Journal of Biochemistry and Molecular Biology, Vol. 34, No. 6, November 2001, pp. 531-536

JOURNAL OF
Biochemistry
and
Molecular Biology

Comparison of Three Substrates (Casein, Fibrin, and Gelatin) in Zymographic Gel

Nack-Shick Choi, Kab-Seog Yoon, Jin-Young Lee, Kyoung-Yoen Han and Seung-Ho Kim[1]
Proteome Research Lab, Korea Research Institute of Bioscience and Biotechnology, Yusong, Taejon 305-600, Korea

Received 6 August 2001, Accepted 17 August 2001

Three zymographic techniques using casein, fibrin, and gelatin as substrates in SDS-PAGE were compared based on three aspects: (1) The proteolytic pattern of extracellular enzymes from the three bacterial strains, *Bacillus* sp. DJ-1, DJ-2, and DJ-3. (2) The enzymatic sensitivity of their activity on zymogram gels. (3) The stability of stained zymogram gels with Coomassie brilliant blue in the destaining solution. There was no significant difference on the pattern of extracellular enzymes from the three strains. The bands in the fibrin gel were clearer and more distinct from the extensive destaining process. It was also shown that the gelatin gel revealed the highest enzymatic sensitivity among the three gels, based on the densitometric analysis. In the casein gel, a trace that could be mistaken as a proteolytic band appeared around 40-50 kDa.

Keywords: *Bacillus*, Casein, Coomassie brilliant blue, Fibrin, Gelatin, Zymography

Introduction

Zymographic techniques are used to detect proteolytic enzymes following electrophoretic separation in gel matrices (Granelli-Piperno and Reich, 1978; Loskutoff *et al.*, 1983; Sprengers *et al.*, 1984). These methods are based on a SDS-polyacrylamide gel that is co-polymerized with protein substrates that are degraded by the proteases that are restored during the incubation period in an enzyme reaction buffer after electrophoretic separation (Kim *et al.*, 1998). Activities in zymograms are visualized as clear zones, where proteolytically active bands have degraded the substrate in the gel.

Various types of protein substrates have been introduced. Casein (Raser *et al.*, 1995), fibrin (Kim *et al.*, 1998; Choi and Kim, 1999, 2000, 2001; Kim and Choi, 1999, 2000), and gelatin (Heussen and Dowdle, 1980; Kleiner and Stetler-Stevenson, 1994; Leber and Balkwill, 1997) are wildly used in the field of protein chemistry. Generally, casein and gelatin zymographic techniques have been used for protease assay, in particular, for the quantification of metalloproteinases (Paech *et al.*, 1994; Kleiner and Stetler-Stevenson, 1994). On the other hand, fibrin zymography has been used to identify fibrinolytic enzymes (Kim *et al.*, 1998; Choi and Kim, 1999, 2001; Kim and Choi, 1999, 2000), or plasminogen activators (Choi and Kim, 2000).

In this article, we compared three zymography techniques (casein, fibrin, and gelatin gels) based on the proteolytic pattern and enzymatic sensitivity of three *Bacillus* strains DJ-1, DJ-2, and DJ-3 from Doen-Jang (Kim *et al.*, 1998). We also compared their stability in the destaining solution as follows: (1) A comparison of stained zymogram gels before the gel running. (2) A comparison of stained gels after gel running without sample loading. (3) A comparison of the proteolytic bands in zymogram gels. This study provides the merits or disadvantages of the three zymogram gels, based on their enzymatic property or sensitivity, as well as the stability of the stained zymogram gels with Commassie brilliant blue in the destaining solution.

Materials and Methods

Materials Bovine casein, porcine gelatin (300 Bloom), and bovine fibrinogen and thrombin (to convert fibrinogen to soft fibrin) used in this work as protein substrates were purchased from Sigma.

***Bacillus* strain and culture** The three bacterial strains that were used in this study were isolated from Doen-Jang, a Korean traditional fermented food, and were identified as the *Bacillus* sp. strain from the Korean Collection for Type Cultures (KCTC) (Kim *et al.*, 1998). We named these three strains *Bacillus* sp. DJ-1, DJ-2, and DJ-3. The cells were grown in a tryptic soy broth (TSB, Difco) at 37°C for 2 days. Then the cells were removed from the culture broth by centrifugation at 10,000 × *g* for 10 min.

