Journal of Microbiology and Biotechnology
Vol. 2, No. 1, 14-20, 1992

Purification and Characterization of Soymilk-clotting Enzyme Produced by *Penicillium* sp.

KOO, SUNG KEUN, SANG-OK LEE, AND TAE-HO LEE*

Department of Microbiology, College of Natural Sciences,
Pusan National University, Pusan 609-735, Korea

Received 6 December 1991 / Accepted 28 February 1992

Some microorganisms isolated from soil, including some bacteria and fungi, were found to secrete an extracellular soymilk-clotting enzyme. Among them, an isolated fungus showed the highest soymilk-clotting activity and the strain was assigned to genus *Penicillium* based on its cultural and morphological characteristics, and designated as *Penicillium* sp. L-151K. Soymilk-clotting enzymes A and B produced by *Penicillium* sp. L-151K were purified by ammonium sulfate precipitation and chromatographies on Sephadex G-25, CM-Sephadex, Sephadex G-100 and phenyl-Toyopearl gel. The two purified enzymes A and B were found to be homogeneous by polyacrylamide gel electrophoresis at pH 9.5. The molecular weights of enzyme A and B were 24,000 and 40,000, respectively, by gel filtration on Sephadex G-100. Enzymes A and B coagulated soymilk optimally at 60℃ and were stable up to 50℃. Both enzymes were most active at pH 5.8 for soymilk coagulation, and were stable with approximately 80% of original activity from pH 3.0 to 5.0. Each enzyme was an acidic protease with an optimum pH of 3.0 for casein digestion. The soymilk-clotting efficiency of these enzymes was improved with $CaCl_2$ or $MgCl_2$ when making soymilk-curd.

It is well known that soybeans contain useful human nutrients, especially protein with a high nutritive value and a high percentage of essential fatty acids. Soybeans have been extensively used as the raw material for Korean traditional foods, such as "Doobu" (soybean curd), "Doenjang" (soybean paste), and "Ganjang" (soybean sauce). However, soybean products have a few disadvantages such as a beany flavor and slight bitterness, which are due to the volatile short chain carbonyl compounds (3).

Recently the functional properties of soybean protein has been improved by enzymatic, physical and chemical modification and soybean protein has been used for cheese and yogurt like foods, such as Domiati cheese (5) and Mozzarella cheese (10). Soymilk-protein is known to be coagulated by inorganic salts (calcium and magnesium salts) and organic acids (acetic acid and lactic acid), and α-D-gluconic lactone as a coagulant and lactic acid fermentation method using *Streptococcus thermophilus* or *Streptococcus lactis* can be used for the production of smooth texture curd. Murata *et al.* (6, 7) reported that acid, neutral and alkaline proteinase originated from microorganisms, plants and animals were capable of coagulating soymilk-protein and that among them alkaline and neutral proteinases from microorganims were suitable for the industrial production of soymilk curd with a smooth texture. They also demonstrated that these proteinases showing high proteolytic activity as to soymilk-protein exhibited high soymilk-clotting efficiency and coagulation increased with the addition of $CaCl_2$ or $MgCl_2$ to the soymilk. Furthermore, comparative investigations were also performed on the functional properties of soymilk curd prepared with a soymilk-clotting enzyme in salts and acids (8). However, studies on purification and characterization of soymilk-clotting enzyme have not been undertaken except for *Bacillus* sp., which produced an alkaline protease (9). This paper deals with the screening of microorganisms producing large amounts of soymilk-clotting enzyme. Purification and some

*Corresponding author

Key words: Soymilk-clotting enzyme, soymilk-coagulation, soymilk-clotting protease

properties of soymilk-clotting enzyme from a selected fungus, *Penicillium* sp. L-151K, are described.

MATERIALS AND METHODS

Materials

CM-Sephadex C-50, Sephadex G-100, Sephadex G-25 and standard proteins were obtained from Sigma Chemical Co. Phenyl-Toyopearl 650 M was purchased from Tosoh in Japan. All other chemicals were of reagent grade.

Preparation of Soymilk

Purchased soybeans were soaked in distilled water overnight and then were disrupted using a grinder. The suspension was boiled for 30 minutes at 100℃ and then filtered through cloth. The filtrate was centrifuged at 2, 800×*g* for 10 minutes and the supernatant was used as soymilk, the substrate of soymilk-clotting enzyme.

