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Purification and Characterization of a Fibrinolytic Enzyme Produced from *Bacillus* sp. strain CK 11-4 Screened from Chungkook-Jang

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***Bacillus* sp. strain CK 11-4, which produces a strongly fibrinolytic enzyme, was screened from Chungkook-Jang, a traditional Korean fermented-soybean sauce. The fibrinolytic enzyme (CK) was purified from supernatant of *Bacillus* sp. strain CK 11-4 culture broth and showed thermophilic, hydrophilic, and strong fibrinolytic activity. The optimum temperature and pH were 70°C and 10.5, respectively, and the molecular weight was 28,200 as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The first 14 amino acids of the N-terminal sequence of CK are Ala-Gln-Thr-Val-Pro-Tyr-Gly-Ile-Pro-Leu-Ile-Lys-Ala-Asp. This sequence is identical to that of subtilisin Carlsberg and different from that of nattokinase, but CK showed a level of fibrinolytic activity that was about eight times higher than that of subtilisin Carlsberg. The amidolytic activity of CK increased about twofold at the initial state of the reaction when CK enzyme was added to a mixture of plasminogen and substrate (H-D-Val-Leu-Lys-ρNA). A similar result was also obtained from fibrin plate analysis.**

Bacillus spp. produce a variety of extracellular and intracellular proteases. An alkaline protease (subtilisin), a neutral metalloprotease, and an esterase are secreted into media, whereas at least two intracellular serine proteases are produced within *Bacillus* spp. (8, 16, 24, 25, 27, 30). In particular, the production of subtilisin protease has been exploited commercially for use in laundry detergents and for other applications (6, 10).

The usage of protease for thrombolytic therapy by oral administration has been assessed (26, 33, 37). Blood clots (fibrin) are formed from fibrinogen by thrombin (EC 3.4.21.5) and are lysed by plasmin (EC 3.4.21.7), which is activated from plasminogen by tissue plasminogen activator (tPA). Although fibrin clot formation and fibrinolysis are maintained in balance by the biological system, thromboses, such as myocardial infarction, occur when clots are not lysed as a result of a disorder of the balance (39).

Intravenous administration of urokinase and streptokinase has been widely used for thrombosis therapy but these enzymes have a low specificity to fibrin and are expensive. tPA has been developed for the treatment of thrombosis because of its efficacy and stronger affinity to fibrin (27).

Oral administration of the fibrinolytic enzyme nattokinase (NK) (32), revealed to be the same as subtilisin NAT (19) and which is produced from *Bacillus* NAT in the traditional Japanese fermented food, Natto, has been reported to enhance fibrinolytic activity in plasma and the production of tPA (31).

A fibrinolytic enzyme produced from *Bacillus subtilis* has also been reported (5), but it did not show the same level of plasminogen activator activity as does NK.

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Bacillus sp. strain CK 11-4, which produces a strongly fibrinolytic enzyme, was screened in our laboratory from Chungkook-Jang, a traditional Korean fermented-soybean sauce, to develop the enzyme for use as a thrombolytic agent. In this paper, we report the purification and some characteristics of the fibrinolytic enzyme produced from *Bacillus* sp. strain CK 11-4.

MATERIALS AND METHODS

Strain. *Bacillus* sp. strain CK 11-4 producing fibrinolytic enzyme (CK) was isolated from Chungkook-Jangs collected from various regions in Korea.

Enzyme production. *Bacillus* sp. strain CK 11-4 was grown on basal medium containing 0.3% beef extract, 0.5% peptone, 1% soytone, and 1% milk casein. The pH was adjusted to 7.0 with 1 M HCl or 1 M NaOH. For the seed culture, one colony per plate was inoculated into 5 ml of basal medium and incubated at 37°C in a shaking water bath for 16 h. The seed culture broth (1 ml) was transferred to 1 liter of basal medium in a jar fermenter and fermented at 40°C, at an air flow rate of 1 vol/vol/min, for 16 h.

Crude enzyme preparation. Cells were separated from the 1-liter culture broth by centrifugation (10,000 × *g*, 15 min), and the supernatant fluid was added to 3 volumes of acetone. The mixture of supernatant and acetone was allowed to stand at 4°C for 1 day. After centrifugation (10,000 × *g*, 15 min) of the mixture, the resultant precipitate was lyophilized and used for the following experiments.

