Isolation and Characterization of an Extracellular Serine Protease with Strong Fibrinolytic Activity from *Bacillus subtilis* CIR110 and its Immobilization onto Cellulose Beads

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Bacillus subtilis CIR110, which secreted a potent fibrinolytic serine protease, was isolated from fermented food. The enzyme was purified to homogeneity from the culture supernatant in high yields by hydrophobic interaction chromatography, cation-exchange chromatography, and gel permeation chromatography. The molecular weight of the enzyme estimated by SDS-polyacrylamide gel electrophoresis and gel filtration was 28 kDa. The isoelectric point (pI) of the enzyme was 8.95. The pH optimum of casein hydrolysis was 10.5, and the enzyme was stable at pH 6-12 and up to 55°C. This enzyme had strong caseinolytic and fibrinolytic activity, and its activity was even more stabilized by Ca^{2+}, but inhibited by phenylmethanesulfonyl fluoride (PMSF) and α_2-macroglobulin. Of the synthetic substrates, the most sensitive substrate was z-Ala-Ala-Leu-*p*NA for subtilisin. On the basis of the results obtained, it was demonstrated that this enzyme was a subtilisin-like serine protease. The purified enzyme was covalently immobilized onto porous cellulose beads by the *N*-hydroxysuccinimide activation method. The immobilized enzyme maintained caseinolytic and fibrinolytic activities. This provides promise for potential applications in antithrombogenic biomaterials used in blood-contacting medical devices and artificial organs as well as thrombolytic agents.

Introduction

Thrombolytic enzymes are becoming more important in practical applications of therapy. Thrombolytic enzymes digest fibrin, the main protein component of blood clots. Various thrombolytic enzymes such as urokinase, streptokinase, and tissue

plasminogen activator have been widely used in the treatment of thrombosis[1]. However, these enzymes are not only very expensive, but also still have problems such as their short halflives in the blood, antigenicity, and lack of stability. The fibrinolytic serine protease, Brinase from *Aspergillus oryzae* has been known for a long time, and assessed in therapeutic attempts as a thrombolytic agent[2)3)4)]. Unlike urokinase and streptokinase which act as plasminogen activators, it can directly lyse fibrin clots. In recent years the thrombolytic therapy by oral administration of fibrinolytic serine proteases, nattokinase from vegetable cheese natto[5)6)], and lumbrokinase from the earthworm, *Lumbricus rubellus* has been investigated by Sumi and his coworkers[7)8)9)]. These two serine proteases also demonstrated fibrinolytic activity irrespective of the presence of plasminogen. The immobilization of lumbrokinase on polyurethane surface has also been reported more recently by Ryu *et al.*[10)].

With advances in medical technology, there has been an increasingly great demand for the development of antithrombogenic biomaterials which can be used for blood-contacting medical devices and artificial organs. As a practical approach to antithrombogenic biomaterials, the immobilization of such serine proteases seems of interest and significance for obtaining an antithrombogenic surface which is capable of dissolving any thrombus that might be developed on the surface.

In this investigation we isolated the bacteria which were capable of producing a strong fibrinolytic enzyme from fermented foods, and obtained purified enzyme preparations in high yields by the sequential chromatographic procedures. The purified enzyme has been characterized and proved to be a subtilisin-like serine protease. The enzyme demonstrated strong fibrinolytic activity in the presence or absence of plasminogen. Furthermore, this enzyme was covalently immobilized onto cellulose beads as a model support, and the fibrinolytic activity of the immobilized enzyme was briefly examined.

Materials and Methods

Materials — Milk casein and bovine fibrinogen were purchased from Nacalai tesque (Tokyo, Japan); soymilk was from Honen Corp. (Tokyo, Japan); pepstatin, *N*-tosyl-L-lysyl-chloromethylketone (TLCK), *N*-tosyl-L-phenylalanyl-chloromethylketone (TPCK), trypsin inhibitor, leupeptin and bovine plasma α_2-macroglobulin (α_2M) were obtained from Boehringer Mannheim Gmbh (Mannheim, Germany); PMSF was from Sigma Chemical Co. (St. Louis, MO); Peptidyl-4-methyl-coumaryl-7-amides (MCA-substrates) and z-Ala-Ala-Leu-*p*NA were from Peptide Institute, Inc. (Osaka, Japan). Activated cellulose beads (dia., ca. 200 μm) were obtained as a gift from Kuraray Co., Ltd., Medical Products Division, Kurashiki, Japan. The gel beads carried 8 atom spacer arms attached to the matrix by epichlorohydrin and activated by *N*-hydroxysuccinimide. The substitution level was approximately 10 μmol NHS-groups/ml gel. Phenyl-Toyopearl and CM-Toyopearl were the products of Tosoh (Tokyo, Japan). The FPLC-system and a Superose 12 column from Pharmacia (Uppsala, Sweden) were used for isolation of the enzyme.