Isolation of Microorganisms Producing a Soymilk-clotting Enzyme

Microorganisms producing a soymilk-clotting enzyme were isolated from soil samples collected throughout Korea.

The first screening: One drop of a soil suspension diluted with sterile water was spread on an agar plate medium (soymilk 5.0%, dextrose 0.2%, peptone 0.2%, yeast extract 0.2%, KH_2PO_4 0.2%, pH 6.1) followed by incubation at 30℃ for 3 days. The colonies growing on the plate were picked up and maintained on Bennet agar slants (dextrose 1.0%, peptone 0.2%, yeast extract 0.1%, beef extract 0.1%, KH_2PO_4 1.0%, pH 6.0) at room temperature.

The second screening: One loop of each isolate from the first screening was inoculated into a test tube containing 10 m*l* of soymilk adjusted to pH 6.0 with 0.2 M potassium phosphate buffer. Followed by cultivation with shaking at 30℃ for 3 days strains which coagulated soymilk were selected as candidates for further screening.

The third screening: One loop of each isolate from the second screening was inoculated into the medium for enzyme production (soymilk 5.0%, dextrose 0.2%, peptone 0.2%, yeast extract 0.2%, K_2HPO_4 0.5%, pH 6.1) followed by cultivation with shaking at 30℃ for 3 days. The culture broth was filtered through Toyo filter paper No. 2 and centrifuged again at 11,100×g, at 4℃ for 10 minutes. The supernatant was used as the enzyme solution and the one strain which showed the highest soymilk-clotting activity was selected for all the following experiments.

Cultivation and Preparation of Crude Soymilk-clotting Enzyme

In order to investigate the effects of carbon and nitrogen sources, inorganic salts, initial pH, cultivation temperature and aeration on soymilk-clotting enzyme production, the selected fungus, *Penicillium* sp. L-151k, was grown at 30℃ for 3 days in 500 m*l* shake flask containing 100 m*l* of each indicated media composition with addition of 2 ml of seed culture. The culture broth was filtered through Toyo filter paper No. 2 and centrifuged again at 11,100×*g*, at 4℃ for 10 minutes. The supernatant was used as the crude enzyme solution.

Enzyme Assay

Soymilk-clotting activity: One m*l* of soymilk adjusted to pH 6.0 with 0.2 M potassium phosphate buffer was preincubated at 60℃ for 10 minutes, then 0.1 m*l* of the appropriately diluted enzyme solution was added to the soymilk. The clotting activity was determined at 60℃ as described by Arima *et al.* (1). One unit of soymilk-clotting enzyme was defined as the amount of enzyme that clotted 1 m*l* of soymilk, in 1 minute under the above conditions.

Proteolytic activity: The assay mixture contained 1 m*l* of 2.0% casein and 1 m*l* of the appropriately diluted enzyme solution. The reaction was done at 37℃ for 30 minutes, and was stopped by addition of 2 m*l* of 0.44 M trichloroacetic acid, followed by incubation at 37℃ for 20 minutes and filtration through Toyo filter paper No. 2. After 5 m*l* of 0.55 M Na_2CO_3 solution was added to 1 m*l* of filtrate, 1 ml of 2-fold diluted Folin reagent was added and incubated at 37℃ for 20 minutes. The absorbance of the resultant mixture was measured at 660 nm with a Shimadzu UV-240 spectrophotometer.

Protein Concentration

Protein concentration was measured by the absorbance at 280 nm.

Homogeneity of the Purified Enzymes

Discontinuous gel electrophoresis was done on a 7% polyacrylamide gel at pH 9.5 at 4℃ by the method of Davis (2). Protein was stained with Amido Black and then destained with 7% acetic acid in 10% methanol.

Molecular Weight Measurement

The molecular weight of the enzyme was measured by gel filtration on Sephadex G-100 (3×100 cm). The standard proteins used for calibration were lysozyme (M.W. 14,000), α-chymotrypsinogen (M.W. 25,700), ovalbumin (M.W. 45,000) and bovine serum albumin (M.W. 66,000).