Enzyme assay. Fibrinolytic activity was determined by both the modified plasminogen-free fibrin plate method and the plasminogen-rich fibrin plate method (1) by using 1 U of plasmin (Sigma, St. Louis, Mo.) per ml as a standard fibrinolytic protease. The fibrinogen (plasminogen-free) solution [2.5 ml of 1.2% (wt/vol) human fibrinogen (Sigma) in 0.1 M sodium phosphate buffer, pH 7.4] was mixed with 0.1 ml of thrombin solution (100 NIH U/ml; Sigma) and 7.4 ml

TABLE 1. Purification steps of CK from *Bacillus* sp. strain CK 11-4

Step	Vol (ml)	Total protein (mg)	Total activity (U)	Sp act (U/mg)	Fold purification	Yield (%)
Culture broth	500	48	920	19.2	1	100
Acetone precipitation	10	45.4	915	20.2	1.1	99.5
CM-cellulose	10	14.2	902	63.5	3.3	98
Toyo-pearl HW 55	1	6.1	874	143.3	7.5	95

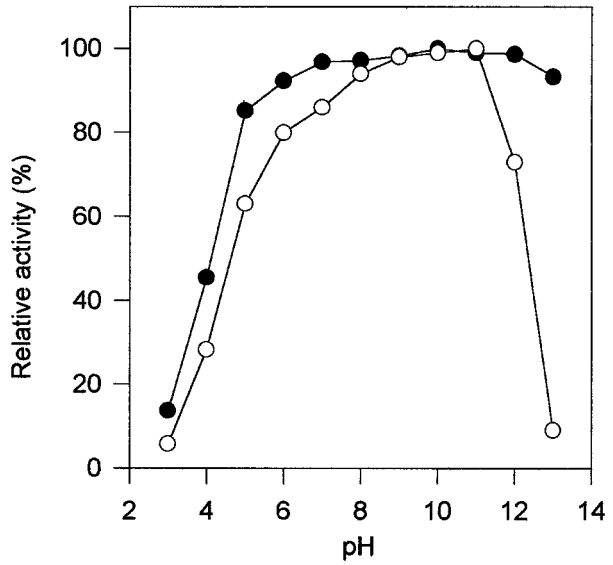

FIG. 1. Effect of pH on fibrinolytic activity and stability of CK from *Bacillus* sp. strain CK 11-4 at 30°C. ●, activity; ○, stability.

of 1% (wt/vol) agarose solution in a petri dish (100 by 15 mm). In the case of plasminogen-rich fibrin plate, 2.0 ml of fibrinogen (1.5% [wt/vol]) and 0.5 ml of plasminogen (10 U/ml) were used. After the dishes were allowed to stand for 30 min at room temperature to form fibrin clots, five holes were made on a fibrin plate by suction by using a capillary glass tube (1-mm-diameter). Two microliters of sample solution was dropped into each hole and incubated at 37°C for 8 h. After measuring the dimension of the clear zone, the number of units was determined according to standard curve by using plasmin.

Caseinolytic activity was assayed by the following procedure. A mixture (1 ml) containing 0.7 ml of 0.1 M sodium phosphate buffer (pH 7.5), 0.1 ml of 2% α-casein, and 0.1 ml of enzyme solution was incubated for 5 min at each temperature, mixed with 0.1 ml of 1.5 M trichloroacetic acid, allowed to stand at 4°C for 30 min, and then centrifuged at room temperature. The A_{275} for the supernatant was measured and converted to the amount of tyrosine equivalent. One unit of caseinolytic activity (CU) was defined as the amount of enzyme releasing 1 μmol of tyrosine equivalent per min.

Amidolytic activity was measured spectrophotometrically by using chromogenic substrates as follows. The reaction mixture (1 ml) contained 20 μl of enzyme solution, 5×10^{-4} M substrate, and 0.1 M sodium phosphate buffer (pH 7.4). After continuous measurement for 5 min at 37°C with a spectrophotometer equipped with a cuvette temperature controller, the amount of ρ-nitroaniline that was liberated was determined from the A_{405}. One unit of amidolytic activity (AU) was expressed as micromoles of substrate hydrolyzed per minute per milliliter by the enzyme. Each value is the mean of three determinations.

Enzyme purification. Crude enzyme was dialyzed against 5 liters of 10 mM glycine-NaOH buffer (pH 10.0, four buffer changes for 12 h each). The dialysate was adjusted to pH 6.0 with 0.2 M HCl, mixed for 2 h with 500 ml of carboxymethyl cellulose equilibrate with buffer A (10 mM sodium phosphate buffer, pH 6.0), and filtered through filter paper (Whatman No. 1). The cellulose was washed three times with 500 ml of buffer A, packed into a glass column (5 by 60 cm) and eluted by a 0~1 N NaCl gradient at a rate of 2.0 ml/min at 4°C. The active fractions (220 ml) were added to 660 ml of acetone and allowed to stand at 4°C for 18 h. The precipitates were collected by centrifugation and then lyophilized. For further purification, gel filtration with Toyo-pearl HW 55 gel equilibrated with 10 mM glycine-NaOH buffer (pH 10.0) was performed. The active fractions were precipitated with acetone and then lyophilized. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done according to Laemmli (15) by using a 10 to 20% gradient polyacrylamide gel and a 4% stacking gel at 4°C.

Effect of pH on the fibrinolytic activity of CK. Because fibrin is insoluble, 800 μl of fibrinogen solution (2%) was added to 100 μl of thrombin solution (100 NIH U/ml). The mixture was incubated at 37°C for 1 h and then centrifuged. The precipitate was washed twice with 1 ml of each buffer and vortexed for dispersion of fibrin formed in the reaction. Purified enzyme was dissolved either in 0.1 M sodium acetate buffer (pH 4.0 to 6.0), sodium phosphate buffer (pH 6.5 to 8.0), or glycine-NaOH buffer (pH 8.5 to 12.0). The enzyme solution (100 μl) was then added to the dispersed fibrin solution. Other reaction conditions were the same as described earlier.