Strains and media—*B. subtilis* CIR110 and CIR120 were isolated from fermented vegetables (cooked pumpkin and soybean) during this study. The characteristics of these strains are described below. *B. subtilis* IFO3936[11] and IFO3335[12] were from the Institute for Fermentation, Osaka, Japan. They were used as the subtilisin-producing strains for comparison with *B. subtilis* CIR110. All these strains were grown in L-medium (1% peptone, 0.5% yeast extract, 0.1% glucose, 0.5% NaCl, pH6.5) or soymilk-medium (2% soymilk, 0.5% yeast extract, 0.1% glucose, 0.5% NaCl, pH6.5).

Purification of the fibrinolytic enzyme—*B. subtilis* CIR110 was grown aerobically at 30°C in L-medium. After 15hr of incubation, cell-free enzyme solutions were prepared by centrifugation at 10,000x*g* for 10min. The culture broth was brought to 1.5M $(NH_4)_2SO_4$, 30mM Tris-HCl (pH8.0), 5mM $CaCl_2$, and was applied to a Phenyl-Toyopearl column chromatography previously equilibrated with a buffer consisting of 1.5M $(NH_4)_2SO_4$, 30mM Tris-HCl (pH8.0), and 5mM $CaCl_2$. Proteins were eluted with a linear gradient from 1.5 to 0M $(NH_4)_2SO_4$ in 30mM Tris-HCl (pH8.0) and 5mM $CaCl_2$. The active fractions were pooled and were brought to 75% saturation with ammonium sulfate and kept at 4°C for overnight. The precipitates were collected by centrifugation at 10,000x*g* for 20min, then dissolved in an equilibration buffer for CM-Toyopearl, consisting of 10mM Tris-HCl (pH7.0), 5mM $CaCl_2$. The sample solution was dialyzed against the same buffer for overnight, then applied to a CM-Toyopearl column. Elution of proteins was conducted with the same buffer, the active fractions were pooled, brought to 0.15M NaCl, concentrated, and applied to a Superose 12column previously equilibrated with a buffer consisting of 20mM Tris-HCl (pH7.5), 5mM $CaCl_2$, 0.15M NaCl. The final preparation was concentrated, stored at −20°C, and used for further experiments.

Enzyme assays—Caseinolytic activity was assayed as follows: an enzyme solution (200μl) suitably diluted in 1.5ml microtube was mixed with 200μl of 1% casein solution in 0.1M glycine-NaOH, pH10.5 at 37°C. After 5min incubation, 600μl of 5% trichloroacetic acid (TCA) solution was added to the reaction mixture. The mixture was further incubated at room temperature for 30min and then centrifuged at 12,000rpm for 15min. Then, 500μl of the supernatant was mixed with 2.5ml of 0.4M Na_2CO_3 and 0.5ml of Folin-Ciocalteau phenol reagent. The reaction mixture was incubated for 10min at 40°C, and an absorbance of the mixture was measured at 660nm. One unit of the protease activity is defined as the amount of the enzyme to produce the digest which is not precipitated by TCA solution and gives absorbance value equivalent to 1μmole of tyrosine per min at 37°C. The fibrinolytic activity was measured using standard bovine fibrin plates in the absence of plasminogen[13]. Measurement of amidolytic activity was done by fluorogenic and chromogenic assays using MCA substrates and z-Ala-Ala-Leu-*p*NA, respectively. The fluorometric assay was conducted in the following manner; enzyme, 125μM substrate, 0.1M NaCl, 50mM Tris-HCl buffer (pH8.0), in a final volume of 1.35ml was incubated at 37°C. After addition of 1.5ml of 17% CH_3COOH, the amount of 7-amino-4-methyl-coumarin (AMC) liberated was measured by a Hitachi 2000 fluorescence spectrophotometer

(Tokyo, Japan). The excitation and emission wavelengths were 380 nm and 460 nm, respectively. The assay using z-Ala-Ala-Leu-*p*NA was done according to the method of Stepanov *et al.*[14].

