Journal of Biochemistry and Molecular Biology, Vol. 34, No. 2, March 2001, pp. 134-138

JOURNAL OF
Biochemistry and Molecular Biology

The Effect of Sodium Chloride on the Serine-type Fibrinolytic Enzymes and the Thermostability of Extracellular Protease from *Bacillus amyloliquefaciens* DJ-4

Nack-Shick Choi and Seung-Ho Kim*

Protein Engineering Group, Korea Research Institute of Bioscience and Biotechnology, Yusong, Taejon 305-600, Korea

Received 10 November 2000, Accepted 18 December 2000

By adding sodium chloride (2.5%) into a *Bacillus amyloliquefaciens* DJ-4 culture broth, two serine-type fibrinolytic proteases with a molecular weight of 29 (subtilisin DJ-4) and 38-kDa were stimulated on the SDS-fibrin zymogram or inhibitor gels. *B. amyloliquefaciens* DJ-4 showed the highest proteolytic activity (5.52 plasmin NIH unit/ml) on the fibrin plate based on the molar ratio when cells were subjected to the 2.5% NaCl. Using a fibrin plate, the secreted protease from this strain in the presence of 5% NaCl showed that about 49% of the enzyme's activity remained after incubation at 60°C for 30 min, but as the salt concentration was increased (10% NaCl) the activity nearly disappeared (0.14 plasmin NIH unit/ml). However, through a fibrin zymography assay, three fibrinolytic enzymes (38, 53 and 80-kDa) from the cells in the presence of 10% NaCl were detected. Also, two salt-activated serine-type fibrinolytic proteases (29 and 38-kDa) showed thermostability from 65 to 70°C for 30 min. Furthermore, these proteases also showed stability, pH 6-11. In particular, 29-kDa (subtilisin DJ-4) was very stable in the pH range of 4-11 at 4°C for 48 h.

Keywords: *Bacillus amyloliquefaciens*, Fibrin zymography, Doen-Jang

Introduction

When a blood vessel is damaged, a hemostatic response occurs in order to stop blood loss. After the hemorrhage reaction, the cross-linked fibrin polymer that is formed from fibrinogen by thrombin is dissolved by endogenous plasmin. In an unbalanced state, such as a myocardial infraction, the clots are not lysed and thus thrombosis occurs (Cho *et al.*, 1999; Kim and Choi, 2000).

*To whom correspondence should be addressed.
Tel: 82-42-860-4162; Fax: 82-42-860-4593
E-mail: shkim@kribb4680.kribb.re.kr

Bacillus sp. strains are well known for secreting several extracellular, such as serine proteases, neutral metalloprotease and esterase (Kim *et al.*, 1996; Park *et al.* 1999; Kim and Choi, 2000). Recently, many fibrinolytic enzymes that were produced by *B.* strains screened from fermented food were reported (Sumi *et al.*, 1987; 1990; Kim *et al.*, 1996; Kim and Choi, 2000).

It was reported that the cations, such as Na^+, K^+ and Mg^{2+}, Ca^{2+} and La^{3+}, increased the serine protease secretion of *Bacillus licheniformis* and *B. subtilis* cells. However, further increase of the salt concentration lowered the stimulating effect (Artemov and Samuilov, 1986).

Five *Bacillus* strains that produce fibrinolytic enzymes were screened from Doen-Jang (a traditional soybean fermented food in Korea) in our laboratory (Kim *et al.*, 1998). We purified subtilisin DJ-4, a fibrinolytic enzyme from *B. amyloliquefaciens* DJ-4, and identified it as a serine-type protease by fibrin zymography (Kim and Choi, 2000).

The purpose of the present work was to study the effect of sodium chloride on the secretion of serine protease, as well as the enzyme's thermostability by *B. amyloliquefaciens* DJ-4 based on the activity assay using fibrin zymography.

Materials and Methods

Materials Fibrinogen and thrombin, from bovine for fibrin zymography and plasmin as a standard proteolytic enzyme for fibrin from bovine and agarose, were purchased from Sigma (St. Louis, USA). Other chemicals were of analytical grade.

***Bacillus* strain and culture** The bacterial strain used in this study was isolated on the fibrin plate from Doen-Jang, a Korean traditional fermented food, and was identified as *Bacillus amyloliquefaciens* DJ-4 (Kim and Choi, 2000) from the Collection for Type Cultures (KCTC). The cells were grown at 37°C in a tryptic soy broth (TSB, Difco) and transferred to 100 ml of fresh media. After 12 h, each indicated concentration of NaCl was added, and the cells were allowed to grow for an additional 12 h. Cells

were removed from the culture broth by centrifugation at 10,000 × g for 10 min, and the enzyme activity was analyzed by fibrin plate or zymography.