Effect of protease inhibitor. Purified and lyophilized enzyme was dissolved in 10 mM glycine-NaOH buffer (pH 10.0) and mixed with each salt solution to give a final inhibitor concentration of 0.1 or 1.0 mM. Enzyme samples were separately incubated at 37°C for 10 min with each of the following inhibitors (3): phenylmethylsulfonyl fluoride (a serine protease inhibitor), EDTA, ε-aminocaproic acid, E64 [an effective cysteine protease inhibitor produced by *Aspergillus japonicus* TPR-64, L-*trans*-epoxysuccinyl-leucylamide-(4-guanidino)-buthan], pepstatin A (a transition state analog that is a potent inhibitor of cadepsin D, pepsin, rennin, and many microbial aspartic proteases), and 2,4-dinitrophenol. Residual activity was then determined.

Determination of the N-terminal amino acid sequence of the enzyme. After SDS-PAGE, purified enzyme on polyacrylamide gel was transferred to a polyvinylidene difluoride membrane by electroblotting (17) and stained with Ponceau S solution containing 5% acetic acid. The stained portion was excised and used for N-terminal sequencing directly.

Restriction enzyme mapping of CK gene. PCR was performed with chromosomal DNA of *Bacillus* sp. strain CK 11-4 by using primer 1, 5′-ATGATGAGG AAAAAGAGTTTTTGGC-3′, and primer 2, 5′-CATCCGACCATAATGGAA CGGATTC-3′. The resultant PCR product was isolated and inserted in pGEM-7Zf(+) vector (Promega) at an *Sma*I site and transformed to *Escherichia coli* JM109. Plasmid containing PCR product was obtained from transformant culture and analyzed with several restriction enzymes.

RESULTS

Purification of fibrinolytic enzyme from *Bacillus* sp. strain CK 11-4. Strain CK 11-4 is an aerobic, spore-forming, gram-positive, motile, rod-shaped, and catalase- and oxidase-producing microorganism. It is clear that the bacterium should belong to the genus *Bacillus*. Temperature and pH ranges for growth were 28 to 55°C and pH 6 to 10, respectively (41).

CK was purified from the culture supernatant of *Bacillus* sp. strain CK 11-4 by the procedure described in Materials and Methods. Its purification procedure is summarized in Table 1. CK was easily and quickly purified by consecutive chromatog-

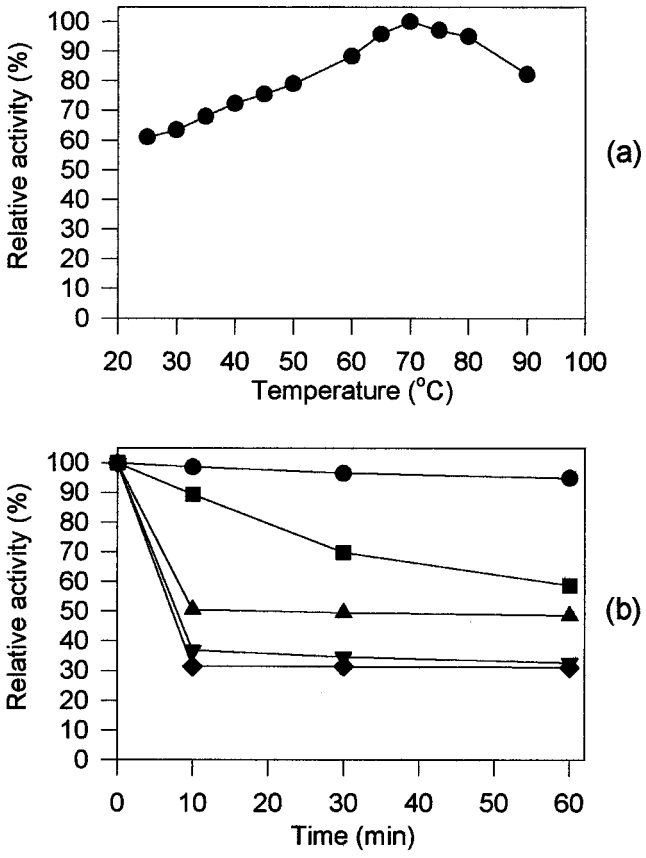

FIG. 2. Effect of temperature on fibrinolytic activity (a) and stability (b) of CK at pH 10.0. (b) ●, 40°C; ■, 50°C; ▲, 60°C; ▼, 70°C; ◆, 80°C.