Immobilization of the enzyme—The enzyme was covalently immobilized on the porous cellulose beads activated by *N*-hydroxysuccinimide ester. A typical immobilization procedure was as follows: 100 mg of the activated beads were suspended in 1 ml 10 mM phosphate buffer (PB) at pH 7.4. To the suspension, a given amount of the enzyme was added under shaking in the cold room, and the mixture was kept at 4 °C for indicated time. Then, the beads were washed with 10 mM phosphate buffer saline (PBS), and the buffer consisting of 0.1 M Tris-HCl (pH 8.0), 0.5 M NaCl, was added to block the remaining active groups.

Analytical procedures—Protein concentration was determined by the method of Bradford with IgG as a standard[15]. The isoelectric points (p*I*) of the enzyme was measured by isoelectric focusing on Ampholine PAG plate from pH 3.5-9.5 (Pharmacia). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was done using 15% polyacrylamide gel by the method of Laemmli[16]. In addition, MALDI TOF-MS (Matrix Assisted Laser Desorption Ionization, Time of Flight Mass Spectrometry) analysis was performed for the measurements of molecular weight of the enzyme using Kompact MALDI I mass spectrometer (Shimadzu-Kratos, Kyoto, Japan).

Results and Discussion

Characterization of isolates—Strains CIR 110 and CIR 120 were identified as *Bacillus subtilis* at the National Collections of Industrial and Marine Bacteria Limited (NCIMB Ltd.), Aberdeen, Scotland. Both strains were strictly aerobic, gram-positive, catalase producing, and endospore forming. The maximum temperature for the growth was 55 °C. The biotin requirement was tested in this work. Both strains were shown to require biotin for growth, therefore these were classified into *Bacillus subtilis (natto)*[17].

Growth and enzyme production—To examine the production of fibrinolytic enzyme(s) by *B. subtilis* CIR 110, the time course of growth and enzyme activities in culture broth of CIR 110 and CIR 120 strains were measured. Furthermore two typical *B. subtilis (natto)* strains, IFO 3936 and IFO 3335 were investigated for comparison. All strains showed almost the same patterns of cell growth in L-medium. Figure 1 shows enzyme production by each strains in the L-medium (A) or soymilk-medium (B). It is noteworthy that of the strains tested, *B. subtilis* CIR 110 produced the protease with the highest caseinolytic and fibrinolytic activity in both media, while other strains produced the fibrinolytic enzyme only when they were cultured in soymilk-medium. Generally, *B. subtilis (natto)* strains have been cultured in the medium containing soybean cake extract (soymilk)[18]. However, in this case, the enzyme purification becomes quite difficult because the separation of the lipids and insoluble small particles of soymilk is necessary. Therefore, it is interesting that *B. subtilis* CIR 110 was capable of producing the highly fibrinolytic protease without soymilk. This advantage may offer promise for possible applications in the large-scale enzyme production.

