Purification and Characterization of Soymilk-clotting Enzymes from *Bacillus* sp. K-295G-7

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Received March 3, 1987

Enzymes I and II, which have a high soymilk-clotting activity, produced from K-295G-7 were purified by chromatographies on Sephadex G-100, CM-cellulose, hydroxylapatite, and 2nd Sephadex G-100.

The two purified enzymes were found to be homogeneous by polyacrylamide gel electrophoresis (PAGE) at pH 4.3. The molecular weights of enzymes I and II were 28,000 and 29,500 by SDS-PAGE, and their isoelectric points were 9.22 and 9.45, respectively. Enzymes I and II coagulated soymilk optimally at 65°C and were stable up to 45°C. Both enzymes were most active at pH 5.8, for soymilk coagulation between pH 5.8 to 6.7, and were stable with about 50~100% of the original activity from pH 5 to 10.

Each of the purified enzymes was a serine protease with an optimum pH of 9.0 for soy protein isolate (SPI) and casein digestions, because these enzymes were inhibited completely by diisopropylfluoro-phosphate (DFP).

The soymilk-clotting activity to proteolytic activity ratio of the enzyme II was 3 times higher than that of enzyme I. Enzymes I and II were more sensitive to the calcium ion concentration in soymilk than bromelain is.

Soybean contains useful human nutrients such as proteins, lipids and sugars. Soybean protein has a high nutritional value, thus the amount of soybean production in the world is about the same as that of meat production.¹⁾ Soybean has been used as material for Japanese traditional foods such as tofu, yuba, miso, and soy sauce from ancient times. Soy protein is now much less used for its functional properties as a food material except as an additive for meat and marine products.²⁾ Although soymilk has been produced as a milk substitute, it has not become very popular in Japan. Fuke and Matsuoka^{3,4)} have investigated the production of a cheese-like food from soymilk by treatment with plant proteases. However, the cheese-like product of bromelain has the problem of being slightly bitter.

In a preceding paper,⁵⁾ we reported the screening of microorganisms producing

soymilk-clotting enzymes, and the production and investigation of some properties of the enzyme. We also reported that the curd made with the enzyme from K-295G-7 had a smooth texture and a mild taste without any bitterness. Recently, we found that the isolated microorganism belonged to the gram-positive *Bacillus* species and it produced an alkaline protease.

We have undertaken the purification of the enzyme having high soymilk-clotting activity. The soymilk-clotting activity was separated into two fractions (enzymes I and II) during the purification steps. This paper deals with the purification and characterization of these two enzymes produced by *Bacillus* sp. K-295G-7.

MATERIALS AND METHODS

Materials. SPI (soy protein isolate) powder was supplied from Nisshin Seifun Co., Ltd. and soymilk was from

Kibun Food Chemifa. Co., Ltd. Bovine serum albumin, ovalbumin, elastin, diisopropyl-fluorophosphate (DFP), and soy bean trypsin inhibitor (STI) were from Sigma. Chymostatin, leupeptin, antipain, E-64, and pepstatin were from the Protein Research Foundation of Japan. Sephadex G-100 was obtained from Pharmacia, CM-52 was from Whatman, ampholite was from LKB, and hydroxylapatite was from Seikagaku Kogyo. All other chemicals were of reagent grade.

Culture Conditions. K-295G-7 was cultured in a 5-l jar fermentor. The medium composition and culture conditions were described previously.⁵⁾

Enzyme assay. Soymilk-clotting activity. Soymilk-clotting activity was measured by the method described previously.⁵⁾

Proteolytic activity. The assay mixture contained 1 ml of 1.2% SPI (or hemoglobin or casein) in 0.1 M Tris-HCl buffer (pH 9.0) and 200 μ l of the enzyme solution. The reaction was done at 50°C for 30 min, and was stopped by the addition of 1 ml of 0.55 M trichloroacetic acid, after which the absorbance of the filtrate was measured at 280 nm. One unit of the activity was defined as the amount of enzyme which increases the absorbance 1.0 at 280 nm under the conditions described above.