TABLE 2. Effect of protease inhibitor on CK activity[a]

Inhibitor[b]	Concn	
	0.1 mM	1.0 mM
DNP	92.6	90.4
ε-ACA	97.8	96.1
EDTA	97.1	78.6
PMSF	41.4	0
E64	96.7	92.6
Pepstatin A	92.4	38.2

[a] Reaction conditions described in Materials and Methods.
[b] DNP, 2,4-dinitrophenol; ε-ACA, ε-aminocaproic acid; PMSF, phenylmethylsulfonyl fluoride.

raphy with carboxymethyl cellulose and Toyo-pearl HW 55. The final specific activity of the purified enzyme increased more than 7.5-fold, and its protein content was about 12.7% on the basis of content in the culture supernatant (Table 1). The purified sample of CK migrated as a single protein band (Fig. 8), and its apparent molecular weight was estimated to be approximately 28,200 by SDS-PAGE. From the results of gel chromatography, activity staining (renature SDS-PAGE; data not shown), and denature SDS-PAGE, CK was considered to be a monomeric protein.

Effect of pH on fibrinolytic activity and stability of CK. The optimum pH for the fibrinolytic activity of CK was around 10 to 12, and the enzyme activity decreased rapidly at levels below pH 6.0. As shown in Fig. 1, the enzyme was very stable in the range of pH 7 to 10.5 at 30°C for 20 h. Above pH 11.0, enzyme stability abruptly decreased.

Although CK showed high activity around pH 10 to 12 in the presence of substrate, its stability was decreased during incubation in the absence of substrate at a pH above 11.0.

Effect of temperature on fibrinolytic activity and stability of CK. The effect of temperature on the fibrinolytic activity of CK was examined at pH 10.0 (Fig. 2a). The temperature showing the maximal enzyme activity was 70°C. After a 60-min incubation, CK was very stable at 40°C, and showed 58.7, 48.6, 32.8, and 31.2% residual activity at 50, 60, 70, and 80°C, respectively (Fig. 2b).

Effect of inhibitors on CK activity. When the enzyme (600 U/ml) was incubated at room temperature for 10 min in 10 mM glycine-NaOH buffer (pH 10.0) with 1 mM phenylmethylsulfonyl fluoride, enzyme activity was completely inhibited. Enzyme activity was partially inhibited by EDTA, ε-aminocaproic acid, E64, and pepstatin A, but 2,4-dinitrophenol did not significantly inhibit CK (Table 2). In view of the effects of pH, temperature, and inhibitors, CK can apparently be classified as a thermophilic alkaline serine protease.

Comparison of CK with other proteases for fibrinolytic activity. The fibrinolytic activity of CK was compared with that of other proteases that were already known. After the activity units of each enzyme were converted to caseinolytic activity for unification of the enzyme unit, fibrinolytic activities were measured and the ratios of fibrinolytic activity to caseinolytic activity were calculated. As shown in Table 3, fibrinolytic activity of CK was 2.6-, 3.2-, and 7.9-fold higher than that of the fungal protease of *Aspergillus oryzae*, trypsin from bovine pancreas, and subtilisin Carlsberg, respectively.

Comparison of CK with other proteases on amidolytic activity on several synthetic substrates. Amidolytic activity of CK was compared with that of NK, subtilisin BPN′, and subtilisin Carlsberg on several chromogenic substrates (Table 4). CK, subtilisin BPN′, and subtilisin Carlsberg showed no activity on the synthetic substrate for trypsin (Bz-DL-Arg-ρNA), but NK showed some activity. CK, NK, and subtilisin BPN′ showed no activity when the synthetic substrate for urokinase (pyro-Glu-Gly-Arg-ρNA) was used, but subtilisin Carlsberg showed some activity. CK had a similar level of amidolytic activity to that of subtilisin BPN′, and a higher specificity for the synthetic substrate for plasmin (H-D-Leu-Lys-ρNA) than did NK.

N-terminal amino acid sequence of CK. After SDS-PAGE, purified enzyme on polyacrylamide gel was transferred to a polyvinylidene difluoride membrane by electroblotting and stained with Ponceau S. The stained portion was cut out and used for N-terminal sequencing.

The N-terminal amino acid sequence of the first 14 residues of CK was analyzed by a protein sequencer. The sequence was Ala-Gln-Thr-Val-Pro-Tyr-Gly-Ile-Pro-Leu-Ile-Lys-Ala-Asp, which is identical to that of subtilisin Carlsberg but different from that of NK, as shown in Fig. 3. Although the N-terminal sequence of the first 14 amino acids of CK and subtilisin Carlsberg are identical, the enzymes showed different substrate specificity.

