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Isolation of psychrotrophic microorganism producing soymilk-clotting enzyme from marine fish

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Abstract : A psychrotrophic microorganism isolated from Alaska pollack (*Theragra chalcoramma*) produced soymilk-clotting enzyme(s) with relatively low proteolytic activity. The isolate No. 268 was tentatively identified as *Pseudomonas* sp. Soymilk-clotting activity of the crude enzyme solution was observed at temperatures ranging from 20 to 60℃ and the optimum temperature was 40℃. When the crude enzyme solution was preincubated for 30 minutes, the clotting activity was stable at temperatures up to 30℃ and 75% of the activity was retained at 40℃. The clotting activity was decreased as the pH of soymilk was increased from 5.8 to 7.3(Received December 24, 1992; accepted January 28, 1993).

Soybean has been extensively used as the raw material for traditional foods such as tofu, soybean paste and soybean sauce, and considered to be one of the most important protein sources in many countries. Recently, methods have been developed to improve the functional properties of soybean protein by enzymatic, physical and chemical modifications,[1-5] and soybean protein has been used for cheese-like and yogurt-like foods.[6-10]

The first step in the manufacture of cheese-like products using soymilk is curd formation. Since Park *et al.*[11] isolated soymilk-clotting enzyme producing *Bacillus* sp. from soil, bacteria such as *Bacillus cereus, B. pumilis* and *B. licheniformis*,[12-14] and molds such as *Aspergillus oryzae, A. tamarii*[15] and a *Penicillium* sp.[16] have been reported to produce soymilk-clotting enzymes.

The acceptability of microbial enzymes for the manufacture of cheese-like products is determined largely by soymilk-clotting activity and the ratio of soymilk-clotting activity to proteolytic activity. Even though coagulation of soymilk protein requires the protein be hydrolyzed by the enzyme to some extent,[16] however, extensive proteolytic activity may cause deterioration of the product during ripening.[17] Other desirable properties of microbial soymilk-clotting enzymes are low optimum temperature for clotting activity and easy inactivation of general proteolytic activity at low temperature. All of the reported microbial soymilk-clotting enzymes have optimum temperatures of 60 to 65℃ and are stable at temperatures up to 50℃.[11-16] The present study is concerned with a psychrotrophic bacterium of marine fish origin capable of producing soymilk-clotting enzyme with a low optimum temperature for clotting.

Materials and Methods

Isolation of psychrotrophic microoganisms from marine fish

Soymilk was obtained from Chung Food Cooperation (Chungju, Choongbuk). Supplemented soymilk broth (SSMB) contained 20.0% soymilk, 0.2% yeast extract, 0.2% glucose, 0.25% K_2HPO_4 and 3.5% NaCl at pH 7.0. Supplemented soymilk agar (SSMA)

Key words : Soymilk-clotting enzyme, psychrotrophic microorganism, *Pseudomonas, Theragra chalcoramma*

was prepared by the addition of 1.6% agar to SSMB.

Each tissue sample of 1.0 g from *Theragra chalcoramma, Pseudosciaena manchurica, Septia esculanta, Clupea pallasi, Acanthodgobius flavinmanus* and *Platycephalus indicus* was added to 100 m*l* of SSMB and was incubated at 10℃ with agitation for 7 days. A loopful of each culture broth was streaked onto SSMA plates, incubated at 10℃ for 7 days, and the colonies were isolated.

Screening for microorganisms producing soymilk-clotting enzyme(s)

Each isolate was incubated into a test tube containing sterilized soymilk containing 3% NaCl adjusted to pH 6.4 with 1.0 M potassium phosphate buffer (pH 4.5), and cultivated at 20℃ with rotary agitation (200 rpm). The isolates which caused soymilk curd formation in less than 3 days were selected and cultivated in enzyme production medium at 20° C for 3 days with rotary agitation (200 rpm). The enzyme production medium contained 5.0% soymilk, 0.2% yeast extract, 0.2% glucose, 0.25% K_2HPO_4 and 3.5% NaCl at pH 7.0. The culture broth was centrifuged at 10,000×g, at 40℃ for 10 minutes and sodium azide at a final concentration of 0.05% was added to the supernatant for preservation at 4℃. Soymilk-clotting activity and proteolytic activity were determined using the supernatant as the enzyme solution.

Assay of soymilk-clotting activity

Soymilk-clotting activity was determined according to the method of Arima *et al.*[18] using soymilk containing 0.5% NaCl adjusted to pH 6.4 with 1.0 M potassium phosphate buffer (pH 4.5) as substrate. One m*l* of the crude enzyme solution and 5.0 m*l* of the substrate, both tempered to reaction temperatures prior to mixing, were mixed and incubated at reaction temperatures in a water bath. Soymilk-clotting time was recorded as the time of intial appearance of solid material on inner surface of the tube while manually rotating the tube in a sharply slanted position. One unit of the milk-clotting activity was defined as the amount of enzyme which clotted 5 m*l* of the substrate in 1 minute under the conditions described above.