Enzyme assay on fibrin plate Enzyme activity was determined by the fibrin plate methods as previously described (Astrup and Müllertz, 1952) with minor modifications. Five-ml 0.6% (wt/vol) fibrinogen solution in a 50 mM sodium phosphate buffer (pH 7.4) was mixed with 5 ml of 2% (wt/vol) agarose solution and 0.1 ml of thrombin solution (10 NIH unit/ml) in a petri dish. The solution in the petri dish was allowed to stand for 1 h at room temperature in order to form a fibrin clot layer. Twenty microliter of the sample solution (3 μg) was dropped into holes previously made on a fibrin plate using a capillary glass tube (5 mm diameter) and incubated at 37°C for 12 h. An equal volume (3 μg) of plasmin solution (1 NIH unit/ml) was incubated into a hole on the fibrin plate as a standard protease. The enzyme activity was estimated by measuring the dimension of the clear zone.

SDS-fibrin zymography Fibrin zymography was done by Kim and Choi's Method (Kim *et al.*, 1998; Choi and Kim, 1999; 2000). A separating gel solution (12%, w/v) was prepared in the presence of 0.12% fibrinogen (w/v) and 100 *ul* of thrombin (10 NIH units/ml). The samples (1 μg) were diluted in a zymogram sample buffer (5 ×), which consisted of 0.5 M Tris-HCl (pH 6.8), 10% SDS, 20% glycerol. Then, 0.03% bromophenol blue was electrophoresed into the fibrin-copolymerized gel at a 15 mA constant current in the cold room (at 4°C).

Protease inhibitor assay on fibrin zymography Classification of the enzymes noted on zymography (Kim and Choi, 1999) was performed by incubating Triton X-100-washed gels in an enzyme reaction buffer containing protease inhibitors, 5 mM PMSF (a known inhibitor of serine proteases) or EDTA (a known inhibitor of metalloproteases).

Enzyme properties on the temperature or pH on fibrin zymography In order to test the effect of temperature on the stability, a fibrin zymogram gel was used. The culture supernatants (1 μg) from 2.5% NaCl were incubated at the indicated temperature for 30 min as described in the Figure Legend. Then the samples were subjected on the fibrin gel. In the case of pH, the samples (1 μg) were incubated in 200 mM of various buffer systems at 4°C for 48 h. After electrophoresis, the gel was stained with Coomassie blue for 2 h and then destained. The digested bands were visualized as the non-stained regions of the fibrin gel. For the quantification, the densities of digested bands on the gel were analyzed by video densitometry using Bio 1D ver. 97.04 (Vilber Lourmat, France) (Kleiner and Stetler-Stevenson, 1994). The protein concentration was determined according to Bradfords Method (Bradford, 1976) using bovine serum albumin (BSA) as a standard.

Results

Strain and cell culture We isolated five *Bacillus* strains, which produce fibrinolytic enzyme, on the fibrin plate from Doen-Jang (Kim *et al.*, 1998), a traditional Korean fermented food. One strain of them, which was used in this study, was identified as *B. amyloliquefaciens* DJ-4 from the Collection for Type Cultures (KCTC) (Kim and Choi, 2000).

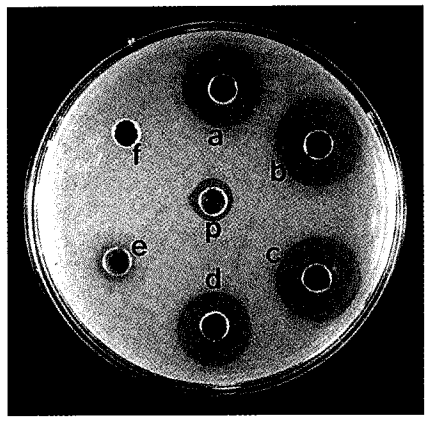

Fig. 1. Fibrinolytic activity assay on the fibrin plate (0.3%) with the culture supernatant of *Bacillus amyloliquefaciens* DJ-4. After 12 h culture, each indicated concentration of NaCl was added. Then the culture supernatants (3 μg/20 μl) were loaded on the holes and incubated at 37°C for 12 h. a-f indicates NaCl concentrations (0, 1, 2.5, 5, 10 and 15%), respectively and p means plasmin (1.0 NIH unit/ml) as a control of fibrinolytic protease. The plasmin unit of enzyme activity was determined by the ratios of the sample to the plasmin activity.