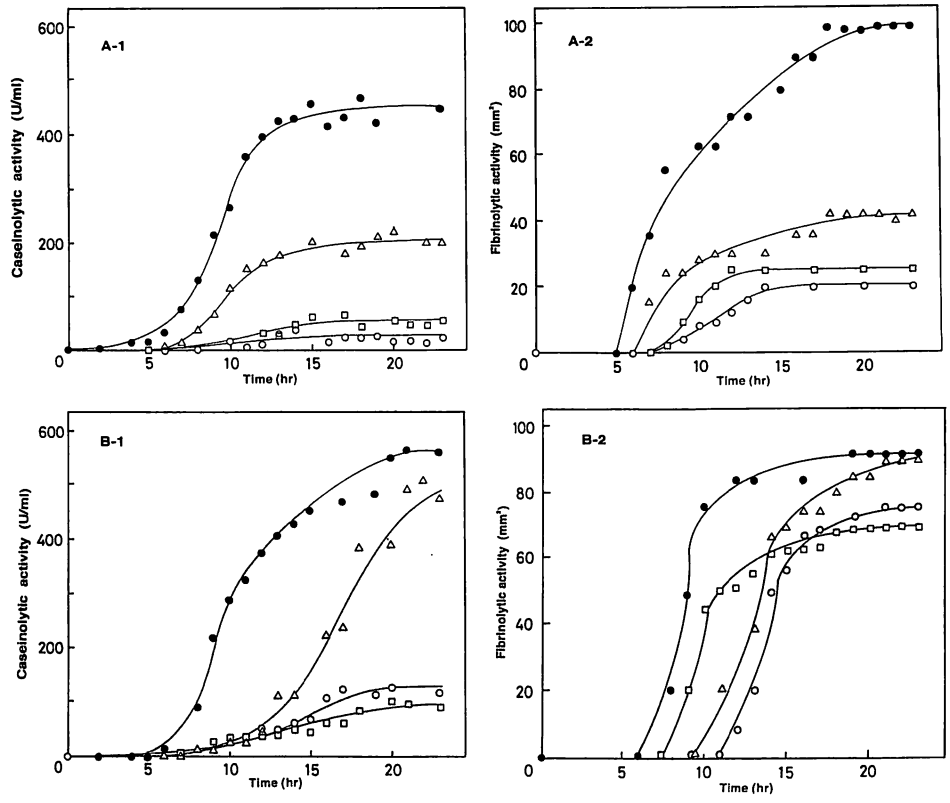

Fig. 1. Time courses of caseinolytic and fibrinolytic enzymes production in L-medium and soymilk medium by *B. subtilis* CIR110 (●), *B. subtilis* CIR120 (○), *B. subtilis* IFO3936 (△), and *B. subtilis* IFO3335 (□). Cells were grown at 30°C in L-medium (A) or soymilk medium (B) on a rotary shaker. The culture supernatants were used for caseinolytic activity assay (A-1, B-1) and fibrinolytic activity assay (A-2, B-2).

Purification of the fibrinolytic enzyme—A strong fibrinolytic serine protease was purified from *B. subtilis* CIR110 through Phenyl-Toyopearl, CM-Toyopearl, and Superore 12 chromatography. Crystallization was performed by ultrafiltration of CM-Toyopearl fraction (1.2mg protein/ml) to a 3-fold concentration and allowed to stand for few days. As summarized in Table 1, protease was about 3.5-fold enriched with 88% yield, and had a specific caseinolytic activity of 3.8×10^3 U/mg protein. This purification method was easily scaled up to at least 10 times in the laboratory. Previously, Sumi *et al.*[6] and Fujita *et al.*[19] demonstrated the presence of a fibrinolytic enzyme (named nattokinase) in the vegetable cheese natto. But they have not described about the purification yield of the enzyme. We have achieved the purification of the strong fibrinolytic enzyme from the culture broth of *B. subtilis* CIR110 in high yields (80-95%), and this purification procedure could easily be scaled up to the industrial preparation systems.

Figure 2 shows SDS-polyacrylamide gel electrophoretograms of the each sample of the purification steps and the microphotograph of the crystalline protease. The final preparation shows the single polypeptide with molecular mass of 28kDa (lane 6 in Fig.

Table 1. Purification of the fibrinolytic serine protease from *B. subtilis* CIR110[a]

Purification step	total volume (ml)	total protein (mg)	total act. (units)	sp. act[b]. (units/mg of protein)	yield (%)	purification (x-fold)
1. Crude fraction	2420	784	8.6×10^5	1.1×10^3	100	1
2. Phenyl-Toyopearl	167	268	8.3×10^5	3.1×10^3	97	2.8
3. Ammonium sulfate precipitation	27	250	8.0×10^5	3.2×10^3	93	2.9
4. CM-toyopearl	178	220	7.7×10^5	3.5×10^3	90	3.2
5. Superose 12	50	200	7.6×10^5	3.8×10^3	88	3.5

a Enzyme activity was assayed at each step of purification procedure in glycine-NaOH buffer, pH 10.5 using casein as substrate.
b Specific activity was measured in micromoles of tyrosine per minute per miligram of protein.

