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Studies on the Characteristics of the Soybean Protein Coagulating Enzyme from Microorganism and the Soy Cheese-Like Food(Curd)

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Abstract

Microorganisms, including the strain IJ-3 isolated from soil, were found to secrete an extracellular soybean protein coagulating enzyme and the strain, IJ-3, was identified as Genus *Bacillus* according to the Bergey's manual. The enzyme coagulated protein in soymilk, thus forming a curd at pHs 5.8~6.4 and at 55~75° C. The optimum temperature for soybean protein coagulating activity was 65~75° C and the enzyme was stable at temperature below 50° C and was found to be stable with about 60~100% of the original activity at a with pH ranges (pH 6~7). The molecular weight of enzyme was estimated to be 28,000 by SDS-PAGE. The curd formed with the enzyme from *Bacillus* sp. IJ-3 has a smooth texture, and a mild taste without any bitterness or a beany flavor.

Key words : soybean protein coagulating enzyme, soy cheese-like food (curd)

INTRODUCTION

With the growing population the seriousness of the world protein supply become conspicuous among the other problems. The use of, therefore, the so-called unconventional sources of protein, such as fish protein concentrates, single cell protein and legume seed protein as substitutes for food protein source has been studied actively. Among them, the remarkable progress which has been achieved in the manufacture of the soybean. Soybeans are well known for variations in colar, size and shape of the seed and other physical properties as well as their chemical composition[1,2].

Soybeans contain about 35% of protein, and the amino acid composition of soybeans is similar to that of meat. Therefore, it has been recognized that soybeans are a food material with a high nutritive value because they contain good quality protein, in addition, high percentage of essential fatty acids. For these reasons, soybeans have been extensively used as "bean curd", "soy sauce", "bean paste" etc., from acient times in Asia.

Most of soybeans, however, have been utilized as traditional raw materials because of their functional properties such as their beany flavor, hydration, foaming, emulsifying etc. and especially it has a little too difficult for food manufacturing of the new rheological cheese-like food.

Several workers[3-9] have tried to improve functional properties (emulsion stability, foam stability, gelation etc.) of soybean protein by physical, chemical and enzymatic modifications.

It has been reported that bromelain, a plant protease, is able to clotted the soybean protein. Mohri and Matsushita[8] and Fuke *et al.*[9] reported that soymilk, acid precipitated soybean protein, and isolated 11S globulin were clotted by bromelain. During the ripening, however, it was found that the curd formed with the bromelain could be produced the bitterness.

The objective of this paper obtained the basic data for production of the soy protein curd different from Tofu(bean curd) by divalent metals through the screening of organism producing a soybean protein coagulating enzyme from soil. The contents will be concentrated as fallows :

1) enzyme production
2) research for enzymatic properties
3) curd formation with microbial enzyme (IJ-3 stra-

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in enzyme)

4) curd ripening and evaluation

MATERIALS AND METHODS

Isolation sources of microorganisms

Soil samples, collected from various districts of southern Korea, were tested for the isolation of microorganisms producing a soybean protein coagulating enzyme.

Isolation of microorganisms

Samples of soil were spread on soymilk agar plates (A in Table 1) and incubated at 30° C so long as to develop distinct colonies to transfer to the slant. Bacteria were stored on agar slant (B in Table 1) at 10° C. For the enzyme production (C in Table 1), after medium was sterilization at 120° C for 15min, one loop of each isolate from the slants was inoculated into the medium, and incubated at 35° C for 7 days on a reciprocal shaker.

Microorganisms

All strains tested were subcultured from the culture collection of our laboratory isolated from soil. After cultivation, the culture broth was centrifuged (10, 000rpm, 10min) and the supernatant was used for enzyme assay. Five bacterial isolates designated as IJ-3, HP-1, KK-5, JY-10 and WS-2 showed relatively high soybean protein coagulating activity.