Comparison of restriction map of the CK gene with that of subtilisin Carlsberg and its isoforms. For the differentiation of CK and subtilisin Carlsberg, PCR was performed five times to eliminate *Taq* DNA polymerase error. Each PCR product was inserted into pGEM-7Zf(+) vector and transformed to *E. coli* JM109. Each plasmid containing the PCR product of the CK gene was digested with several restriction enzymes, and the same results were obtained from all of the restriction maps. A comparison with the restriction enzyme maps of subtilisin Carlsberg and its isoforms, shown in Fig. 4, indicates that the restriction enzyme sites of the CK gene are different from that of *Bacillus licheniformis* NCIB 6816 (subtilisin Carlsberg) (9) at the *Pst*I, *Acc*I, and *Hin*dII sites. Differences are also evident with *B. licheniformis* 11594 (20) at the *Nco*I and *Acc*I sites, with *B. licheniformis* 14353 (21) at the *Pst*I site, and with *B. licheniformis* 15413 (22) at the *Hin*dII, *Eco*RI and *Nde*I sites. On the restriction enzyme map, CK was most similar to that of *B.*

TABLE 3. Comparison of CK with other proteases for fibrinolytic activity[a]

Protease	Caseinolytic activity (U)	Fibrinolytic activity (U)	Fibrinolytic/caseinolytic activity (%)
Protease from *Bacillus* sp. strain CK 11-4	352	257	73.0 (100)
Protease from *B. licheniformis* (type VIII)	423	41	9.7 (13.3)
Protease from *A. oryzae* (type XIII)	127	35	27.6 (37.8)
Trypsin from bovine pancreas (type I)	334	75	22.5 (30.8)
Protease (K) from *Tritirachium album* (type XXVIII)	426	129	15.3 (20.9)
Protease from *Streptomyces griseus* (type XXI)	395	80	20.3 (27.8)
Subtilisin BPN′ (type XXVII)	438	142	32.4 (44.4)
Subtilisin Carlsberg (type VIII)	325	30	9.2 (12.6)

[a] Reaction conditions described in Materials and Methods.

TABLE 4. Comparison of specificity of CK with nattokinase, subtilisin BPN′, and subtilisin Carlsberg with chromogenic synthetic substrates

Substrate	Enzyme activity[a]			
	CK (μmol/min/ml)	Nattokinase[b] (nmol/min/ml)	Subtilisin BPN′ (μmol/min/ml)	Subtilisin Carlsberg (μmol/min/ml)
H-D-Val-Leu-Lys-ρNA[c]	424.3 (100)	68.5 (100)	119.7 (100)	462.5 (100)
Bz-DL-Arg-ρNA[d]	0 (0)	18.0 (26.3)	0 (0)	0 (0)
H-D-Phe-Pip-Arg-ρNA[e]	21.7 (5.1)	14.0 (20.4)	6.8 (5.7)	50.3 (10.9)
H-D-Val-Leu-Arg-ρNA	16.9 (4.0)	13.5 (19.7)	3.4 (2.6)	25.9 (5.6)
pyro-Glu-Gly-Arg-ρNA[f]	0 (0)	0 (0)	0 (0)	19.6 (4.2)

[a] Values in parentheses are percentages calculated on the basis of enzyme activity to H-D-Val-Leu-Lys-ρNA.
[b] Reference 27.
[c] Synthetic substrate for plasmin.
[d] Synthetic substrate for trypsin.
[e] Synthetic substrate for thrombin.
[f] Synthetic substrate for urokinase.

licheniformis 14353, but the first amino acid of the N-terminal amino acid sequence of CK is alanine rather than glycine, as it is for the *B. licheniformis* 14353 protease. These results demonstrate that CK is not identical to subtilisin Carlsberg or its isoforms.

Effect of plasminogen addition on amidolytic activity of CK. When CK was added to the mixture of substrate solution (H-D-Val-Leu-Lys-ρNA) and plasminogen, amidolytic activity increased about twofold at the initial stage of the reaction compared with when CK alone was added to the substrate solution (Fig. 5).

A similar result was obtained from fibrin plate analysis (Fig. 6, Table 5). In plasminogen-free fibrin plate, the fibrinolytic activity of a mixture of CK and plasminogen showed a 1.6-fold increase above the level of fibrinolytic activity with CK alone. In plasminogen-rich fibrin plate, the fibrinolytic activity of CK was 1.5-fold higher than the activity of CK in plasminogen-free fibrin plate.

When the activity was spectrophotometrically measured on the range of 0.000~0.035 with a small amount of CK (0.1 U) added to the mixture of plasminogen and substrate, a stair-like reaction curve, with repeated and rather random increasing reaction-rate and decreasing reaction-rate stages, was obtained (Fig. 7). This phenomenon apparently appeared in the initial state of the reaction, and disappeared by degrees during further incubation time until the reaction rate was similar to that of the control.

The mixture of CK and plasminogen was analyzed by SDS-PAGE after incubation for 2 min (Fig. 8). Plasminogen was degraded by CK, and the bands of degraded products showed approximate molecular weights of 25,600, 34,600, and 37,600 in comparison with a CK band of 28,200. In the products from plasminogen, a product with a molecular weight of about 28,200 was newly produced when that was compared with the addition amount of CK.

DISCUSSION

This article describes the purification and characterization of CK produced from *Bacillus* sp. strain CK 11-4 for assessment of its application as a thrombosis agent. As mentioned above, intravenous administration of urokinase and streptokinase has been widely used for thrombosis therapy. Fibrinolytic therapy by oral drug administration has been recently investigated in animal models in which enteric-coated urokinase capsules were given to normal and experimental dogs with saphenous vein thrombosis (34). Sumi et al. reported that intravenous administration did not show any clear thrombolytic effect, but oral administration enhanced fibrinolytic activity. In another study, Sumi et al. (31) reported that when NK was given to human subjects by oral administration, fibrinolytic activity and the amounts of tPA and fibrin degradation product in plasma increased about twofold.