Enzyme assay on the fibrin plate Extracellular protease of *Bacillus amyloliquefaciens* DJ-4 was assayed by fibrin plate analysis. The *B. amyloliquefaciens* DJ-4, as shown in Fig. 1, showed the highest enzyme activity (5.52 plasmin NIH unit/ml) on the fibrin plate based on the molar ratio when cells are subjected to 2.5% NaCl.

We examined the stability under the temperature or pH of this enzyme that was obtained from the culture broth containing various concentrations of NaCl. The secreted protease from *B. amyloliquefaciens* DJ-4, cultured in the presence of 5% NaCl, showed that about 54% of the enzymes activity remained after incubation at 60°C for 30 min (Fig. 2). However, as the salt concentration was increased (10% NaCl), the activity nearly disappeared (less than 0.14 plasmin NIH unit/ml) (Fig. 1 and Fig. 2). This enzyme, as shown in Fig. 3, also showed stability in the pH range of 4-11 at 4°C for 48 h.

Fibrin zymography assay To identify the extracellular protease from *Bacillus amyloliquefaciens* DJ-4, a fibrin zymography analysis was done. We found that *B. amyloliquefaciens* DJ-4 secretes four extracellular fibrinolytic enzymes (80, 53, 38 and 29-kDa) in culture broth on the fibrin zymography (Fig. 4A). When the cells are subjected to 10% NaCl, it was difficult to estimate the enzyme activity on the fibrin plate (less than 0.14 plasmin NIH unit/ml) (Figs. 1 and 2). However, based on the zymogram gel, three fibrinolytic enzymes (38-, 53- and 80-kDa) were detected (Fig. 4A).

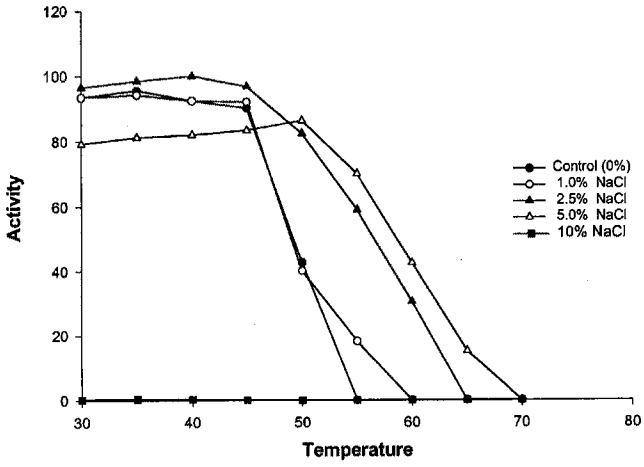

Fig. 2. Thermostability of enzymes from *Bacillus amyloliquefaciens* DJ-4 cultured in the presence of various concentrations of NaCl. After incubation at the indicated temperature for 30 m, the samples (3 μg/20 μl) were subjected to fibrin plate (0.3%), then incubated at 37°C for 12 h. The residual enzyme activities were determined as the plasmin unit (100% equal to 5.52 plasmin NIH unit/ml).

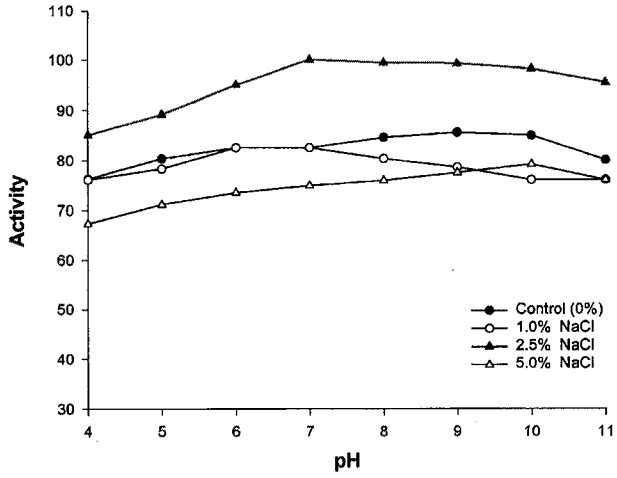

Fig. 3. Effect of pH on the enzymes from *Bacillus amyloliquefaciens* DJ-4 cultured in the presence of various concentrations of NaCl. After incubation of each enzyme over the pH range for 48 h at 4°C, the samples (3 μg/20 μl) were subjected to fibrin plate (0.3%), then incubated at 37°C for 12 h. The residual enzyme activities were determined as the plasmin unit (100% equal to 5.52 plasmin NIH unit/ml).