RESULTS

Selection and Identification of Soymilk-clotting Enzyme-producing Strain

From the third screening one fungal isolate, designated L-151K showed the highest soymilk-clotting activity among various strains. This strain was used as the representative strain. Based on its cultural and morphological characteristics we assigned this fungus to genus *Penicillium* and designated it as *Penicillium* sp. L-151K.

Culture Conditions for Soymilk-clotting Enzyme Production

Carbon, nitrogen, inorganic source, effect of soymilk concentration, initial pH, cultivation temperature and aeration were investigated in order to establish optimum culture medium and conditions for efficient enzyme production. The proper culture conditions for soymilk-clotting enzyme production are summarized in Table 1.

Time Course of the Enzyme Production

The time course of the enzyme production during cultivation was also examined. A seed culture was grown in a 500 ml shake flask with continuous reciprocal shaking for a day and was inoculated at a concentration of 2% (v/v) into 3 *l* of culture medium containing optimal compositions. The main culture was performed in a 5 *l* fermentor (B. Braun Melsungen Co.) at 32℃. At suitable time intervals small amounts of culture broth were withdrawn and centrifuged and the enzyme activity in the supernatant was assayed together with changes in cell growth and broth pH during cultivation. The results are shown in Fig. 1. Maximum soymilk-clotting enzyme activity and cell growth were observed at 3 to 4 days after the beginning of cultivation.

Purification of Soymilk-clotting Enzyme

Solid ammonium sulfate was gradually added to the crude enzyme solution to 80% saturation with continuous stirring. After it was left overnight the resultant precipitate was collected by centrifugation (Hanil H 50 A-6) at 11,100×*g* for 10 minutes and dissolved in a small volume of 0.01 M sodium acetate buffer (pH 4.0). In order to eliminate the ammonium sulfate present in the enzyme solution dialysis against 0.01 M sodium acetate buffer was performed. However, dialysis was impossible because the dialysis tube was dissolved, probably by the cellulase present in the crude enzyme solution. The elimination of ammonium sulfate in the enzyme solution was carried out by gel filtration on a Sephadex G-25 column (5×30 cm) previously equilibrated with the same buffer. The eluate from the column was fractionated by 10 ml and the active fraction was pooled. The enzyme solution was applied to a CM-Sephadex C-50 column (3×30 cm) previously equilibrated with 0.01 M sodium acetate buffer, pH 4.9. After the column was eluted with the same buffer, a linear gradient of NaCl (0 M-0.5 M) in the buffer was applied. As shown in Fig. 2, the soymilk-clotting activity was separated into two fractions, one of which was eluted without adsorption on the column and designated as enzyme A, the other of which was adsorbed on the column and eluted at concentration of 0.2 M NaCl and designated as enzyme B. Enzyme A and B seem to have different ionic properties and each enzyme was independently pooled for the following stage. The specific activities of both

Table 1. The proper culture condition for soymilk-clotting enzyme production

Medium	Soymilk	5%
	Dextrose	0.2%
	Polypeptone	0.2%
	Yeast extract	0.2%
	K_2HPO_4	0.5%
	Initial pH	6.0%
Other conditions	Temperature	32℃
	Culture time	3 days
	Agitation	120 Rev. stroke (reciprocal)

100 ml of medium per 500 ml shaking flask

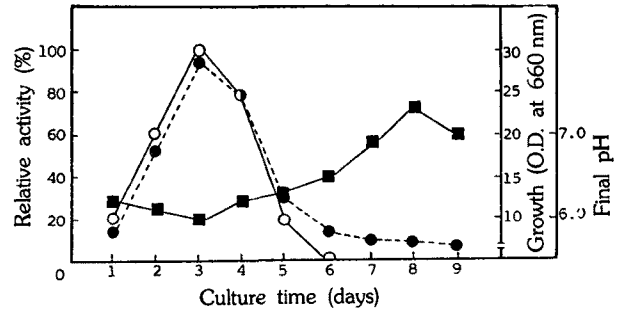

Fig. 1. Time course of growth, final pH and soymilk-clotting enzyme activity.
—○—: soymilk-clotting activity, —●—: growth, —■—: final pH.