Assay for soymilk-clotting activity

For assay of the enzyme activity, 5ml of the substrate solution (soymilk adjusted to pH 6.0 with phosphoric acid) was added to 0.5ml of enzyme solution of the suitable dilution at 65° C. The time to make the fragment of curd was measured with stop watch. The amount of enzyme that clotted 5ml of soymilk in 1min under the above conditions was defined as 1 soymilk-clotting unit according to the Arima *et al.*[10].

Polyacrylamide gel electrophoresis

Analytical gel electrophoresis was performed at pH 4.3 using 7.5% gel according to the method of Davis[11]. Protein was stained with Coomassie Brilliant Blue R and then destained with 7% acetic acid in 10% methanol.

Molecular weight determination

SDS-PAGE was done in 0.1% SDS-0.1M sodium phosphate buffer solution (pH 7.2) at 8mA per gel by the method of Weber and Osborn[12], and molecular weight markers (BDH Biochemicals, 14,300-71,500) were used.

Soy cheese-making procedure

Making of soy cheese-like food was tried according to the procedure described by Fuke *et al.*[9]. The soymilk (3 liters) was held at 30° C and the stater was added in the proportion of 2 % of the total volume of soymilk. After 1~2 hour reaction, temperature was increased to 65° C for enzyme action and coagulant (IJ-3 enzyme) was added to the vat when the acidity reached to 0.2%. After cutting and cooking, the whey was drained. The obtained curd block was milled, salted, hooped and pressed. The blocks were sealed with paraffin and ripened at 12° C. Samples were analyzed at 0, 2, 4 and 6 months of ripening.

Chemical analysis of soy cheese-like food samples

This included determinations of moisture by heating at 105° C to constant weight, pH, and NaCl according to the IDF standard[13], and fat by ether extraction. The nitrogen soluble at pH 6.0 (SN), the nitr-

Table 1. Composition of media (%)

Medium	Soymilk	Glucose	Peptone	Yeast ex.	KH_2PO_4	Agar	$CaCl_2$	pH
A	5	0.2	0.2	0.2	0.2	1.5	0.01	6.0
B		1.0	0.2	0.2	1.0	1.5		
C	5	0.5	0.2	0.2	0.5		0.01	6.0

A ; Composition of medium for 1st screening B ; Composition of medium for slant
C ; Composition of medium for enzyme production

ogen soluble in 12.5% TCA (NPN, non-protein nitrogen) and total nitrogen (TN) were determined by the Kjeldahl method.

Organoleptic tests

Soy cheese-like food samples were evaluated for flavor quality as well as body and texture.

RESULTS AND DISCUSSION

Results of screening tests

The bacteria which clotted soymilk within 30min. were listed in Table 2.

Isolated strain No. IJ-3 produced the enzyme which was characterized by its strong soybean protein coagulating activity and stability of enzyme production among the 5 strains, so that further tests on the other strains were abandoned.

Table 2. Results of screening tests for soybean protein coagulating enzyme

Bacteria strain No.	pH of broth	Coagulating time (min)
IJ-3	8.3	3.5
HP-1	8.2	5.0
KK-5	7.9	8.5
JY-10	8.0	15.0
WS-2	8.1	30.0

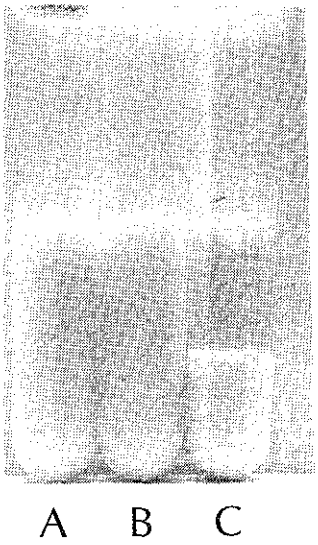

Fig. 1. Coaulation of soymilk by enzyme reaction.
A : Water, B : Inactivated enzyme
C : Strain IJ-3 enzyme

Production of soybean protein coagulating enzyme with the strain IJ-3

One half ml of enzyme solution was added to 5ml of soymilk adjusted to pH 6.0 with phosphoric acid. After incubating at 65° C for 5min., the resultant coagulum was centrifuged at 3000rpm for 5min. Fig. 1 shows that soymilk was clotted by microbial enzyme produced by strain IJ-3 (C in Fig. 1), while no coagulum was observed either by addition of water (A in Fig. 1) or by the addition of the enzyme treated at 100° C for 5 min (B in Fig. 1).

