Production and Properties of a Soymilk-clotting Enzyme System from a Microorganism

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Some microorganisms, including some bacteria isolated from soil, were found to secrete an extracellular soymilk-clotting enzyme. Among them, strain No. K-295G-7 showed the highest soymilk-clotting activity and stability of the production of the soymilk-clotting enzyme. The enzyme system (culture filtrate) coagulated protein in soymilk, a curd being formed at pH 5.8~6.7 and at 55~75°C. The optimum temperature for the soymilk-clotting activity was 75°C and the enzyme system was stable at temperatures below 50°C down to 35°C. About 80~100% of the original activity remained after 1 hr at pH 5~7 and 35°C.

It has been recognized that soybeans are a food material with a high nutritive value because they contain good quality protein and a high percentage of essential fatty acids. For this reason, soy-beans have been extensively used as the raw material for traditional foods, such as "Tôfu" (bean curd), "Miso" (bean paste), "Shôyu" (soy sauce) and so on, since ancient times in Japan. In many countries, soybeans have also been regarded as one of the major important sources of protein for human beings. Soybeans, however, have a few shortcomings such as their beany flavor,^{1,2)} and water absorption and retention in the gel used for cheese-like foods.³⁾ The simple elimination of these properties would be beneficial for the further utilization of soybean protein.

Several workers^{4~10)} have attempted to improve the functional properties of soybean protein by enzymatic, physical and chemical modifications. Lately, it has been reported that Bromelain, a plant protease, is capable of clotting the protein in soymilk, soymilk curd thereby being formed. Fuke and Matsuoka^{3,4)} and Mohri and Matsushita⁵⁾ reported that soymilk, acid-precipitated soy-bean protein, and isolated 11S globulin were clotted by Bromelain. They also investigated the proper-

ties of the curd and the clotting mechanism involved.

No report, however, has appeared on the efficiency of a microbial enzyme to clot soybean protein. This paper deals with the screening of organisms producing a soymilk-clotting enzyme, and the production and investigation of some properties of the enzyme.

MATERIALS AND METHODS

Soymilk. Soymilk, "Kibun Tōnyu Muchōsei (pH 6.7)," was purchased from Kibun Co., Ltd. (Japan).

Isolation sources of microorganisms. Soil samples, collected from various districts of Japan, were the sources for isolation of microorganisms producing a soymilk-clotting enzyme.

Isolation of microorganisms producing a soymilk-clotting enzyme. The first screening was performed as follows: one drop of a soil suspension diluted 100- to 1,000-fold with sterilized water was spread on an agar plate containing medium A (Table I), followed by incubation at 30°C for 3 days. The colonies growing on the plate were picked up and maintained on Bennet agar slants (B in Table I) at ambient room temperature. About 300 colonies of bacteria and about 150 colonies of fungi were isolated from 300 soil samples by this method.

The second screening was performed as follows: about 10 ml of the soymilk adjusted to pH 6.0 with 1 M potassium

TABLE I. MEDIUM COMPOSITIONS (%)

	Soymilk	Dextrose	N.Z. Amine	Peptone	Yeast ex.	Beef ex.	KH ₂ PO ₄	Agar	pH
A	5	0.2		0.2	0.2		0.2	1.3	6.1
B		1.0	0.2		0.1	0.1	1.0	1.5	5.6
C	5	0.2		0.2	0.2		0.5		6.1

A: Composition of medium for 1st screening.

B: Composition of medium for slants.

C: Composition of medium for enzyme production.

phosphate buffer (pH 4.5) was placed in a test tube with a cotton plug, and sterilized at 120 °C for 15 min. One loop of each isolate from the first screening was inoculated into the soymilk, followed by incubation at 35 °C for 2 days. By this method, 70 bacterial isolates and 60 fungal isolates, which were observed to coagulate soymilk, were selected and maintained on agar slants (B in Table I).