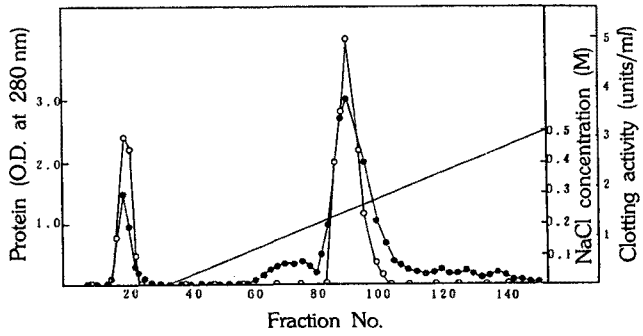

Fig. 2. Elution profile of ion exchange chromatography on CM-Sephadex C-50.
—●—: absorbance at 280 nm, —○—: enzyme activity.

enzymes were increased by approximately two-fold when compared to the previous stage. Enzymes A and B were concentrated by ultrafiltration through a CX-10 membrane and the respective enzyme concentrate was put on a Sephadex G-100 column (2.5×85 cm) equilibrated with 0.01 M acetate buffer, pH 4.0. On elution of the column with the same buffer, the enzyme showing soymilk-clotting activity was eluted as a single peak, but not with a constant ratio of protein to enzyme activity in each fraction. The active fractions were pooled and used for the following step. The specific activities of enzymes A and B were increased by 13-fold and 9-fold, respectively, by this purification procedure. In order to separate impure proteins based upon hydrophobic difference, solid ammonium sulfate was added independently to the pooled active fraction of enzymes A and B to 40% saturation. Each enzyme was applied onto a column of phenyl-Toyopearl 650 M (1.5×4 cm) equilibrated with 0.01 M sodium acetate buffer containing 40 % ammonium sulfate. After the column was washed with the same buffer containing 40% ammonium sulfate, adsorbed proteins were eluted by applying a linear gradient of ammonium sulfate (40%~20%) in this buffer. The active fraction of both enzymes A and B was shown as a single peak with a constant ratio of protein to enzyme activity in each fraction.

Homogeneity of Enzymes A and B

The homogeneity of purified enzymes A and B was examined by polyacrylamide disc gel electrophoresis at pH 9.5. As shown in Fig. 3, both enzymes were homogeneous, migrating as a single band on each gel. Table 2 summarizes the purification procedure and the yield during the purification. Purified enzymes A and B were obtained with recoveries of 4.8% and 14.0%, respectively.

Properties of the Purified Enzymes

Molecular weight of enzymes A and B: The molecular weights of enzymes A and B were estimated to be approximately 24,000 and 40,000, respectively, from the elution volume of gel filtration on a Sephadex G-100 column.

Optimal pH: The soymilk-clotting activities of enzymes A and B were measured at different pHs ranging from 5.8 to 6.8 at 60℃. The soymilk-clotting activity test could not be done lower than pH 5.8, since the protein in soymilk was coagulated without the addition

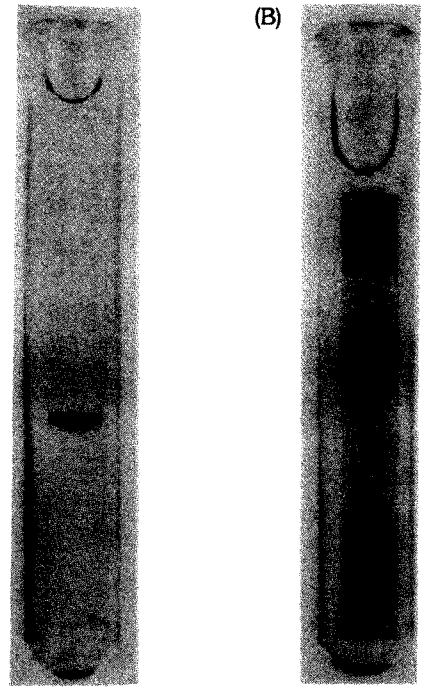

Fig. 3. Polyacrylamide disc gel electrophoresis of the purified soymilk-clotting enzyme A and B.