On the basis of these reports, strains producing fibrinolytic enzyme were isolated from Chungkook-Jangs, a traditional Korean soybean-fermented food, obtained from various regions of Korea. Among them, strain CK 11-4 showed the strongest fibrinolytic activity and was identified as a *Bacillus* spp. CK was purified from supernatant of *Bacillus* sp. strain CK 11-4 culture broth and showed thermophilic-hydrophilic and strong fibrinolytic activity.

The N-terminal sequences of the first 14 amino acids of CK

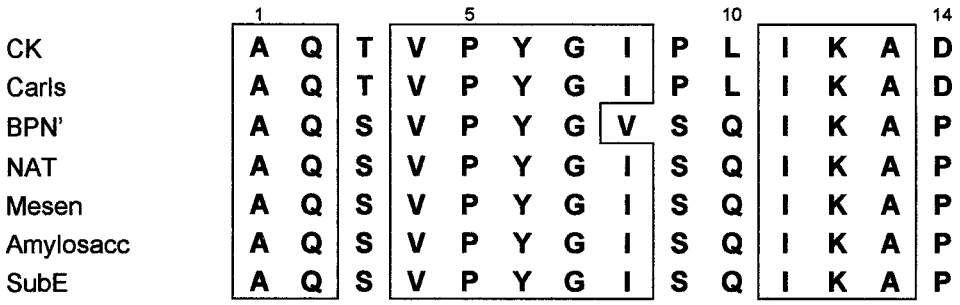

FIG. 3. Comparison of N-terminal amino acid sequence of CK with those of other proteases. CK, extracellular protease from *Bacillus* sp. strain CK 11-4 (this study); Carls, subtilisin Carlsberg (extracellular protease from *B. licheniformis* [24]); BPN′, subtilisin BPN′ (extracellular protease from *B. amyloliquefaciens* [34]); NAT, subtilisin NAT [NK, extracellular protease from *B. subtilis* (*natto*) (16)]; Mesen, extracellular protease from *Bacillus mesentericus* (32); Amylosacc, extracellular protease from *Bacillus amylosaccharitiicus* (38); SubE, subtilisin E (extracellular protease from *B. subtilis* [31]).