Assay of proteolytic activity

Proteolytic activity was determined according to Chen and Levin[19] using 1.0% solubilized casein in 0.05 M phosphate buffer, pH 6.8. One m*l* of the crude enzyme solution and 5.0 m*l* of the substrate, both tempered to reaction temperatures prior to mixing, were mixed and incubated at reaction temperatures for 20 minutes in a water bath. After the reaction was stopped by the addition of 5.0 m*l* of 10% trichloroacetic acid (TCA), the tubes were allowed to stand at room temperature for 15 minutes and were then centrifuged at 10,000×g for 10 minutes. Absorbance at 280 nm of the supernatant was measured against a blank prepared by adding TCA to the substrate prior to the addition of the crude enzyme solution. One unit of the proteolytic activity was defined as the amount of enzyme which increases absorbance at 280 nm by 1.0 under conditions described above.

Characterization of the isolates

Milk-clotting enzyme producing psychrotrophs were identified according to Bergey's Manual of Systematic Bacteriology[20] and Manual of Methods for General Bacteriology.[21]

Results and Discussion

Isolatioon of milk-clotting enzyme producing psychrotrophs

Twenty among 400 isolates caused curd formation in less than 3 days when the isolates were cultivated in soymilk containing 3.0% NaCl adjusted to pH 6.4 at 20℃. NaCl at a concentration of 3.0% was added to all the media used in the isolation to retard the development of pseudomonads which is known to produce notably heat resistant proteases.[22] When supernatants of the culture broths of these isolates were added to soymilk adjusted to pH 6.8, 6.4 or to 6.1, no soymilk-clotting activity was observed in 3 hours at 30, 40, 50 or 60℃. When 0.5% NaCl was added to soymilk, crude enzyme

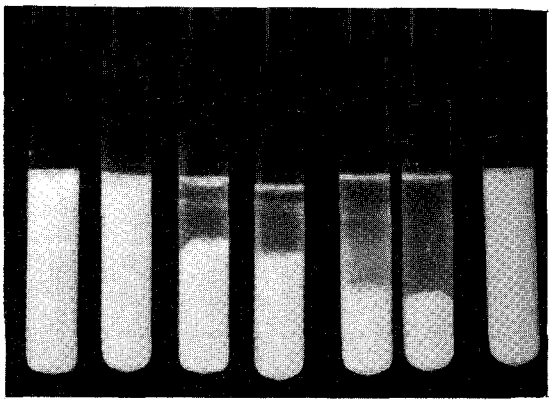

Fig. 1. Clotting of soymilk by crude enzyme solutions. A, Blank (distilled water); B, Isolate No. 71; C, Isolate No. 37; D, Isolate No. 180; E, Isolate No. 45; F, Isolate No. 268; G, Same as F but inactivated at 100℃ for 10 minutes.

Table 1. Soymilk-clotting and proteolytic activities of the isolates

Isolate	SMCA (U/m*l*)[a]	PA (U/m*l*)[b]	SMCA/PA
No. 37	0.08	0.08	1.00
No. 45	0.20	0.14	1.43
No. 94	0.09	0.10	0.90
No. 101	0.18	0.14	1.29
No. 140	0.06	0.05	1.20
No. 163	0.11	0.09	1.22
No. 180	0.15	0.12	1.25
No. 222	0.08	0.13	0.62
No. 268[c]	0.20	0.08	2.50
No. 276	0.07	0.09	0.78
No. 308	0.11	0.12	0.92
No. 317	0.05	0.06	0.83
No. 345	0.10	0.06	1.67
No. 367	0.06	0.06	1.00

[a] Soymilk-clotting activity. One unit of SMCA was defined as the amount of enzyme that clotted 5 m*l* of soymilk in one minute at 40℃

[b] Proteolytic activity. One unit of protease activity was defined as the amount of enzyme which increases absorbance at 280 nm by 1.0 in 20 minutes at 40℃

[c] Isolated from Alaska pollack and used for further experiments.