The organisms were subjected to a third screening as follows: twenty-five ml of the medium for enzyme production (C in Table I) was placed in 100 ml shaking flasks (Sakaguchi flasks). After sterilization at 120 °C for 15 min, one loop of each isolate from the second screening was inoculated into the medium, followed by incubation at 35 °C for 3 days on a reciprocal shaker (125 oscillations per min). The resultant culture broth was centrifuged at $9,000 \times g$ for 20 min to remove insoluble materials including the cells. The supernatant liquid, thus obtained, was used as the enzyme solution (enzyme system). Three bacterial isolates designated as W-18a, W-18b and K-295G-7 showed relatively high soymilk-clotting activity.

Assay for soymilk-clotting activity. One half ml of the enzyme solution was added to 5 ml of soymilk adjusted to pH 6.1 with 1 M potassium phosphate buffer (pH 4.5), and the clotting activity was determined at 65 °C as described by Arima *et al.*¹¹⁾ The amount of enzyme that clotted 5 ml of soymilk in 1 min under the above conditions was defined as 1 soymilk-clotting unit.

RESULTS

Evidence of soymilk coagulation by the enzyme system

One half ml of the enzyme solution was added to 5 ml of soymilk adjusted to pH 6.1 with 1 M potassium phosphate buffer (pH 4.5). After incubation at 65 °C for 5 min, the resultant coagulum was centrifuged at $1,100 \times g$ for 5 min.

Figure 1 shows that soymilk was coagulated by the enzyme system produced by the W-18a, W-18b and K-295G-7 strains, while no coagulation was observed either on the addition of

FIG. 1. Coagulation of Soy-milk by the Enzyme Reaction.

A, water; B, inactivated enzyme; C, K-295G-7; D, W-18a; E, W-18b.

water (A in Fig. 1) or the enzyme system treated at 100 °C for 5 min (B in Fig. 1). These observations indicate that the soymilk-clotting was caused by the enzyme reaction. The coagula (Tôfu-like), which were obtained on enzyme treatment, were separated into curd and whey after centrifugation.

Soymilk-clotting enzyme production by K-295G-7

In this work, we used K-295G-7 as a representative strain for further study of the soymilk-clotting enzyme, because the enzyme

produced by this strain exhibited the highest activity and stability of production among the three strains. Three liters of the enzyme production medium (C in Table I), containing a small amount of Tōshiba silicone defoamer, was poured into a 5-liter Jar fermentor, followed by sterilization at 120°C for 15 min in an autoclave. The seed culture (200 ml) of K-

295G-7, which had been grown in the same medium in 500 ml shaking flasks at 35°C for 2 days on a reciprocal shaker, was inoculated into the fermentor. The cultivation was carried out at 35°C at an aeration rate of 1.5 liters/min with agitation at 650 rpm. At suitable time intervals, a small amount of the culture broth was withdrawn and centrifuged, and then the enzyme activity in the supernatant was assayed. Figure 2 shows the time course of cultivation. The maximum soymilk-clotting activity, which was 0.16 units/ml, was observed at 3 to 4 days after the beginning of cultivation.

FIG. 2. Time Course of Cultivation.
--○--○--, pH; —●—●--, clotting activity.

Effects of temperature and pH on curd formation

In this experiment, an ammonium sulfate fraction was used as the enzyme solution. Ammonium sulfate was added to the supernatant obtained on cultivation of K-295G-7 to 80% saturation with the salt. The resultant

FIG. 3. Coagulation of Soy-milk by the Crude Enzyme from K-295G-7.

A: Effect of Temperature on Soymilk-clotting at pH 6.1. Reactions were carried out at various temperatures for 5 min.

B: Effect of pH on Soymilk-clotting at 65°C. Reactions were carried out at different pHs for 5 min.

FIG. 4. Some Properties of the Soymilk-clotting Enzyme from a Microorganism (K-295G-7).

A: Effect of Temperature on Soymilk-clotting Activity. Reaction mixtures containing 5 ml of soymilk, adjusted to pH 6.1, and 0.5 ml of enzyme solution were incubated at various temperatures for 30 min.