Soybean protein coagulating enzyme production by *Bacillus* sp. IJ-3

The strain, IJ-3, was identified as Genus *Bacillus* according to the Bergey's manual[14] (Table 3).

One and half liters of the enzyme production medium (C in Table 1), containing a small amount of silicone defoamer, was poured into a 3-liter jar fermentor, and sterilized at 121° C for 15min in an autoclave. The seed culture (250ml) of *Bacillus* sp. IJ-3, which grown in the same medium in 500ml Erlenmeyer flask at 35° C for 2 days on a shaker, was innoculated into the fermentor. After appropriate culture with aeration, culture broth was centrifuged and the clear supernatant was assayed for soybean protein coagulating activity.

Fig. 2 shows the time course of cultivation for enzyme production. The result showed that the maximum soybean protein coagulating activity, 0.3~0.4

Table 3. Characteristics of IJ-3 strain isolated from soil

Properties		Properties	
Gram staining	+	*Production of*	
Shape	Rod	yellow pigment	+
Motility	+	urease	−
Catalase	+	Decomposition of	
Oxidase	−	casein	+
Acid from glucose	+	starch	+
		Utilization of sugars	
O-F test	O+	glucose	+
	F−	arabinose	+
V-P test	+	xylose	−
Indole formation	−	sorbose	−

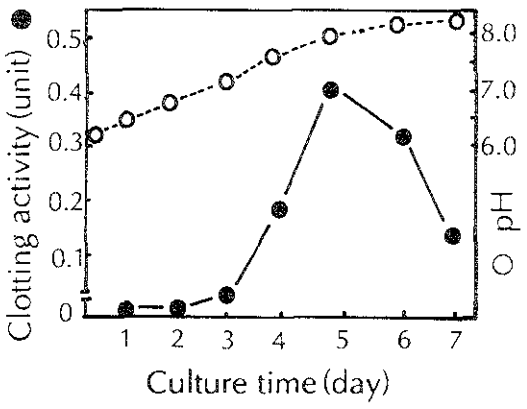

Fig. 2. The time course of cultivation by strain IJ-3.

unit/min, was observed after about 5 day of cultivation.

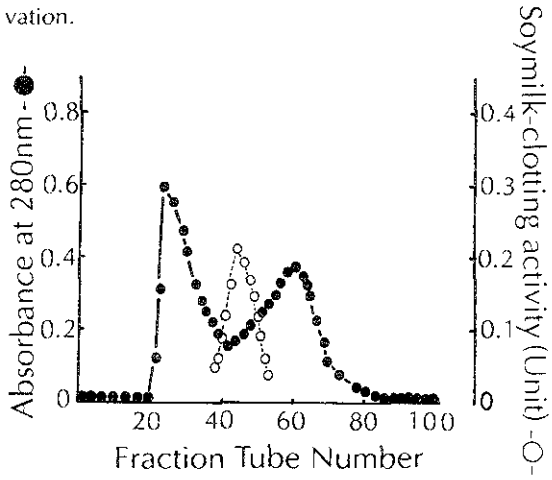

Fig. 3. Elution profile of soybean protein coagulating enzyme on sephadex G-100 column chromatography.