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

Table 1. Preparation of substrate-containing SDS zymogram gels

Component	Volume (mL)		
Separating gel 12%: 10 mL for minislabs (Mini-PROTEIN II, Bio-Rad)	Fibrin	Casein	Gelatin
Acrylamide-bisacrylamide, 30%: 0.8%	4	4	4
1.5 M Tris-HCl buffer (pH 8.8)	2.5	2.5	2.5
Bovine fibrinogen (0.012 g/mL)	1		
Bovine thrombin (1NIH unit/mL)	0.1		
Casein (0.012 g/mL)		1	
Gelatin (0.012 g/mL)			1
10% SDS	0.1	0.1	0.1
10% (w/v) Ammonium persulfate	0.1	0.1	0.1
TEMED	0.04	0.04	0.04
Distilled water	2.2	2.3	2.3
Total volume		10 mL	
Stacking gel 5%: 2 mL for minislabs			
Acrylamide-bisacrylamide, 30%: 0.8%		0.33	
1.0 M Tris-HCl buffer (pH 6.8)		0.25	
10% SDS		0.02	
10% (w/v) Ammonium persulfate		0.02	
TEMED		0.002	
Distilled water		1.38	
Total volume		2 mL	

Zymography Casein, gelatin, and fibrin zymograms were carried out as described previously (Kim *et al.*, 1998). Briefly, casein, gelatin, or bovine fibrinogen (0.12%, w/v) that were dissolved in a 20 mM sodium phosphate buffer (pH 7.4) were co-polymerized with 12% (w/v) acrylamide, 0.32% (w/v) bisacrylamide, and 375 mM Tris-HCl (pH 8.8) in order to make a running gel. In the case of fibrin gel, 100 μl of bovine thrombin (10 NIH unit/ml) was added in the fibrinogen solution to make a soft fibrin. Then 4% (w/v) acrylamide, 0.11% (w/v) bisacrylamide, and 330 mM Tris-HCl (pH 6.8) (no substrate) were used for the stacking gel, then poured into a mini-gel cast (Bio-Rad) (Table 1). The samples (1 *ug*) that were obtained from the culture supernatant of *Bacillus* sp. DJ-1, DJ-2, and DJ-3 were prepared by diluting the culture supernatant 5-fold with a zymogram sample buffer (0.5 M Tris-HCl, pH 6.8, 10% SDS, 20% glycerol, and 0.5% bromophenol blue) (Kleiner and Stetler-Stevenson, 1994). After the prepared samples were loaded into the wells, an electrophoresis (Laemmli, 1970) was carried out in the cold room at a constant current of 12 mA. After the electrophoresis was completed, the gel was incubated for 30 min at room temperature on a rotary shaker in 50 mM Tris-Cl (pH 7.4), which contained 2.5% Triton X-100. The gel was washed with distilled water to remove Triton X-100, then incubated in a zymogram reaction buffer (30 mM Tris-HCl, pH 7.4, 200 mM NaCl, 10 mM $CaCl_2$, and 0.02% Brij-35) at 37°C for 12 h. The gel was stained with Coomassie brilliant blue (0.5%) for 30 min and then destained in the fixed volume (100 ml) of the destaining solution that contained 10% methanol and 5% acetic acid for a limited period of time. For quantification, the densities of the digested bands on the zymograms 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 Bradford's method (Bradford, 1976) using bovine serum albumin (BSA) as a standard.

Comparing the staining intensity of three zymographic gels before the run After polymerization of each gel, part of it was cut into disks, stained, and put into a destaining solution. Every 12 h a disk was drawn and kept in distilled water. In order to quantify the amount of unbounded dye that was released from the stained gel, the aliquot of the destaining solution from each gel was taken every 3 h. The absorbance was measured at 595 nm.

Comparing the staining intensity of three zymographic gels after the run without a sample After the running of each gel without a sample, part of it was cut vertically into stripes, stained, and put into a destaining solution. Every 12 h a stripe was drawn and kept in distilled water. In order to quantify the amount of released dye from the stained gel, the aliquot of the destaining solution from each gel was taken every 3 h. The absorbance was measured at 595 nm.

Results

Zymographic assay Three zymographic gels (casein, fibrin, and gelatin) were applied to the extracellular proteases from *Bacillus* sp. DJ-1, DJ-2, and DJ-3 that were isolated from Doen-Jang, a Korean traditional fermented food (Kim *et al.*, 1998). The same pattern of molecular sizes of five, two, and six extracellular proteases (ECP) from *Bacillus* sp. DJ-1,

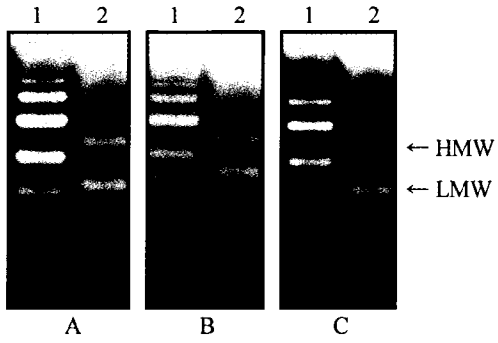

Fig. 1. Comparison of three substrates (casein, fibrin, and gelatin) in zymographic gel with the culture supernatant of *Bacillus* sp. DJ-1 (1) and DJ-2 (2), isolated from Doen-Jang, a Korean traditional fermented food. A, B, and C are gelatin, casein, and fibrin gels, respectively. HMW and LMW mean high molecular weight and low molecular weight.