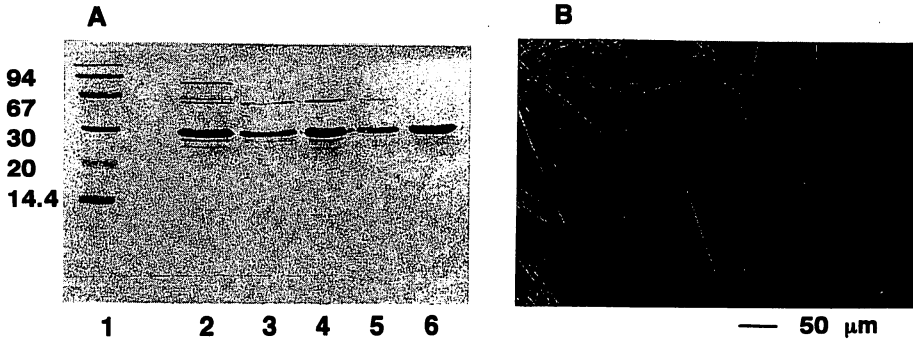

Fig. 2. SDS-PAGE (A) and microphotograph (B) of the crystalline protease. A. Coomassie stain (15% gel): Lane 1, molecular mass standards (in kilodaltons; Pharmacia electrophoresis calibration kits); lane 2, crude fraction; lane 3, Phenyl-Toyopearl fraction; lane 4, $(NH_4)_2SO_4$ precipitate; lane 5, CM-Toyopearl fraction; lane 6, Superose 12 fraction.

2A). In addition, the molecular weight measured by TOF-MS analysis was 27,683.

Catalytic properties of the enzyme—The physical characterization was mainly studied by casein hydrolysis.

(A) pH optimum. The pH profile of enzyme activity is shown in Fig. 3. The pH optimum was 10.5, and the enzyme was stable between pH 6-12 at least 20 hours at 25°C (data not shown).

(B) Substrate specificity. Table 2 showed the substrate specificity of the enzyme for various synthetic substrates. The highly sensitive substrates for the enzyme were z-Ala-Ala-Leu-*p*NA for subtilisin and Suc-Ala-Ala-Pro-Phe-MCA for chymotrypsin. However, it had little or no activity toward the thrombin and urokinase substrate.

(C) Effect of inhibitors on enzyme activity. Effect of inhibitors on enzyme activity was studied both in caseinolysis and fibrinolysis. The results obtained with various inhibitors are summarized in Table 3. *B. subtilis* CIR110 protease was fully inhibited by PMSF, an inhibitor of serine proteases. α_2-Macroglobulin (α_2M), a major protease inhibitor of blood plasma, also inhibited caseinolytic and fibrinolytic activity to a significant extent. However, TPCK, TLCK, and Leupeptin had no effect on both

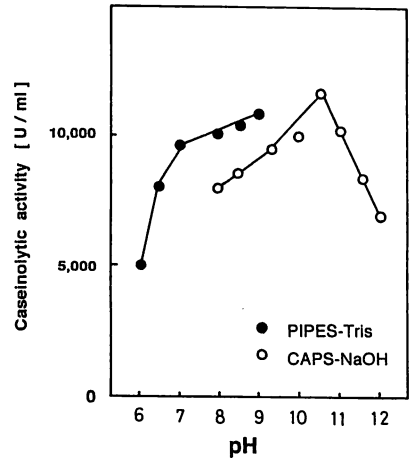

Fig. 3. pH Profile of the enzyme activity. An aliquot (6 μg protein) was assayed for proteolytic activity with casein as the substrate at various pH values. Each value was the mean of triplicate determinations. Symbols: ●, Pipes-Tris buffer; ○, CAPS-NaOH buffer.