Protease inhibitor assay on fibrin zymography We examined whether or not these four extracellular fibrinolytic enzymes from *Bacillus amyloliquefaciens* DJ-4 were applied to the enzyme activity inhibitor assay. These enzymes were classified with a protease inhibitor (5 mM PMSF or EDTA) on the zymogram gels. Two bands (29 and 38-kDa) were inhibited by 5 mM PMSF (Fig. 4B). We classified these enzymes as a serine protease. However, in the presence of 5 mM EDTA, four bands were unaffected (Fig. 4C). Also, as shown in Figure 4 A, two serine-type fibrinolytic proteases (29 and 38-kDa) were activated by 2.5% NaCl. In particular, 29-kDa (subtilisin DJ-4 by Kim and Choi, 2000) was induced about three times more by 2.5% NaCl, based on the densitometric analysis. However, the further increase in the salt concentration (5% NaCl) lowered the activating effect (Fig. 4A).

Enzyme properties on the temperature or pH on fibrin zymography We tested the effect of temperature on the stability of these four extracellular fibrinolytic proteases from *Bacillus amyloliquefaciens* DJ-4 on the fibrin zymogram gels. The culture supernatants (1 μg) from 2.5% NaCl were incubated at each indicated temperature for 30 min, or pH at 4°C for 48 h as described in the figure legends. Two salt-activated proteases (29 and 38-kDa) (Fig. 4B) and 53-kDa showed thermostability from 65 to 70°C for 30 min, when cells were subjected to 2.5% NaCl (Fig. 5B, C and D). In particular, using densitometric analysis, two serine-type proteases (29 and 38-kDa) were observed. About 58 and 42% of the enzyme's activities remained after incubation at 70°C for 30 min, respectively (Fig. 5C and D).

We also analyzed the effect of pH on the stability of these

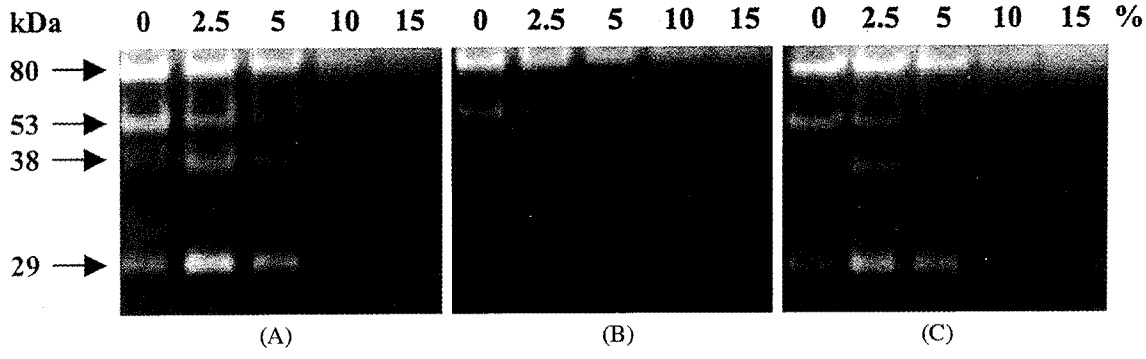

Fig. 4. Fibrin zymography and protease inhibitor assays of culture supernatant of *Bacillus amyloliquefaciens* DJ-4 cultured in the presence of various concentrations of NaCl. After 12 h culture, each indicated concentration of NaCl was added. Then the culture supernatants (1 μg) were loaded to SDS-PAGE containing fibrin (0.12%) as a protein substrate. Protease inhibitor assay was carried out in the presence of 5 mM of PMSF (B) or EDTA (C). A is control (without any protease inhibitors). 1-4 indicates NaCl concentrations (0, 2.5, 5 and 10%), respectively.