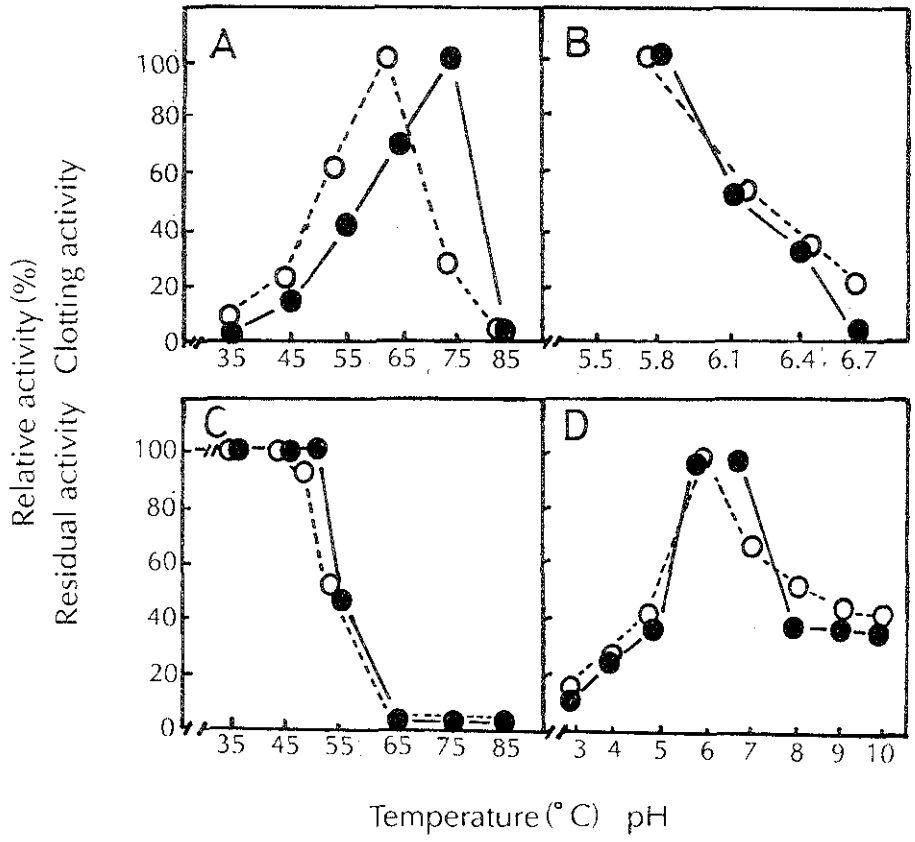

Fig. 4. **Effect of temperature and pH on activity and stability of soybean protein coagulating enzyme IJ-3.**
A ; Effect of temperature on the soybean protein coagulating activity.
B ; Effect of pH on the soybean protein coagulating activity.
C ; Effect of temperature on the stability of the enzyme.
D ; Effect of pH on the stability of the enzyme.
—●—, crude enzyme ; —○—, purified enzyme

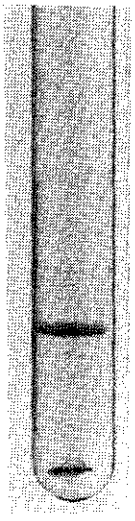

Fig. 5. Polyacrylamide gel electrophoresis of strain IJ-3 enzyme.
The electrophoresis was done at 3mA per tube for 3hr.

Properties of enzyme from *Bacillus* sp. IJ-3

Using the ammonium sulfate fractionation and Sephadex G-100 (Fig. 3), extracellular protein from *Bacillus* sp. IJ-3 was concentrated and gel filtrated. The active fractions after gel filtration were collected and load to the CM-Cellulose Column Chromatography. The enzyme, finally, was purified about 7.1-fold with overall yield of 33.1% through the 2nd gel filtration.