Table 2. Substrate specificity of the purified enzyme[a]

Substrate		Rate of hydrolysis (nmol/min. mg protein)	Km (mM)
Suc-Ala-Ala-Pro-Phe-MCA	(Chymotrypsin)	420	0.17
Boc-Ile-Glu-Gly-Arg-MCA	(Factor Xa)	150	
Boc-Phe-Ser-Arg-MCA	(Trypsin)	60	0.25
Boc-Glu-Lys-Lys-MCA	(Plasmin)	56	
Boc-Leu-Ser-Thr-Arg-MCA	(Activated Protein C)	37	
Boc-Val-Leu-Lys-MCA	(Plasmin)	5.3	
Boc-Val-Pro-Arg-MCA	(α-Thrombin)	7.7	
Boc-Gln-Ala-Arg-MCA	(Trypsin)	4.5	
Leu-MCA	(Aminopeptidase)	n.d.	
z-Phe-Arg-MCA	(Kallikrein)	n.d.	
Bz-Arg-MCA	(Trypsin)	n.d.	
Glt-Gly-Arg-MCA	(Urokinase)	n.d.	
z-Ala-Ala-Leu-*p*NA	(Subtilisin A)	2880	0.5

a The enzyme activity was assayed by the method described in materials and methods.

Table. 3. Effects of various inhibitors on the caseinolytic and fibrinolytic activity of purified enzyme[a]

Inhibitor	Concentration	Relative Activity (%)	
		casein hydrolysis	fibrin hydrolysis
None		100	100
PMSF	2mM	2	0
α_2-Macroglobulin	30 μM	26	0
Pepstatin	2 μM	92	93
TLCK	270 μM	97	94
TPCK	570 μM	105	86
Trypsin inhibitor	10 μM	100	100
Leupeptin	2 μM	98	94

a The preincubation of the enzyme with each inhibitor was carried out at 37 °C for 30 min, and the aliquot was assayed for casein and fibrin hydrolysis. Each value was the mean of triplicate measurements.

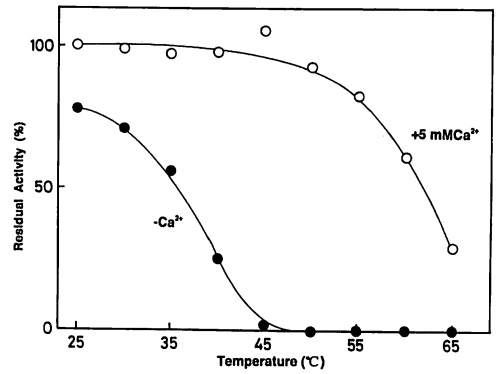

Fig. 4. Effect of Ca^{2+} and temperature on the stability of the enzyme. The enzyme dissolved in the Tris-HCl buffer (pH 7.5), in the presence or absence of 5 mM Ca^{2+} was incubated at the indicated temperatures for 1 hr, and the residual proteolytic activities were measured with casein as substrate. Symbols: ○, in the presence of Ca^{2+}; ●, in the absence of Ca^{2+}.

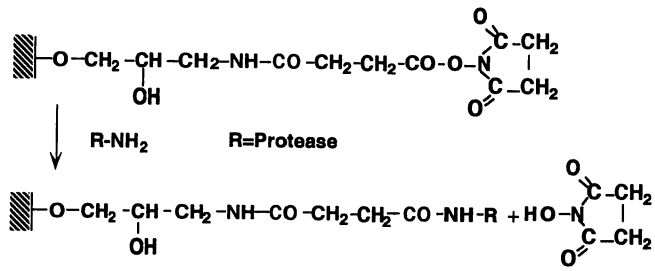

Fig. 5. Covalent coupling of the enzyme to cellulose beads activated with *N*-hydroxysuccinimide ester.

activities. As a whole, these results indicated that the enzyme was a subtilisin-like protease, being consistent with its ability to cleave z-Ala-Ala-Leu-*p*NA. The inhibition of serine proteases by α_2M is well known and explained as due to the entrapment of the proteases within the α_2M molecule to form the α_2M-protease complex[20]. This inhibition should be taken into account for use of the enzyme as a thrombolytic agent.

(D) Effect of Ca^{2+} on enzyme activity and stability. The enzyme was considerably stabilized by 5 mM Ca^{2+}. As shown in Fig. 4, the residual activity was 100% when incubated at 25-50°C for 1 hour in the presence of Ca^{2+}, and 0% at 45°C in the absence of Ca^{2+}.

Isoelectric point of the enzyme — The purified enzyme was homogeneous on isoelectric focusing and the isoelectric point was 8.95 (data not shown). This value was different from 9.8 of subtilisin NAT[21] and 8.5 of subtilisin AM[22].