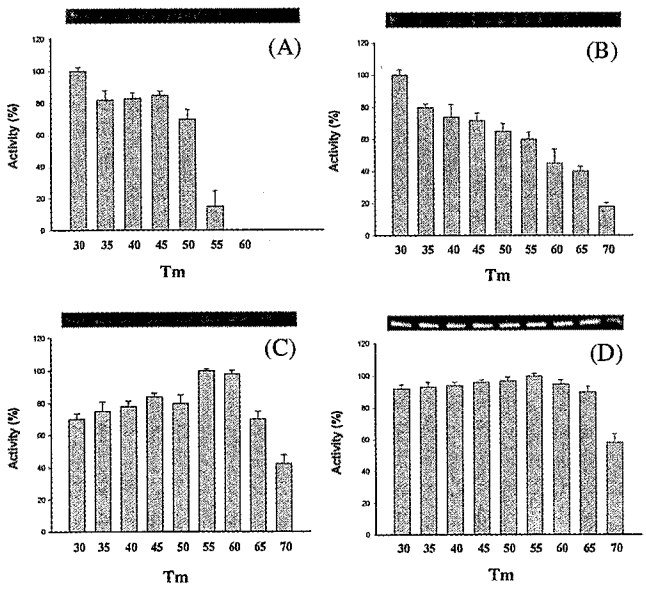

Fig. 5. Thermostability of four extracellular proteases from *Bacillus amyloliquefaciens* DJ-4 cultured in the presence of 2.5% NaCl on the fibrin zymogram gel, and densitometric analysis. After incubation of the culture supernatants (1 μg) at the indicated temperature for 30 min, the samples were loaded on the fibrin gel and the densities of digested bands were analyzed by densitometer. A-D represents 80, 53, 38 and 29-kDa, respectively. Every bar means are based on the relative value to maximum activity of each concentration of NaCl. Every value is the mean±S.E.M (N = 5).

four extracellular proteases by using fibrin zymography. The two bands, 53 and 80-kDa, showed enzyme activity in the pH range of 6-11 at 4°C for 48 h (Fig. 6 A and B). In particular, 29-kDa (subtilisin DJ-4) was very stable in the range of 4-11 (Fig. 6 D).

Discussion

Since there is growing medical interest in thrombosis agents, great attention has been directed towards a search for thrombolytic agents of various organisms. The fibrinolytic agents, such as urokinase, tPA (tissue type plasminogen activator) and streptokinase, have been used as thrombosis therapy. However, these agents have a low specificity to fibrin and are very expensive (Nakajima *et al.*, 1993). Sumi *et al.* reported that the oral administration of urokinase enhanced fibrinolytic activity (Sumi *et al.*, 1985). They also demonstrated that NK (nattokinase) from natto, a traditional soybean fermented food in Japan, activated fibrinolytic activity and the amounts of tPA as well as the fibrin degradation product in plasma increased about two-fold when NK was given to humans by oral administration (Sumi *et al.*, 1990).

In view of these reports, CK 11-4, showing a fibrinolytic activity, was purified from the *Bacillus* sp. strain that was isolated from Chungkook-Jang, a traditional Korean soybean

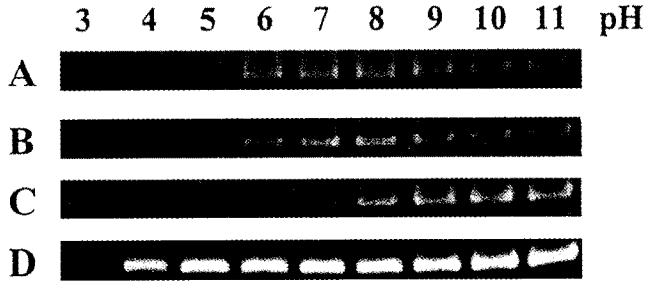

Fig. 6. Effect of pH on the enzyme from *Bacillus amyloliquefaciens* DJ-4 cultured in the presence of 2.5% NaCl on the fibrin zymogram gel. After incubation of the enzyme (1 μg) at the indicated pH at 4°C for 48 h, the residual enzyme activities of the samples were assayed on the fibrin gel. A-D represents 80, 53, 38 and 29-kDa, respectively.

fermented food (Kim *et al.*, 1996). Five *Bacillus* strains that produce fibrinolytic enzymes were screened from Doen-Jang (a traditional soybean fermented food in Korea) in our laboratory (Kim *et al.*, 1998). Doen-Jang is widely used in the Korean daily diet, and several bacterial strains have been identified. We also purified subtilisin DJ-4, a fibrinolytic enzyme from *B. amyloliquefaciens* DJ-4, and identified it as a serine-type protease by fibrin zymography (Kim and Choi, 2000).