Table 2. Purification of soymilk-clotting enzymes A and B

Procedure	Volume (ml)	Total protein (O.D. at 280×ml)	Total activity (units)	Specific activity (units/O.D. at 280 nm)	Recovery (%)
Culture broth	4000	12800.0	1600.0	0.1	100.0
Ammonium sulfate	100	1029.5	1544.3	1.5	96.5
Sephadex G-25 column chromatography	280	490.0	1306.0	2.7	81.6
CM-Sephadex C-50	A; 40	42.5	255.0	6.0	15.9
column chromatography	B; 20	120.0	780.0	6.5	48.7
Sephadex G-100	A; 20	1.7	129.0	75.0	6.5
column chromatography	B; 30	9.0	540.0	60.0	27.0
Phenyl-Toyo-pearl	A; 12	0.9	96.0	100.0	4.8
column chromatography	B; 24	3.6	280.0	77.8	14.0

A: soymilk-clotting enzyme A, B: soymilk-clotting enzyme B.

of enzyme solution lower than pH 5.8. Fig. 4-A shows the effects of pH on soymilk-clotting activity. The activities of both enzymes decreased as the pH increased from 5.8 to 6.8.

pH stability: Each enzyme was treated at different pHs, from 2 to 8 for 1 hour at 35℃ and then the remaining activities were measured. As shown in Fig. 4-B, approximately 80 % of the original activity of both enzymes remained after treatment in the range of pH 3 to 5.

Optimal temperature: The soymilk-clotting activities of enzymes A and B were measured at various temperatures (30℃ to 90℃) at pH 6.0. Fig. 5-A shows the effects of temperature on soymilk-clotting activity. Both enzymes A and B had their optimal activity at 60℃ with no activity observed below 30℃.

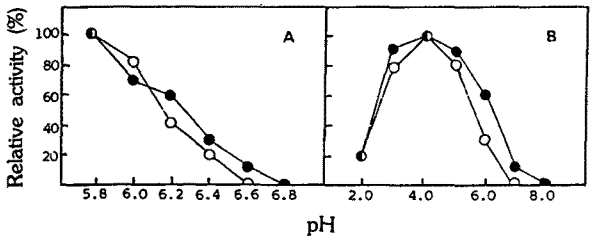

Fig. 4. Effect of pH on activity and stability of soymilk-clotting enzymes A and B.
A: Effect of pH on the soymilk-clotting activity. Soymilk-clotting activity was measured at different pHs, ranging from 5.8 to 6.8, at 60℃. B: Effect of pH on the enzyme stability. One half ml of the enzyme solution and 0.5 ml of buffer were mixed and stored at 37℃ for 1 hour. After adjustment to pH 6.0, the residual activity was measured. —○—: soymilk-clotting enzyme, A, —●—: soymilk-clotting enzyme B.

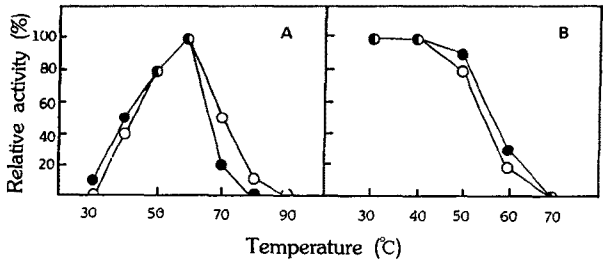

Fig. 5. Effect of temperature on activity and stability of soymilk-clotting enzymes A and B.
A: Effect of temperature on the soymilk-clotting activity. Reaction mxitures containing 5 ml of soymilk and 0.5 ml of enzyme solution were incubated at various temperatures. B: Effect of temperature on the enzyme stability. The enzyme solution was left at various temperatures for 30 min and then the residual activity was measured. —○—: soymilk-clotting enzyme A, —●—: soymilk-clotting enzyme B.

Temperature stability: The thermostability of enzymes A and B at pH 6.0 was measured by 30 minutes of preincubation of each enzyme at various temperatures before the the enzyme assay. As shown in Fig. 5-B, both enzymes are stable up to 50℃, but are rapidly inactivated at temperatures above 50℃.