Fig. 4 shows the properties of enzyme from *Bacillus* sp. IJ-3. The enzyme exhibited maximum activity at arround 65°C at pH 6.0, and no activity was observed at 35°C or 85°C (Fig. 4-A). On the other hand, the activity decreased as the pH increased from 5.8 to 6.7 (Fig. 4-B). However, the soybean protein coagulating activity test could not be carried out

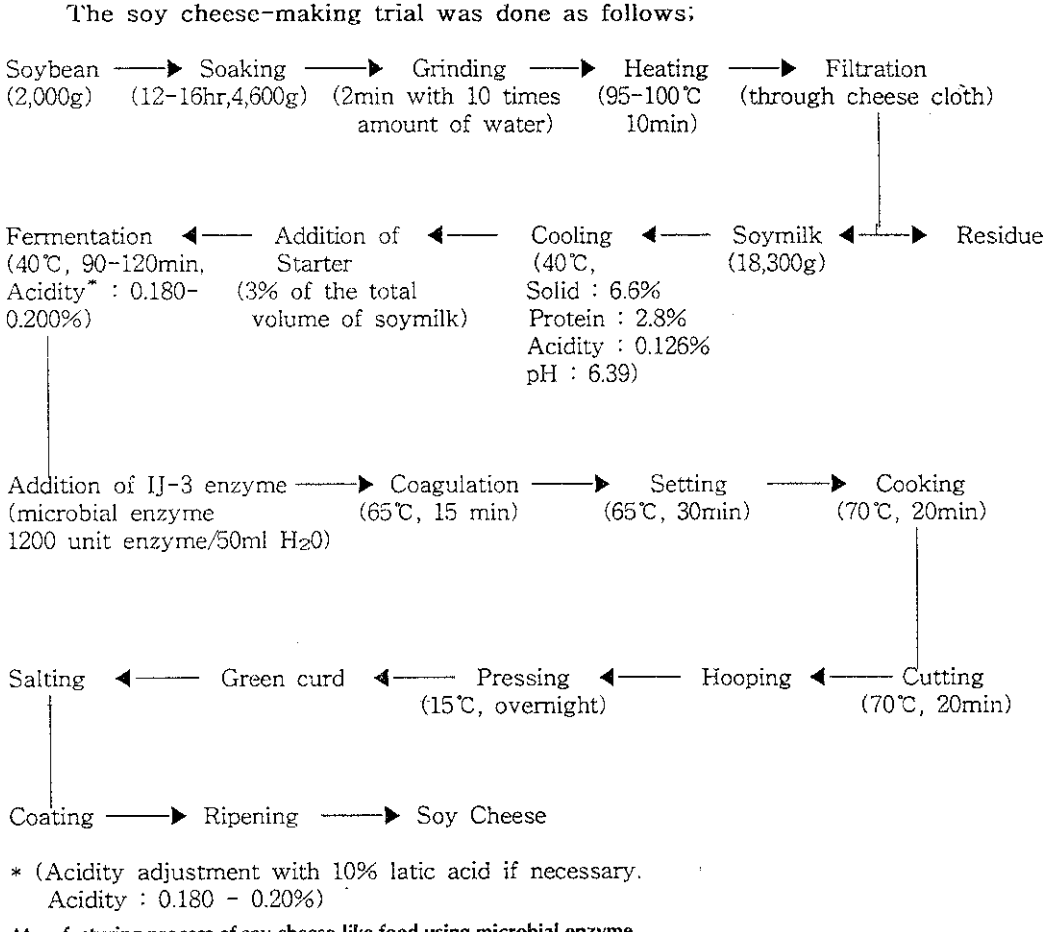

Fig. 6. Manufacturing process of soy cheese-like food using microbial enzyme.

Table 4. **Composition of soy cheese-like food produced using IJ-3 enzyme as soymilk coagulant**

Ripening time (months)	Moisture (%)	Fat (%)	Protein (%)	pH	NaCl (%)
0	42	21.6	25.7	6.0	2.1
2	41.3	21.6	25.7	6.18	2.2
4	40.1	21.5	25.8	6.20	2.2
6	37.6	21.4	25.8	6.22	2.3