B: Effect of pH on Soymilk-clotting Activity. Soymilk-clotting activity was determined at different pHs, ranging from 5.8 to 7.0, under the conditions of 65°C for 30 min.

C: Effect of Temperature on Stability. The enzyme solution (pH 6.1) was stood at various temperatures for 30 min. Then the residual activity was determined.

D: Effect of pH on Stability. One half ml of the enzyme solution and 0.5 ml of McIlvaine buffer were mixed and stood at 35°C for 1 hr. After adjustment to pH 6.1, the residual activity was determined.

precipitate was collected by filtration on Tôyô-Roshi No. 2 filter paper and dissolved in a small amount of water. The enzyme solution, thus obtained, was dialyzed against a 20-fold volume of 0.04 M potassium phosphate buffer (pH 6.1). In order to examine the effect of temperature on curd formation, reactions were carried out at various temperatures at pH 6.1 for 5 min and the resultant coagula were centrifuged at $1,100 \times g$ for 5 min. As shown in A in Fig. 3, a curd was clearly formed at 55°C and 65°C, but not at 45°C or 75°C. On the other hand, the effect of pH on curd formation was investigated at different pHs ranging from 5.8 to 7.0 under the conditions of 65°C for 5 min. B in Fig. 3 shows that after the enzyme reaction, the soymilk was easily separated into curd and whey at pHs 5.8, 6.1 and 6.4.

Properties of the enzyme from K-295G-7

An ammonium sulfate fraction was also used as the enzyme solution in this experiment. The pH and temperature dependency profiles of the soymilk-clotting activity are shown in A and B in Fig. 4. The enzyme exhibited maximum activity at around 75°C at pH 6.1, and no activity was seen at 35°C and 85°C (A in Fig. 4). On the other hand, the activity decreased as the pH increased from 5.8 to 6.7 (B in Fig. 4). However, the soymilk-clotting activity test could not be carried out below pH 5.8 because soymilk coagulated without the addition of the enzyme solution. The thermal and pH-stabilities are illustrated in C and D in Fig. 4. The enzyme was stable at temperatures below 50°C down to 35°C when treated at pH 6.1 for 30 min. However, treatment at 55°C under the same conditions resulted in the loss of 53% of the original activity. On the other hand, the enzyme solution retained soymilk-clotting activity at 35°C for 1 hr in McIlvaine buffer ranging from pH 3~10. About 80~100% of the original activity remained after treatment in a pH range of 5~7, while 47% of the activity remained even at pH 10.0.

DISCUSSION

It was not known whether microorganisms secrete a soymilk-clotting enzyme. This is the first paper which deals with the efficient production of soymilk curd with the aid of a soymilk-clotting enzyme from a microorganism. The authors were able to isolate some bacteria capable of coagulating soymilk protein, from 300 soil samples. This suggests that microorganisms having such a property are widely distributed in nature.

It was obvious that the coagulation of protein in soymilk was due to the reaction caused by the enzyme from K-295G-7, because the phenomenon was observed at pH 6.1 with an ammonium sulfate or acetone or ethanol fraction (data not shown), but not on heating the culture filtrate to 100°C for 5 min (Fig. 1). However, it was not clear whether the coagulation was due to a single enzyme action or the

combined action of several enzymes. Further characterization of the enzyme system and the mechanism of coagulation would be interesting from enzymological viewpoints, and may be accomplished by investigating the properties of the purified enzyme. Work along these lines including the identification of K-295G-7 is now progress in our laboratory.

It is well known that soymilk curd is produced by means of isoelectric point precipitation and chemical coagulation due to divalent metals or glucono- δ -lactone. Recently, it was reported that plant proteases, such as Bromelain and Ficin,³⁾ coagulate protein in soymilk, and the resulting curd has a soft and smooth texture. The curd made with the enzyme system from K-295G-7 also has a smooth texture, and a mild taste without any bitterness or a beany flavor. This result shows that the curd obtained through the enzymatic action can serve as a material for further development of food items, and the procedure

may be widely applicable to food processing.

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