Immobilization of the enzyme — The fibrinolytic enzyme was covalently immobilized on the porous cellulose beads through the reaction scheme as shown in Fig. 5. The immobilization reaction was complete within 7 h, independent of the concentration of the enzyme, while the saturated caseinolytic and fibrionolytic activity of the immobilized enzyme was dependent on the initial enzyme concentration of the reaction

mixture. The amount of immobilized enzyme was almost proportional to the initial enzyme concentration, at least, in the low concentration range examined (Fig. 6). Figure 7 showed the fibrinolytic activity of the immobilized and free enzyme. The immobilized enzyme was less affected by protease inhibitors than non-immobilized enzyme in terms of caseinolysis and fibrinolysis (data not shown). We have found that the immobilization and/or chemical modifications of the enzyme could play an important role in retaining its fibrinolytic activity. The properties of the enzyme immobilized on several different carriers are now under investigation. The preliminary experiments of enzyme immobilization showed the good results for the enzyme stability and the resistance to the protease inhibitors[23]. The detailed study will be reported elsewhere. The fibrinolytic enzyme of *B. subtilis* CIR110 may have promise for potential

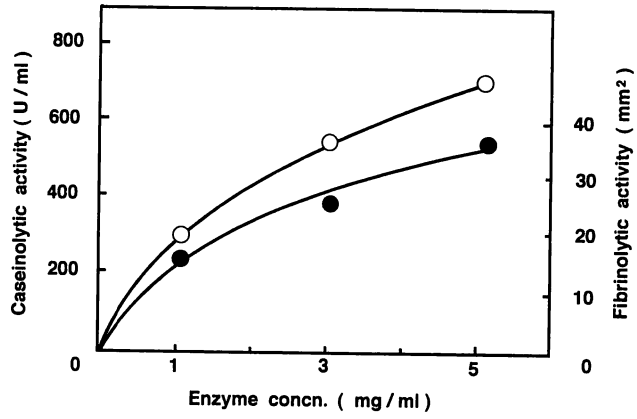

Fig. 6. Fibrinolytic and caseinolytic activity of the enzyme immobilized on cellulose beads. The activated cellulose beads were incubated with a given amount of the enzyme in 10mM phosphate buffer (pH 7.4) at 4°C under slow constant shaking for 8 hours. Then, 40 μl of beads suspension were used for enzyme assay. Symbols: ○, caseinolytic activity; ●, fibrinolytic activity.

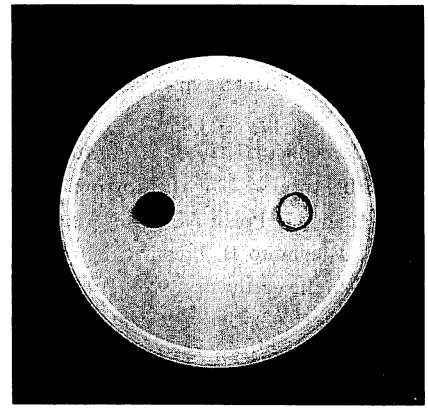

Fig. 7. Fibrinolytic activity of the free (left) and immobilized enzymes (right). The purified free enzyme (10 U) and immobilized enzyme (15 U/25 mg beads) were separately placed on a fibrin plate, and the fibrinolytic activity was measured after incubation for 1 hr at 37°C.

Table. 4. Comparison of the N-terminal amino acid sequences of the enzyme with that of bacterial serine proteases

Subtilisins[a]	1	10	20	30	Homology
CIR110	AQSVPYGISQIKAPALHSQGYTGSNVKVAV				
NAT	AQSVPYGISQIKAPALHSQGYTGSNVKVAV				100% [15]
AM	AQSVPYGISQIKAPALHSQGYTGSNVKVAV				100% [25]
BPN'	AQSVPYGVSQIKAPALHSQGYTGSNVKVAV				97% [23]
Carlsberg	AQTVPYGISLIKADKVQAQGFKGANVKVAV				67% [8]

a Subtilisins shown above were produced by: CIR110, *B. subtilis* CIR110; NAT, *B. subtilis* (natto); AM, *B. subtilis* var. *amylosacchariticus*; BPN', *B. amyloliquefaciens*; Carlsberg, *B. licheniformis*.

applications in antithrombogenic biomaterials and thrombolytic agents.

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