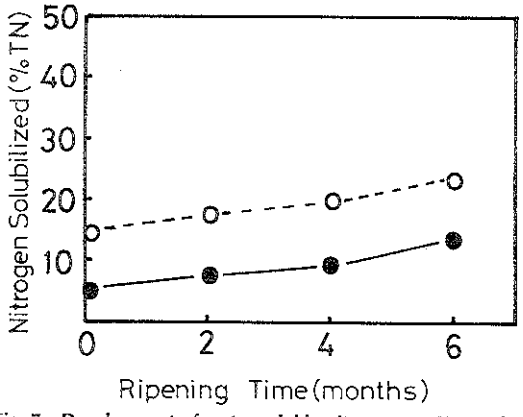

Fig. 7. **Development of water soluble nitrogen at pH 6.0 (SN) and non-protein nitrogen (NPN) during ripening.**
—●—, SN ; —○—, NPN

below pH 5.8 because soymilk coagulates without any addition of the enzyme solution. The thermal and pH-stabilities are shown in C and D in Fig. 4. The enzyme was stable at temperatures below 50°C when treated at pH 6.0 for 30min (Fig. 4-C). On the other hand, about 100% of original activity remained after treament in a pH 6.0 at 35°C for 1hr (Fig. 4-D).

Homogeneity and molecular weight of soybean protein coagulating enzyme

The homogeneity of enzyme was examined by PAGE at pH 4.3. As shown in Fig. 5, enzyme was found to be homogeneous which migrated as single band on the gel. The molecular weight of enzyme was determined to be 28,000 by SDS-PAGE. The molecular weight of enzyme is similar to those of subtilisin BPN' and subtilisin Carlsberg[15].

Production of soy cheese-like food (curd) using the microbial enzyme

To evaluate the enzyme from *Bacillus* sp. IJ-3 as a coagulant of soy protein, a soy cheese-making trial was carried out (Fig. 6).

A coagulant added to the soymilk was 1200 soybean protein coagulating unit and clotting times of IJ-3 was 15min. The soy protein curd particles made with IJ-3 were smaller than the milk curd particles and showed stronger water absorption than the milk curd during cooking. The moisture of the curd decreased to about 4% and pH increased slightly during ripening (Table 4).

The rate and extent of proteolysis was estimated by the level of soluble nitrogen formed during ripening.

As shown in Fig. 7, IJ-3 curd showed regular increases in the NPN and SN with ripening period.

Organoleptic evaluation

Neither bitterness nor rancidity developed even after 6 months of ripening. As to the texture, there was no significant problem, although IJ-3 curd was slightly more mealy than the milk cheese.

It is well known that soymilk curd is produced by means of isoelectric point precipitation and chemical coagulations by divalent metals or glucono-δ-lactone. This is the novel report which deals with the production of soy protein curd formed by using a soybean protein coagulating enzyme from a microorganism. Fuke *et al.*[9] reported that bromelain and ficin coagulate protein in soymilk, and the resulting curd has soft and smooth texture. The curd formed with bromelain, however, has a problem of being slightly bitter. On the other hand, the curd made with the enzyme from *Bacillus* sp. IJ-3 was suggested to be more useful as food items than those made with coagulants such as divalent cations, acid and plant protease, etc. because of its mild taste without any bitterness.

From this result, the curd obtained by enzyme action serves as a material for further development of food items, and the procedure may be widely applicable in food processing.

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미생물에서 얻어지는 대두단백응고효소의 성질 및 대두 치즈화 식품(커드)에 대한 연구

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요 약

토양에서 분리한 균주 IJ-3를 포함하는 미생물이 균체외의 두유응고 효소를 분비하였다. 균주 IJ-3는 Bergey's manual에 의해 *Bacillus*속의 균체로 확인되었다. 두유응고 효소는, pH 5.8~6.4 및 55~75°C 이하에서 커드를 형성하였다. 두유응고 활성에 대한 최적온도는 65~75°C였고, 효소는 50°C 이하의 범위의 온도에서 안정하였으며, pH 범위(pH 6~7)에서 효소고유 활성의 60~100%의 안정성을 보였다. 효소의 분자량은 SDS-PAGE에서 28,000으로 추정하였다. *Bacillus* sp. IJ-3 효소로 커드는 부드러운 조직과 쓴맛이나 콩비린내가 없는 순수맛을 보였다.