Protein concentration. Protein was measured by the absorbance at 280 nm, assuming that the absorbance at the concentration of 1 mg/ml is 1.0.

Polyacrylamide gel electrophoresis. Analytical gel electrophoresis was done at pH 4.3 using 7.5% gel by the method of Davis.⁶⁾ Protein was stained with Coomassie brilliant blue R and then destained with 7% acetic acid in 10% methanol.

Molecular weight measurement. The molecular weight of the enzymes was measured with Sephadex G-100 column (3 $\times$ 100 cm) chromatography. Cytochrome *c* (12,500), ovalbumin (45,000), and bovine serum albumin (68,000) were used as the molecular weight markers. SDS-polyacrylamide gel electrophoresis was done in 0.1% SDS-0.1 M sodium phosphate buffer, pH 7.2, at 8 mA per gel by the method of Weber and Osborn⁷⁾ and molecular weight markers (BDH Biochemicals, 14,300 ~ 71,500) were used.

Isoelectric focusing. Isoelectric focusing was done in a 110-ml column with ampholyte pHs 3 ~ 10 and 6 ~ 9 for 48 hr at 720 V and 4°C. After the run, 1.0-ml fractions were collected and absorbance at 280 nm, pH, and soymilk-clotting activity were measured.

Amino acid analysis. Each of enzymes I and II was hydrolyzed *in vacuo* in 6 N HCl at 110°C for 24 hr. The amino acid compositions of the enzymes were identified on

a Durrum amino acid analyzer model D-500.

RESULTS AND DISCUSSION

Purification of soymilk-clotting enzyme

All operations were done at 4°C throughout the purification steps.

Step I. Ammonium sulfate fractionation. Ammonium sulfate, 1.96 kg, was added with continuous stirring to 3.5 l (total activity; 760 units) of the culture broth of K-295G-7 to bring 80% saturation. After it was left overnight, the resultant precipitate was collected by filtration through a Buchner funnel with Toyo-Roshi No. 2 filter paper, and the enzyme was dissolved in a small quantity of water. The filtrate was dialyzed against 0.04 M potassium phosphate buffer, pH 6.1, concentrated to 40 ml by ultrafiltration and used in further steps.

Step II. First gel filtration. Twenty ml of the concentrated sample was put on a column of Sephadex G-100 equilibrated with 0.04 M potassium phosphate buffer (pH 6.1), and chromatographed. The milk-clotting enzyme fraction obtained was pooled and concentrated to 100 ml by ultrafiltration.

Step III. CM-cellulose chromatography.

FIG. 1. Elution Profile of Soymilk-clotting Enzymes on CM-Cellulose Column.

The concentrate from step II, 100 ml (total activity: 559 units), was put on a CM-52 column (2.5 $\times$ 45 cm) equilibrated with 0.04 M potassium phosphate buffer (pH 6.1). After the column was washed with the same buffer, the enzyme was then eluted with a linear gradient from 0 to 0.15 M NaCl in the same buffer (300 ml each) at the flow rate of 25 ml/hr.

—●—, absorbance at 280 nm; ---○---, soymilk-clotting activity; —, NaCl concentration.

The concentrate from step II was chromatographed on a CM-cellulose column. As shown in Fig. 1, the milk-clotting activity was separated into two fractions which were designated as enzyme I and enzyme II in the order of elution. Enzyme I and enzyme II were independently pooled, and dialyzed against 5 mM sodium phosphate buffer, pH 6.8.

Step IV. Hydroxylapatite chromatography. Enzyme I and enzyme II were separately put on a column (1.5 × 30 cm) of hydroxylapatite equilibrated with 5 mM sodium phosphate buffer (pH 6.8). After the column was washed with the same buffer, the enzyme was eluted with a linear gradient from 0 to 0.2 M sodium phosphate buffer (300 ml each) at the flow rate of 15 ml/hr. The eluent was fractionated into 7.6 ml portions. The fractions of enzyme I and II were pooled, dialyzed against 0.04 M potassium phosphate buffer (pH 6.1) and concentrated to 10 ml by ultrafiltration.