Table 2. Characteristics of the isolate No. 268

Characteristics	
Cell form	Straight rod
Cell diameter (μm)	0.5~0.7
Cell length (μm)	1.5~2.0
Gram reaction	−
Motility	+
Catalase test	+
Oxidase test	+
O/F metabolism	Oxidative
Sensitivity of O/129[a]	Resistant
Growth at pH 4.5	−
Growth with 0.5% NaCl	−
Growth with 3.5% and 7.0% NaCl	+
Growth with 10% NaCl	+(very weak)
Growth at 4℃	+
Growth at 40℃	−
Hydrolysis of casein	+
Hydrolysis of starch	−
Gelatin liquefaction	−
Nitrate reduction	−
Urease production	−
Acid from glucose	−
Fluorescent pigment formation	−

[a] Vibriostatic agent (2,4-diamino-6,7-diisopropylpteridine phosphate)

solutions of 14 isolates caused curd formation in less than 2 hours at 30℃ and 40℃ (Fig. 1). Even though the minimum concentration of NaCl for soymilk-clotting activity was not determined, the isolates failed to grow with the addition of just 0.5% NaCl to the medium. The isolates showed the best growth in the presence of 3.5% and 7.0% NaCl. No other microbial soymilk-clotting enzymes has been known to require NaCl. Enzyme solutions heated for 10 minutes at 100℃ showed no soymilk-clotting activity (Fig. 1).

Soymilk-clotting and proteolytic activities of crude enzyme solutions of the 14 isolates are shown in Table 1. Isolated No. 268, which showed the highest soymilk-clotting activity and the highest ratio of soymilk-clotting activity to proteolytic acitivity, was used for further experiments. Isolate No. 45, which produced as much soymilk-clotting activity as isolate No. 268, was not used in further studies because of its high proteolytic activity. Even though soymilk-clotting activity is related to proteolytic activity,[16] extensive general proteolytic activity of milk-clotting enzyme leads to formation of bitter peptides, weakening of curd strength, and eventual

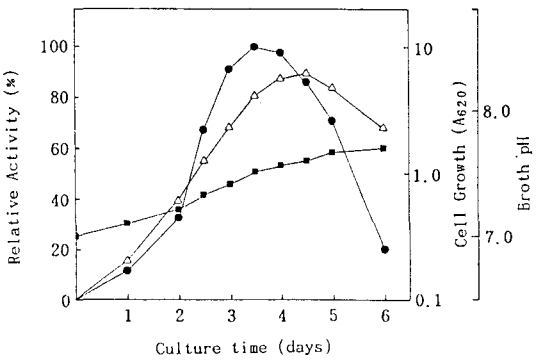

Fig. 2. Time course of growth, broth pH, and soymilk-clotting enzyme activity. ●—●, Soymilk-clotting activity; △—△, Cell growth; ■—■, Broth pH

dissolution of the clot during cheese repening.[17]

Characteristics of Isolate No. 268

Isolate No. 268 was isolated from Alaska pollack (*Theragra chalcoramma*). Some morphological and cultural characteristics are shown in Table 2. Isolate No. 268 was tentatively identified as *Pseudomonas* sp., however, the exact affiliation of the isolate is not known at this time.

Time course of the enzyme production

The time course of the enzyme production, cell growth and broth pH during cultivation of isolate No. 268 in the enzyme production medium at 20℃ is shown in Fig. 2. Maximum soymilk-clotting enzyme activity was obtained at 3.5 days, and the enzyme production was closely related to the cell growth. The pH of the culture broth was steadily increased during cultivation.

Effects of temperature on enzyme activity and stability

The effect of temperature on soymilk-clotting and protease activities of isolate No. 268 is shown in Fig. 3. Maximum clotting activity was observed at 40℃ with a temperature range of 20 to 60℃. The optimum temperature for protease activity of isolate No. 268 was 40℃ and no proteolytic activity was observed at temperature above 60℃. It is thought that the low optimum temperature is due to the psychrotrophic nature of the isolate.

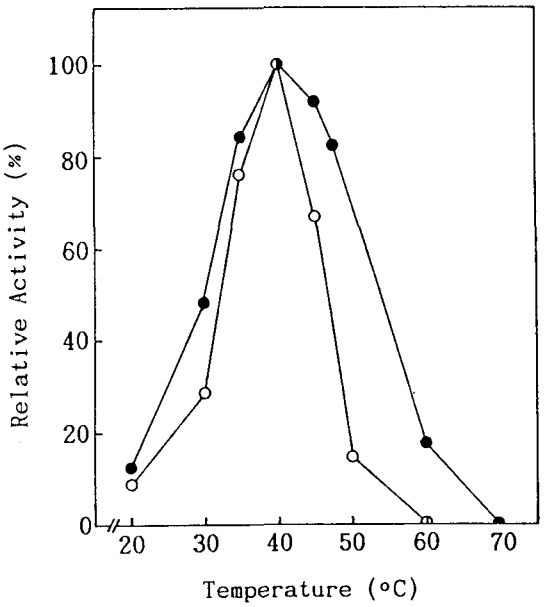

Fig. 3. Effect of temperature on soymilk-clotting and protease activities. ●—●, Soymilk-clotting activity; ○—○, Protease activity

The effect of temperature on stability of soymilk-clotting and protease activities is shown in Fig. 4. The thermostability was measured by preincubating the crude enzyme solution for 30 minutes at various temperatures before the enzyme assay. Both soymilk-clotting and protease activities were stable up to 30℃, and at the optimum temperature for enzyme activity (40℃), 75% of the clotting activity and 65% of the proteolytic activity were retained.

