2PO_4{\cdot}2H_2O$ (3 g/L), $Na_2HPO_4{\cdot}12H_2O$ (3 g/L) in a 250-ml flask, and then were incubated at 37 °C, pH 7.0 with shaking at 150 rpm for 21 h. The culture medium was centrifuged to remove the bacterial cells, and then the levan was harvested by precipitation with the addition of cold 95% ethanol from the culture broth, followed by dialysis through a membrane with 10 kDa cut-off. For selective production of γ-PGA by *B. subtilis* (natto) Takahashi, the bacteria inoculums were grown in Medium E composed of glutamic acid (20 g/L), citric acid (12 g/L), glycerol (80 g/L), NH_4Cl (7 g/L), $MgSO_4{\cdot}7H_2O$ (0.5 g/L), $FeCl_3{\cdot}6H_2O$ (0.04 g/L), K_2HPO_4 (0.5 g/L), and $CaCl_2{\cdot}7H_2O$ (0.15 g/L). The γ-PGA was extracted and purified by a method described previously [36]. The products were characterized by ^1H NMR, ^{13}C NMR and gel permeation chromatography (GPC).

2.3. Assay for milk-clotting activity

Milk-clotting activity was determined according to the method of Arima [3], which is based on the visual evaluation of the appearance of the first clotting flakes, and expressed in terms of Soxhlet units (SU). One SU is defined as the amount of enzyme which clots 1 ml of a solution containing 0.1 g skim milk powder and 0.00111 g calcium chlorides in 40 min at 35 °C. In brief, 0.5 ml of tested materials was added to a test-tube containing 5 ml of reconstituted skim milk solution (10 g dry skim milk/100 ml, 0.01 M $CaCl_2$) pre-incubated at 35 °C for 5 min. The mixture was mixed well and the clotting time T (s), the time period starting from the addition of test material to the first appearance of clots of milk solution, was recorded and the clotting activity was calculated using the following formula: $SU = 2400 \times 5 \times D/T \times 0.5$; T = clotting time (s); D = dilution of test material. The test materials include liquid extract of crude enzyme from SSF, liquid solution of crude enzyme from LSF, levan, γ-PGA and commercial rennet of *M. miehei*. The test solutions of the leven, γ-PGA and commercial rennet of *M. miehei* were prepared by dissolving 1 g of compound in 100 ml distilled water.

2.4. Assay of protease activity

The proteolytic activity was determined at pH 6.0 by the casein digestion method described previously [37]. In brief, 5 ml of 1.2% of casein solution in 0.05 M phosphate buffer (pH 6.0) was added 1 ml of enzyme solution, and the mixture was incubated at 35 °C for 10 min. After incubation, 5 ml (0.44 M) of tri-chloroacetic acid (TCA) was added to quench the reaction, which was followed by filtration. 2 ml of the above filtrate was added 5 ml NaOH (0.28 N) solution and 1.5 ml phenol reagent (Folin-Ciocalteu phenol solution:water = 1:2). After the mixture was kept at 35 °C for 15 min, optical density (OD) at 660 nm was measured with a Hitachi spectrophotometer. Ratio of the milk-clotting activity to proteolytic activity is expressed as milk-clotting units (SU) per the OD_{660}'s obtained in the proteolytic measurements. Protein determination was according to the method of Lowry et al. [38].