Effects of Metal Ions and Inhibitors on Soymilk-clotting and Proteolytic Activity of Enzymes: The effects of some metal ions and inhibitors on the soymilk-clotting activity and on proteolytic activity of enzymes A and B were investigated. Each enzyme activity was measured with each compound at final concentration of 1 mM. Table 3 summarizes the results. $FeCl_2$, $CaCl_2$, $SnCl_2$, $MnSO_4$ and $Pb(NO_3)_2$ enhanced the clotting activity of enzyme B by two to three times while the clotting activity of enzyme A was enhanced by these compounds to a lesser degree. Enzyme A was most enhanced by $MgCl_2$. It was impossible to examine the effect of the metals such as $HgCl_2$, $ZnSO_4$, $BaCl_2$, $Cu(CH_3COOH)_2$, $CoCl_2$ and $AgNO_3$ beacuse these metals coagulated the soymilk without enzymatic action. The proteolytic activity of enzymes was measured with casein as the substrate. $AgCl_2$ and iodoacetic acid increased the proteolytic activity of enzyme A but enzyme A was inhibited by $HgCl_2$ and $ZnSO_4$ significantly, whereas enzyme B was not affected so much by these metals.

Table 3. Effect of metal compounds and inhibitors on soymilkclotting activity and proteolytic activity of enzymes

Compound	Relative soymilk-cloting activity (%)		Relative proteolytic activity (%)	
	Enzyme A	Enzyme B	Enzyme A	Enzyme B
None	100	100	100	100
KCl	92	95	94	93
$FeCl_2$	128	250	95	110
$CaCl_2$	140	200	97	102
$SnCl_2$	140	220	97	92
$MnSO_4$	120	300	101	91
$Pb(NO_3)_2$	100	120	98	82
$MgCl_2$	210	280	79	104
$HgCl_2$	–	–	50	93
$ZnSO_4$	–	–	51	90
$BaCl_2$	–	–	92	99
$Cu(CH_3COOH)_2$	–	–	88	85
$CoCl_2$	–	–	101	90
$AgNO_3$	–	–	172	92
EDTA	90	100	96	98
Cysteine	80	95	97	104
Iodoacetic acid	98	105	135	160

–: not determined

Proteolytic Activity on Casein: The proteolytic activities of enzymes A and B on casein were measured at different pHs. As results, both enzymes showed highest activity at pH 3.0 (data not shown) and these enzymes seemed to be typical acidic proteases.

Effects of $CaCl_2$ and $MgCl_2$ on Soymilk-clotting Activity: The effects of $CaCl_2$ and $MgCl_2$ concentration on soymilk-clotting activity of enzyme A and B were examined and Fig. 6 shows the results. Both enzymes were sensitive to calcium and magnesium ions for soymilk coagulation. As the concentration of $CaCl_2$ and $MgCl_2$ increased, the coagulability of soymilk was also increased. Measurement at concentrations of more than 3 mM was impossible because soymilk was coagulated at these concentrations without the addition of an enzyme solution.

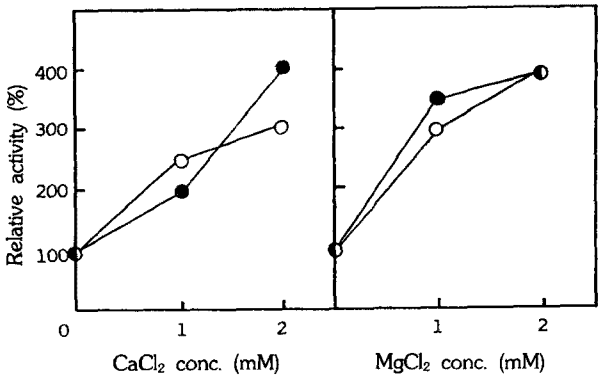

Fig. 6. Effect of $CaCl_2$ and $MgCl_2$ concentration on soymilk-clotting activity.
—○—: soymilk-clotting enzyme A, —●—: soymilk-clotting enzyme B.

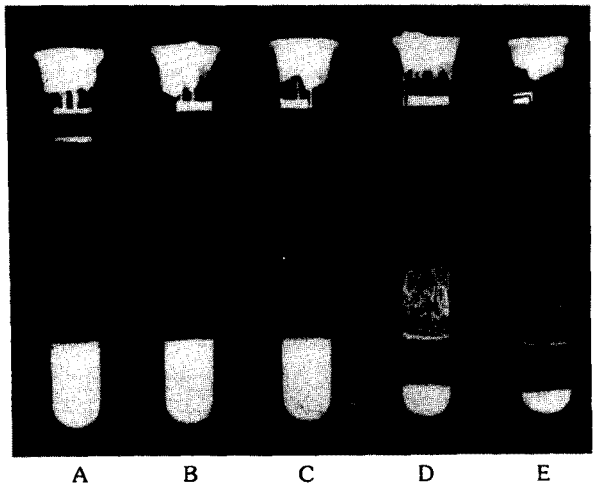

Fig. 7. Coagulation of soymilk by the enzyme reaction.
A: water, B: inactivated enzyme A, C: inactivated enzyme B, D: soymilk-clotting enzyme A, E: soymilk-clotting enzyme B.