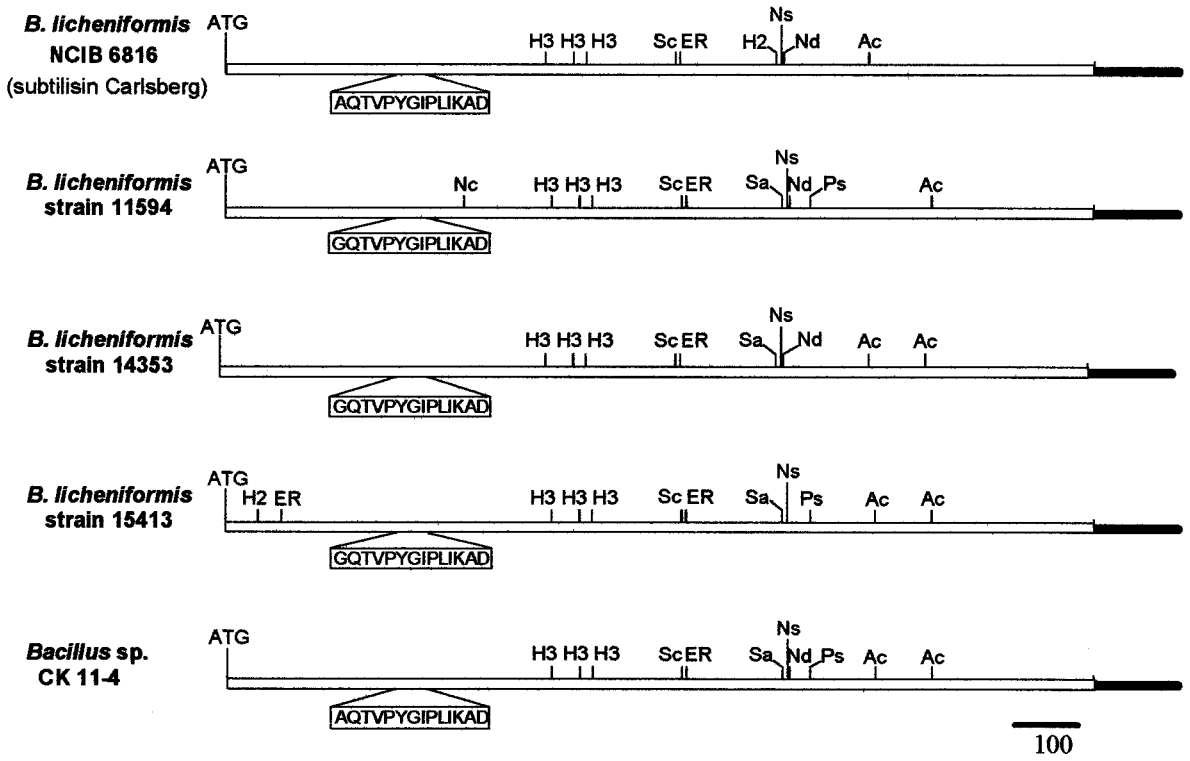

FIG. 4. Comparison of restriction enzyme map of CK with that of subtilisin Carlsberg and its isoforms. Square box shows sequence of first 14 amino acids. H3, *Hin*dIII; Sc, *Sca*I; ER, *Eco*RI; Sa, *Sal*I; Ns, *Nsi*I; Nd, *Nde*I; Ps, *Pst*I; Ac, *Acc*I; H2, *Hin*dII; and Nc, *Nco*I. Bar, 100 kb.

and subtilisin Carlsberg are identical. But because the enzymes showed different substrate specificity and because the level of CK activity to fibrin was about eight times higher than that of subtilisin Carlsberg, these enzymes are not considered to be identical, in spite of the high homology of their amino acid sequences.

For the differentiation of CK and subtilisin Carlsberg, a restriction enzyme map of the CK gene was compared with that of subtilisin Carlsberg and its isoforms. The restriction enzyme sites of the CK gene were different from those of *B. licheniformis* NCIB6816 (subtilisin Carlsberg) at the *Pst*I and *Acc*I sites, as mentioned above. Full sequencing of the CK gene is proceeding, and a partial sequence (about 400 bp) has been obtained. This sequence is different from that of any other protease, although a high homology is evident (data not shown).

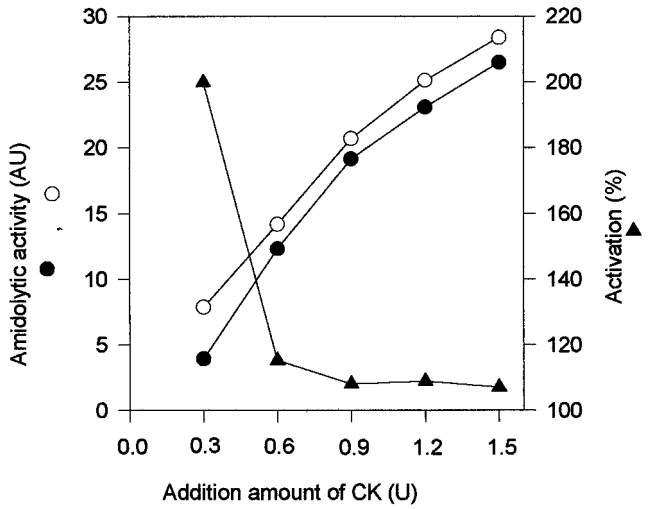

FIG. 5. Amidolytic activity of CK and a mixture of CK and plasminogen with chromogenic substrate (H-D-Val-Leu-Lys-pNA). ●, CK; ○, CK and plasminogen (0.5 U).

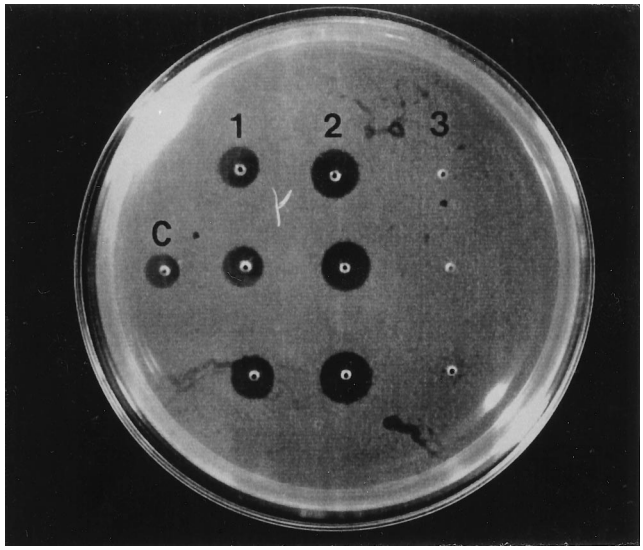

FIG. 6. Fibrinolytic activity of CK, plasminogen, and a mixture of CK and plasminogen in a plasminogen-free fibrin agarose plate. C, 0.1 U of plasmin as control; 1, 0.33 U of CK; 2, mixture of CK (0.33 U) and plasminogen (0.5 U); 3, 0.5 U of plasminogen. The resultant data are shown in Table 5.

TABLE 5. Fibrinolytic activity of CK, plasminogen, and CK-plasminogen mixture in fibrin agarose plate[a]

Fibrin agarose plate	Sample	Fibrinolytic activity (U)	Fold
Plasminogen-free	CK	0.331	1
	CK + plasminogen[b]	0.536	1.6
Plasminogen-rich[c]	CK	0.494	1.5

[a] See also Fig. 6.
[b] A total of 0.5 U of plasminogen was used.
[c] Plasminogen-rich fibrin plate contained 5 U (0.5 U/ml) of plasminogen per plate.