Step V. Second gel filtration. Enzyme I and enzyme II from step IV were independently put on the column of Sephadex G-100 equilibrated with 0.04 M potassium phosphate buffer (pH 6.1), and chromatographed with the same buffer. These enzymes were eluted with single activity peaks which coincided well with the protein profile (data not shown).

The purification steps are summarized in Table I. Purified enzymes I and II were obtained with recoveries of 24.1% and 39.5%, respectively.

Homogeneity of enzymes I and II

The homogeneity of purified enzymes I and II was examined by polyacrylamide gel electrophoresis at pH 4.3. As shown in Fig. 2, both enzymes were homogeneous, migrating as single bands on each gel.

Molecular weight of enzymes I and II

The molecular weight (MW) of enzymes I and II was 24,000 by Sephadex G-100 gel filtration. The MW of both enzymes was also measured by SDS-polyacrylamide gel electrophoresis. Enzymes I and II had MWs of 28,000 and 29,500, respectively. The molecular weights of both enzymes are similar to those of subtilisin BPN' and subtilisin Carlsberg.⁸⁾

Isoelectric point

Enzymes I and II had isoelectric points of pHs 9.22 and 9.45, respectively. These values closely resembled those of bacterial DFP-

FIG. 2. Polyacrylamide Gel Electrophoresis of Enzymes I and II.

The electrophoresis was done at 3 mA per tube for 3 hr.

TABLE I. PURIFICATION OF SOYMILK-CLOTTING ENZYMES

Steps	Total activity (units)	Total protein (mg)	Specific activity (units/mg protein)	Recovery (%)
Culture broth	760	—	—	100
(NH ₄) ₂ SO ₄ precipitation	758	743	1.0	100
Sephadex G-100	743.3	336	2.2	97.8
CM-Cellulose I	290.1	46.6	6.2	38.3
II	330.7	53.0	6.2	43.6
Hydroxylapatite I	234.6	32.4	7.2	30.9
II	308.3	29.2	10.6	40.6
Sephadex G-100 I	183.0	21.4	8.5	24.1
II	299.5	23.5	12.7	39.5

—, not measured.

TABLE II. AMINO ACID COMPOSITIONS OF ENZYMES I AND II

Amino acid	Residues per molecule			
	Enzyme I	Enzyme II	Subtilisin BPN'	Subtilisin Carlsberg ⁸⁾
Asp	25	29	28	28
Thr	27	32	13	13
Ser	32	25	37	32
Glu	16	17	15	16
Pro	9	11	14	9
Gly	39	36	33	35
Ala	25	24	37	41
Val	18	18	30	31
Cys	2	1	0	0
Met	5	5	5	5
Ile	19	21	13	10
Leu	8	8	15	16
Tyr	14	18	10	13
Phe	8	9	3	4
Lys	13	14	11	9
His	4	2	6	5
Arg	8	9	2	4
Total	272	279	275	274

The numbers of residues indicated are the averages of the analyses on the automatic amino acid analyzer.

sensitive alkaline proteases including subtilisin.⁹⁾

Amino acid composition

The amino acids of enzymes I and II were analyzed to compare the amino acid composition with those of subtilisin BPN' and subtilisin Carlsberg.⁸⁾ As shown in Table II, enzymes I and II had similar amino acid composition profiles. Both of these enzymes had a high content of aspartic acid, threonine, serine, glycine, and alanine, and a low content of cysteine. On the other hand, a large difference between both enzymes and subtilisin BPN' or subtilisin Carlsberg was recognized in the content of threonine, valine, isoleucine, and leucine. The occurrence of cysteine in both of enzymes I and II is particularly noteworthy. Thus, it is suggested from the above result that the species of *Bacillus* sp. K-295G-7 is different from *Bacillus subtilis* taxonomically.

Enzymatic properties

Thermal-activity profile. The soymilk-

FIG. 3. Effects of temperature and pH on Activity and Stability of Soymilk-clotting Enzymes I and II.

A: Effects of temperature on the 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.

B: Effects of temperature on the stability of the enzymes. The enzyme solution (pH 6.1) was left at various temperatures for 30 min. Then the residual activity was measured.