Soymilk Coagulation by Enzymes A and B: A 0.1 ml enzyme solution was added to 2 ml of soymilk, adjusted to pH 6.0 with 0.5 M potassium phosphate buffer. After incubation at 60℃ for 5 minutes, the resultant coagulum was centrifuged at 1,100×g for 5 minutes. Fig. 7 shows that soymilk was coagulated by the soymilk-clotting enzymes A and B (Fig. 7-D and 7-E) while no coagulation was observed either for the addition of distilled water (Fig. 7-A) or for the enzymes treated at 100℃ for 5 minutes (Fig. 7-B and 7-C). These observations indicated that soymilk-clotting was caused by the enzyme reaction.

Hydrolysis of Soymilk-protein by the Soymilk-clotting Enzymes: One ml of soymilk, adjusted to pH 6.0 with 1 M sodium phosphate buffer, was preincubated at 60℃ for 10 minutes and 0.1 ml solution of soymilk-clotting enzymes A and B was added and incubated at the same temperature. At suitable time intervals, a small amount of the reaction mixture was withdrawn and the curd was removed by centrifugation. The soluble peptide in the supernatant was estimated by Lowry's method. Fig. 8 shows the time course of hydrolysis of soymilk-protein by enzymes A and B. The amount of released peptides increased as reaction time increased

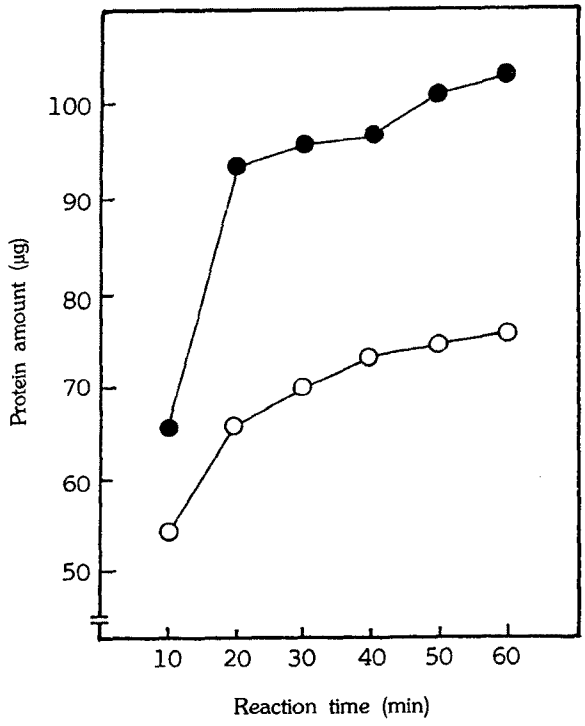

Fig. 8. Hydrolysis of soymilk-protein by enzyme A and B.
—○—: soymilk-clotting enzyme A, —●—: soymilk-clotting enzyme B.

but it rapidly leveled off after 1 hour. The results show that soymilk-clotting efficiency is related to proteolytic activity, and that coagulation of soymilk protein requires that the protein be hydrolyzed by the enzymes to some extent. This result is in approximate agreement with results obtained from plant proteinases, such as bromelain and ficin, as reported by Fuke and Matsuoka (4). The soluble protein produced by the enzyme reaction was an extremely small amount (about 0.2% of the total protein in soymilk is solubilized) and it confirmed that most of soymilk-protein was precipitated by the enzymes. Thus, it was shown that the enzymes from *Penicillium* sp. precipitated the soymilk-protein efficiently.

DISCUSSION

Studies on the coagulation of soymilk-protein by commercial proteinases originating from microorganisms, plants and animals have been carried out. However, the studies on isolates from soil samples which secrete soymilk-clotting enzymes have not been reported, except for *Bacillus* sp. K-295G-7 (9). We were able to isolate fungi capable of coagulating soymilk-protein from 200 soil samples. This suggests that fungi having such a property are widely distributed in nature.