The optimum temperature for soymilk-clotting activity of isolate No. 268 was lower than those of other reported microbial enzymes and the proteolytic activity of the isolate was easily inactivated at low temperatures. All of the reported microbial soymilk-clotting enzymes have optimum temperatures of 60 to 65℃ and are stable at temperatures up to 50℃.[11,16] The use of a microbial enzyme with a low optimum temperature for clotting activity and a low inactivation temperature for protease activity in cheese-like product manufacture might be desirable since less energy is required for curd formation and for inactivation of proteolytic activity at the end of curd formation to prevent deterioration of the product during ripening.

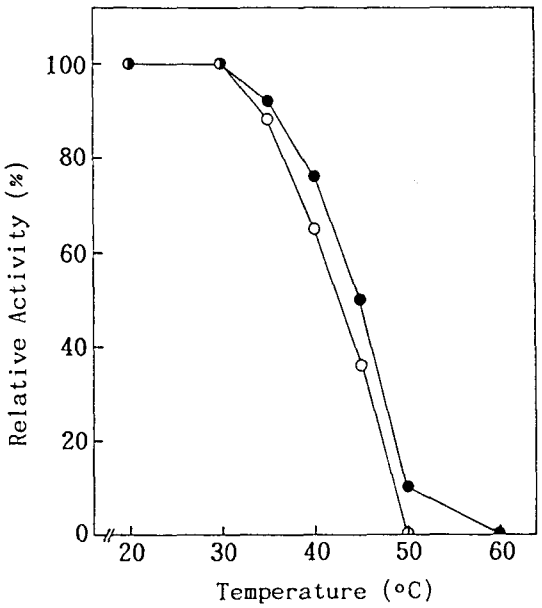

Fig. 4. Effect of temperature on stability of soymilk-clotting and protease activities. ●—●, Soymilk-clotting activity; ○—○, Protease activity

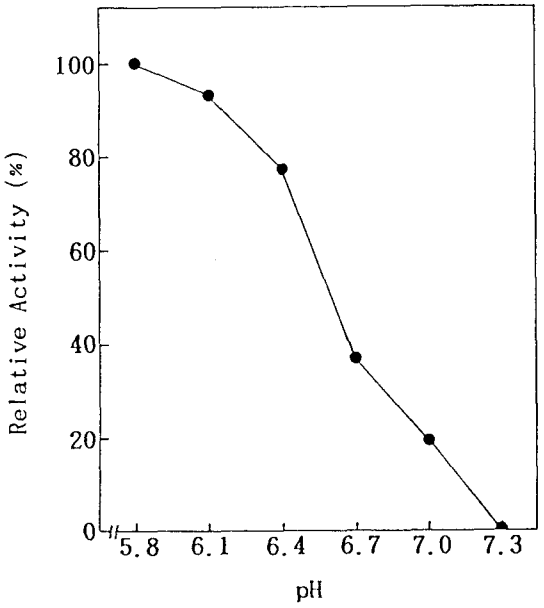

Fig. 5. Effect of pH on soymilk-clotting activity.

Effect of pH on soymilk-clotting activity

The influence of pH on soymilk-clotting activity of isolate No. 268 is shown in Fig. 5. Clotting activities at various pHs were measured by using soymilk with 0.5% NaCl adjusted to pH 5.8 to 6.7 with potassium phosphate buffer or to pH 7.0 and 7.3 with Tris buffer. The activity decreased as the pH increased from 5.8 to 7.3. At pHs lower than 5.8, soymilk was clotted in the absence of the enzyme.

Acknowledgement

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생선으로부터 분리한 두유 응고 효소 생산 호냉성 미생물

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초록 : 생선 시료로부터 대두유를 배지원으로 10℃에서 증식배양하고, 조효소액의 효소활성을 조사하여 대두유 응고활성이 높고 단백질 분해활성이 낮은 호냉성 세균을 최종 선발하여 *Pseudomonas* sp.로 잠정 동정하였다. 조효소액의 대두유 응고활성은 20~60℃에서 관찰되었고 최적온도는 40℃였으며, 단백질 분해활성의 최적온도는 40℃였고 30℃이하 및 60℃ 이상에서는 단백질 분해활성이 나타나지 않았다. 조효소의 활성은 pH 5.8~7.3 범위에서 pH가 증가함에 따라 감소되었다.