C: Effects of pH on the soymilk-clotting activity. Soymilk-clotting activity was measured at different pHs, ranging from pH 5.8 to 7.0, at 65°C.

D: Effects of pH on the stability of the enzymes. One half ml of the enzyme solution and 0.5 ml of McIlvaine buffer were mixed and stored at 37°C for 1 hr. After adjustment to pH 6.1, the residual activity was measured.

—●—, enzyme I; ---○---, enzyme II.

clotting activity of enzymes I and II was measured at various temperatures (35~85°C) at pH 6.1. Figure 3A shows the effects of temperature on soymilk-clotting activity. Both enzymes I and II had their optimum activity at 65°C.

Thermal-stability profile. Enzymes I and II were separately maintained at various temperatures (35~85°C) for 30 min at pH 6.1, and then the remaining activities were measured. As shown in Fig. 3B, both enzymes were stable up to 45°C.

pH-Activity profile. The soymilk-clotting activity of enzymes I and II was measured at

different pHs ranging from 5.8 to 6.7 at 65°C. The soymilk-clotting activity test could not be done below pH 5.8, since the protein in soymilk coagulated without the addition of the enzyme solution below pH 5.8. Figure 3C shows the effects of pH on soymilk-clotting activity. Both enzyme activities decreased as the pH increased from 5.8 to 6.7.

pH-Stability profile. Each of enzymes I and II was treated at different pHs (3~10) for 1 hr at 35°C; after that the remaining activities were measured. As shown in Fig. 3D, about 50~100% of the original activities of both enzymes remained after treatment in the range of pH 5~10.

The milk-clotting enzyme was separated into two fractions, that is, enzymes I and II, and each enzyme coagulated the protein in soymilk. This fact indicates that the coagulation of the protein was due to a single enzyme

action, not the combined action of two enzymes. On the other hand, enzymatic properties, namely thermal-activity and -stability profiles or pH-activity and -stability profiles, were in excellent agreement between enzymes I and II. This suggests that the two enzymes can coagulate the protein efficiently under the same optimum conditions, *i.e.* at pH 5.8 and 65°C.

Proteolytic and soymilk-clotting activities

The optimum pH for proteolysis of enzymes I and II was pH 9.0 on soy protein isolate (SPI), and 10.0 on hemoglobin (data not shown). This indicates that both of the enzymes are of alkaline proteases. On the other hand, the optimum temperature for proteolysis of the two enzymes was 50°C on hemoglobin (data not shown).

Table III shows the relative activity toward

TABLE III. RELATIVE PROTEOLYTIC ACTIVITY TOWARD VARIOUS PROTEINS

The enzymatic reaction was done at pH 9.0 and 50°C for 30 min.

Substrate	Relative activity (%)	
	Enzyme I	Enzyme II
SPI	100	100
Casein	78	41
Hemoglobin	37	33
Ovalbumin	6	25
BSA	25	66
Elastin	4	6

TABLE IV. RATIO BETWEEN SOYMILK-CLOTTING ACTIVITY AND PROTEOLYTIC ACTIVITY

Soymilk-clotting activity was measured as described in MATERIALS AND METHODS. Proteolytic activity was determined with SPI as the substrate at pH 6.1 and at 50°C for 10 min.

Enzyme	Soymilk-clotting activity/ Proteolytic activity
Crude enzyme	49.5
Enzyme I	61.9
Enzyme II	207.6
Bromelain	93.0

TABLE V. EFFECTS OF METAL IONS ON SOYMILK-CLOTTING ACTIVITY AND ON PROTEOLYTIC ACTIVITY OF ENZYMES I AND II

Compound	Relative soymilk-clotting activity (%) ^a		Relative proteolytic activity (%) ^b	
	Enzyme I	Enzyme II	Enzyme I	Enzyme II
None	100	100	100	100
KCl	92	97	105	104
FeCl ₂	128	249	96	100
CaCl ₂	142	204	90	99
PbCl ₂	100	130	90	75
SnCl ₂	148	243	71	82
MnCl ₂	238	230	97	94
MgCl ₂	209	212	87	94
HgCl ₂	—	—	60	84
ZnCl ₂	—	—	51	86
BaCl ₂	—	—	82	85
CuCl ₂	—	—	81	94
CoCl ₂	—	—	105	89

^a Reaction mixture contained 1 ml of soymilk containing 1 mM of metal ions, adjusted to pH 6.1, and 0.1 ml of enzyme solution, and the enzyme reaction was done at 65°C.