In this report, we found that *Bacillus amyloliquefaciens* DJ-4 secreted four extracellular fibrinolytic proteases (80, 53, 38 and 29-kDa) through the fibrin zymography. Among them, two bands (38 and 29-kDa) were induced in the presence of 2.5% NaCl, and these enzymes were also classified as a serine protease on the fibrin inhibitor zymogram gels. Also, two salt-activated serine-type fibrinolytic proteases (29 and 38-kDa) showed thermostability from 65 to 70°C for 30 min. Furthermore, salt-activated subtilisin DJ-4 was very stable in the pH range of 4-11.

References

Artemov, A. and Samuilov, V. D. (1990) Effect of polyelectrolytes on serine proteinase secretion by *Bacillus subtilus*. *FEBS Lett.* **262**, 33-35.

Astrup, T. and Müllertz, S. (1952) The fibrin plate method for estimating fibrinolytic activity. *Arch. Biochem. Biophys.* **40**, 346-351.

Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.* **72**, 248-254.

Cho, S. Y., Hahn, B. S. and Kim, Y. S. (1999) Purification and characterization of a novel serine protease with fibrinolytic activity from *Tenodera sinensis* (Chinese mantis) egg cases. *J. Biochem. Mol. Biol.* **32**, 579-584.

Choi, N. S. and Kim, S. H. (1999) Application of fibrin zymography for determining the optimum culture time for protease activity. *Biotechnol. Techniq.* **13**, 899-901.

Choi, N. S. and Kim, S. H. (2000) Two fibrin zymography methods for analysis of plasminogen activators on gels. *Anal. Biochem.* **281**, 236-238.

Kim, S. H. and Choi, N. S. (1999) Electrophoretic analysis of protease inhibitors in fibrin zymography. *Anal. Biochem.* **270**, 179-181.

Kim, S. H. and Choi, N. S. (2000) Purification and characterization of subtilisin DJ-4 secreted by *Bacillus* sp. strain DJ-4 screened from Doen-Jang. *Biosci. Biotechnol. Biochem.* **64**, 1722-1725.

Kim, S. H., Choi, N. S. and Lee, W. Y. (1998) Fibrin zymography: A direct analysis of fibrinolytic enzymes on gels. *Anal. Biochem.* **263**, 115-116.

Kim, S. H., Choi, N. S., Lee, W. Y., Lee, J. W. and Kim, D. H. (1998) Isolation of *Bacillus* strains secreting fibrinolytic enzymes from Doen-Jang. *Kor. J. Microbiol.* **34**, 87-90.

Kim, W., Choi, K., Kim, Y., Park, H., Choi, J., Lee, Y., Oh, H., Kwon, I. and Lee, S. (1996) Purification and characterization of a fibrinolytic enzyme produced from *Bacillus* sp. strain CK 11-4 screened from Chungkook-Jang. *Appl. Environ. Microbiol.* **62**, 2482-2488.

Kleiner, D. E. and Stetler-Stevenson, W. G. (1994) Quantitative zymography: detection of picogram quantities of gelatinases. *Anal. Biochem.* **218**, 325-329.

Nakajima, N., Taya, N. and Sumi, H. (1993) Potent fibrinolytic enzyme from the lysate of *Katsuwonus pelamis* digestive tract (Shiokara): purification and characterization. *Biosci. Biotechnol. Biochem.* **57**, 1604-1605.

Park, Y. S., Cha, M. H., Yong, W. M., Kim, H. J., Chung, I. Y. and Lee, Y. S. (1999) The purification and characterization of *Bacillus subtilis* tripeptidase (pepT). *J. Biochem. Mol. Biol.* **32**, 239-246.

Sumi, H., Hamada, H., Nakanishi, K. and Hiratani, H. (1990) Enhancement of the fibrinolytic activity in plasma by oral administration of nattokinase. *Acta. Haematol.* **84**, 139-143.

Sumi, H., Hamada, H., Tsushima, H., Mihara, H. and Muraki, H. (1987) A novel fibrinolytic enzyme (nattokinase) in the vegetable cheese Natto: a typical and popular soybean food in the Japanese diet. *Experientia* **43**, 1110-1111.

Sumi, H., Seiki, M., Morimoto, N., Tsushima, H., Maruyama, M. and Mihara, H. (1985) Plasma fibrinolysis after intraduodenal administration of urokinase in rats. *Enzyme* **33**, 121-127.