It is interesting that when CK was added to a mixture of plasminogen and substrate, fibrinolytic activity was increased relative to the control level, although plasminogen did not show activity. Was CK activated by digest products produced from plasminogen as stimulators? In that case, because CK reacted with two substrates, chromogenic substrate and plasminogen, the activity must be less than that for one chromogenic substrate reaction. When albumin or casein was added to the reaction mixture, the chromogenic activity decreased (data not shown).

As shown in Fig. 7, at the reaction rate-increasing stage (arrow a), active product seemed to be produced from plasminogen by CK, but it was then degraded or inactivated (arrow b). For the fractionation, purification, and N-terminal amino acid sequencing of active product(s) in the reaction mixture of CK and plasminogen, zymogram electrophoresis (13) was used. But except for an active band of CK, no other active band was found. When gel filtration chromatography with high performance liquid chromatography and capillary electrophoresis was used, an active band was not found either. So, as the stability of the active product was low and the quantity produced was small, the active product is likely to be difficult to detect. To our knowledge, this phenomenon has not been reported before.

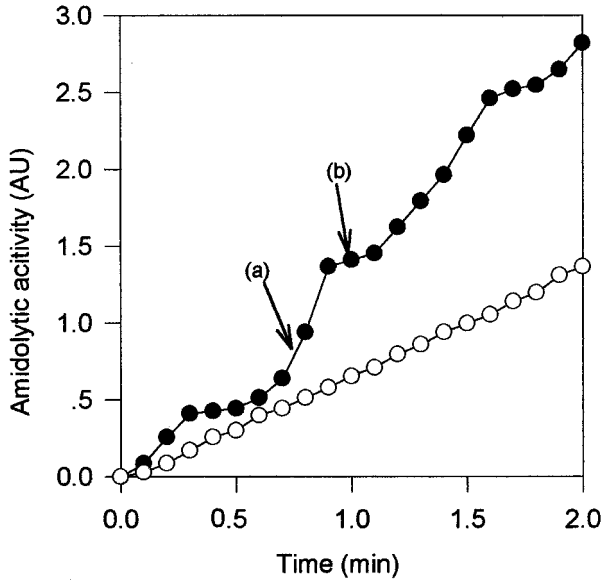

FIG. 7. Amidolytic activity of CK and a mixture of CK and plasminogen. 0.1 U of CK and 1.0 U of plasminogen were used in a narrow spectrometric range. ○, CK; ●, CK plus plasminogen; (a), reaction rate-increasing stage in which plasminogen was seemingly activated by CK; (b), reaction rate-decreasing stage in which activated plasminogen was seemingly degraded by CK.

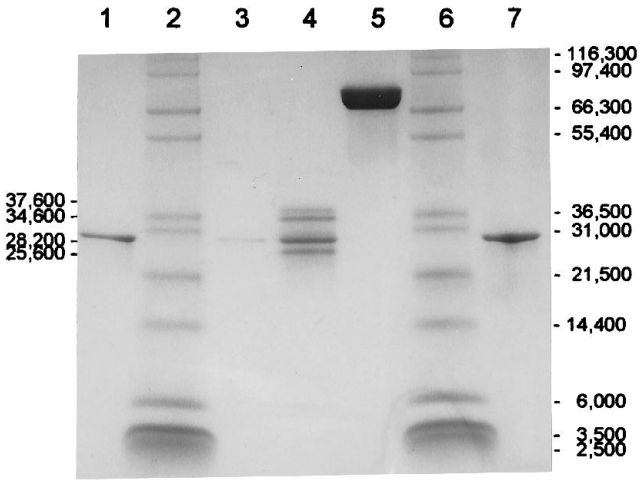

FIG. 8. PAGE gel of purified CK, plasminogen, and the products after reaction of CK and plasminogen. Lane 1, CK (2 μg); lanes 2 and 6, molecular weight markers; lane 3, CK (1 μg); lane 4, mixture of plasminogen (20 μg) and CK (1 μg) after 1-min incubation; lane 5, plasminogen (20 μg); lane 7, CK (3 μg).

There are several studies that have reported on the intestinal absorption of serum albumin (40), lipase (23), ^{131}I-elastase (11), and *Serratia* protease (18). On the basis of perfusion experiments in dogs and rats, Kitaguchi et al. (12), Hijikata et al. (7) and Klöcking et al. (14) have reported that several serine proteases release plasminogen activators. Bernik and Oller (2) observed activation of a plasminogen proactivator of the human kidney by trypsin treatment.

In view of these reports, it can be suggested that CK can be given orally for use as a thrombolytic agent. *Bacillus* spp. have been recognized as being safe for humans (4). Future studies will test CK in vivo, and the cloning, sequencing, and expression of the CK gene from chromosomal DNA of *Bacillus* sp. strain CK 11-4 is proceeding.

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