^b The assay mixture contained 1 ml of 1.2% hemoglobin in 0.1 M Tris-HCl buffer, pH 9.0, 1 mM of metal ions, and 200 μ l of the enzyme solution. The enzyme reaction was done at 50°C for 10 min.

—, not measured.

various proteins when enzymes I and II acted under the above optimum conditions. The highest rates of proteolysis, among the proteins examined, were obtained with SPI. Both of the enzymes also hydrolyzed casein, hemoglobin, *etc.* at different rates.

The ratio of soymilk-clotting activity to proteolytic activity

As shown in Table IV, each ratio of enzymes I and II was about 1.3 and 4 times higher than that of the crude enzyme, respectively. Bromelain was about 2 times higher than that

FIG. 4. Effects of Calcium Concentration on Soymilk-clotting Activity.

Soymilk-clotting activity was measured at 65°C with the indicated concentration of calcium chloride in soymilk. —●—, enzyme I; ---○---, enzyme II; ---●---, bromelain.

TABLE VI. EFFECTS OF VARIOUS INHIBITORS ON SOYMILK-CLOTING ENZYMES

Reagent	Concentration (mM)	Remaining activity (%)	
		Enzyme I	Enzyme II
None		100	100
EDTA	10	98	100
DFP	2.5	0	0
Antipain	0.1	98	100
Pepstatin	0.1	100	100
Leupeptin	0.1	95	100
Chymostatin	0.1	86	68
Phosphoramidon	0.1	92	100
E-64	0.1	86	90
Cysteine	1	86	90
STI	1	95	100

of the crude enzyme. This result indicates that the enzyme II coagulates soymilk most effectively among these enzymes.

Effects of metal ions on soymilk-clotting activity

Table V summarizes the effects of metal ions on the soymilk-clotting activity and on the proteolytic activity of enzymes I and II. With the addition of 1 mM metal ions such as Fe^{2+} , Ca^{2+} , Sn^{2+} , Mn^{2+} , and Mg^{2+} , clotting activity of enzyme II was enhanced by two times while only Mn^{2+} and Mg^{2+} were effective for enzyme I. With metal ions such as Hg^{2+} , Zn^{2+} , Ba^{2+} , Cu^{2+} , and Co^{2+} , the soymilk coagulated at 65°C without enzymatic action. The proteolytic activity was measured with hemoglobin as the substrate, because soybean protein isolate (SPI) was easily precipitated by the addition of 1 mM various metal ions at 50°C. The proteolytic activity of enzymes I and II was affected by metal ions in various degrees. For instance, enzyme I was inhibited by Hg^{2+} and Zn^{2+} significantly, whereas enzyme II was not so much affected by the metal ions including Hg^{2+} and Zn^{2+} . These results suggest that the effects of metal ions such as Fe^{2+} , Ca^{2+} , Sn^{2+} , Mn^{2+} and Mg^{2+} is not on the enzymatic activity but on the soymilk coagulation process to a different degree.

Figure 4 shows the effects of calcium concentration on the soymilk-clotting activity of enzymes I and II, compared with that on bromelain. Both enzymes were more sensitive to calcium ion concentration for soymilk coagulation than bromelain. Raising the calcium concentration increased the soymilk coagulability to about 350% on enzyme II. When the calcium concentration exceeded 3 mM, soymilk coagulation occurred without the addition of enzyme.

Effects of enzyme inhibitors

As shown in Table VI, both enzymes I and II were completely inhibited by DFP and partially by chymostatin. The inhibition by DFP suggests that both enzymes belong to the group of serine proteases.

Acknowledgment. This work was supported in part by a grant from the Foundation for Promotion of Food Science and Technology.

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