Penicillium sp. produced two enzymes A and B, which are separated into two fractions during CM-Sephadex chromatography. Each purified enzyme could coagulate the soymilk-protein, but the enzymes heated to 100℃ for 5 minutes could not. It is apparent that the coagulation of soymilk protein was due to the reaction caused by the enzymes from *Penicillium* sp. and, furthermore, it is clear that the coagulation was not due to the combined action of two or more enzymes but due only to a simple enzyme action. However, even with differences in ionic properties and enzymatic efficiency between enzymes A and B, enzymatic properties of enzymes A and B, such as thermal activity and stability profiles or pH activity and stability profiles, were in agreement between the two enzymes. Judging from these results it seems that the two enzymes can coagulate protein efficiently under the same optimum conditions, that is, at pH 5.8 and 60℃.

Enzymes I and II produced by *Bacillus* sp. K-295G-7 (9) showed similar molecular weights and isoelectric points and both were alkaline proteases. *Penicillium* sp. L-151K of this study produced two isozymes with greatly different molecular weights and ionic properties. Both enzymes were acidic proteases with an optimum pH of 3.0 for casein digestion.

The curd made with the enzyme system from *Penicillium* sp. L-151K has a smooth texture and a mild taste without bitterness or a beany flavor. Most of the soymilk-protein was precipitated by the enzymes (about only 0.2% of the total protein is solubilized) and the enzymes were very efficient in coagulating soymilk. Especially, the addition of $CaCl_2$ or $MgCl_2$ remarkably increased the coagulation of soymilk. These results suggest that curd obtained through this enzymatic action can serve as a material for further development of food items, and the procedure may be widely applicable to food processing.

Acknowledgement

This paper was supported by NON DIRECTED RESEARCH FUND, Korea Research Foundation, 1990. The support is deeply appreciated.

REFERENCES

1. **Arima, K., S. Iwasaki, and G. Tamura.** 1967. Milk clotting enzyme from microorganisms. *Agric. Biol. Chem.* **31**: 540-545.
2. **Davis, B.J.** 1964. Disc electrophoresis-II. Method and application to human serum proteins. *Ann. N. Y. Acad. Sci.* **121**: 404-427.
3. **Fujimaki, M., S. Arai, N. Kirigawa, and Y. Sakurai.** 1965. Studies on flavor components in soybean. *Agric. Biol. Chem.* **29**: 855-863.
4. **Fuke, Y., and H. Matsuoka.** 1984. Preparation of fermented soybean curd using stem bromelain. *J. Food Sci.* **49**: 312-313.
5. **Metwalli, N.H., S.I. Shalabi, A.S. Zahran, and O. EL-Demerdashi.** 1982. The use of soybean milk in soft cheese making: Organoleptic and chemical properties of Domiati cheese made from a mixture of soybean milk and whole milk. *J. Food Technol.* **17**: 297-305.
6. **Murata, K., I. Kusakabe, H. Kobayashi, M. Akaike, Y.W. Park, and K. Murakami.** 1987. Studies on the coagulation of soymilk-protein by commercial proteinases. *Agric. Biol. Chem.* **51**: 385-389.
7. **Murata, K., I. Kusakabe, H. Kobayashi, H. Kiuchi, and K. Murakami.** 1987. Selection of commercial enzymes suitable for making soymilk-curd. *Agric. Biol. Chem.* **51**: 2929-2933.
8. **Murata, K., I. Kusakabe, H. Kobayashi, H. Kiuchi, and K. Murakami.** 1988. Functional properties of three soymilk curds prepared with an enzyme, calcium salt and acid. *Agric. Biol. Chem.* **52**: 1135-1139.
9. **Park, Y.W., H. Kobayashi, I. Kusakabe, and K. Murakami.** 1987. Purification and characterization of soymilk-clotting enzymes from *Bacillus* sp. K-295G-7. *Agric. Biol. Chem.* **51**: 2343-2340.
10. **Yang, C.S., and M.V. Taranto.** 1982. Textural properties of Mozzarella cheese analogs manufactured from soybeans. *J. Food Sci.* **47**: 906-910.