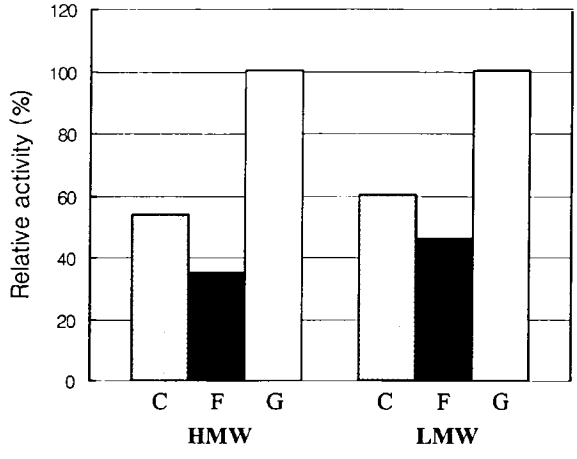

Fig. 2. Densitometric analysis of HMW and LMW (from Figure 1) of *Bacillus* sp. DJ-2. The densities of the digested bands were scanned by densitometer. C, F, and G are the initials of casein, fibrin, and gelatin, respectively. HMW and LMW mean high molecular weight and low molecular weight. Every bar is based on the relative value to the maximum activity (gelatin gel). The bar of each gel was also considered to be the value of the background densities.

DJ-2, and DJ-3 were detected in the three gels, respectively (Figs 1 and 5). We compared the enzymatic sensitivity of the two ECPs, high molecular weight (HMW) and low molecular weight (LMW), of *Bacillus* DJ-2 on the three zymographic gels. Based on the densitometric analysis, the sensitivities of the HMW (and LMW) on the gelatin gel were 2.03 (1.85) and 2.43 (1.95) times higher than those of casein and fibrin gels, respectively (Fig. 2). Gelatin (Fig. 1A) and fibrin (Fig. 1C) gels showed a better resolution of the proteolytic bands than the casein gel (Fig. 1B).

Comparing the staining intensity of three zymographic gels before the run The gelatin gel was destained after 24 h and showed a pale blue color (Fig. 3A(a)). The casein gel was

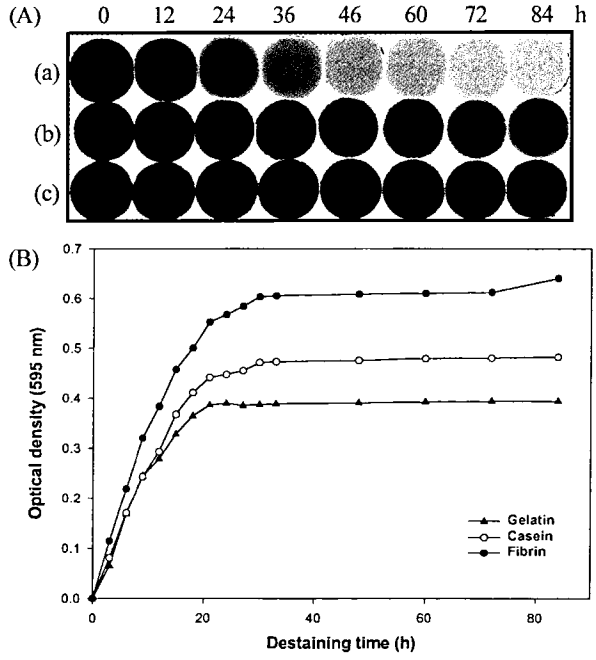

Fig. 3. Comparison of the intensity of stained three zymographic gels before the gel running. Dye stained gel discs were destained for limited periods and kept in distilled water (A). The optical density of the destaining solution of each gel was measured at 595 nm every 3 h (B). (a), (b) and (c) represent gelatin, casein, and fibrin gels, respectively.

destained after 24 h and maintained a fairly blue color (Fig. 3A(b)). When contrasted to the other gels, the fibrin gel was destained only a little and maintained a dark blue color (Fig. 3A(c)). The absorbance of the destaining solution revealed that the gelatin gel lost unbounded dye quickly in 24 h. Also, the amount of unbound dye was high compared to the other gels (Fig. 3B). The fibrin gel showed the same time course for the releasing of unbounded dye, but the amount of unbounded dye was the lowest among the three gels.

Comparing the staining intensity of three zymographic gels after the run without samples The results of the destaining of the gel after the run is shown in Fig. 4. The gelatin gel was destained after 12 h and showed a pale blue color in all of the stripes (Fig. 4A(a)). The casein gel was destained after 12 h and maintained a fairly blue color only on the upper part of the stripe (Fig. 4A(b)). In particular, in the casein gel a trace that could be mistaken as a proteolytic band appeared around 40-50 kDa (arrow marked, Fig. 4A(b)). The fibrin gel was destained only a little and maintained a dark blue color on the whole stripe (Fig. 4A(c)). The absorbance of the destaining solution revealed that the gelatin gel lost unbounded dye quickly in 12 h, and that the amount of unbound dye was high compared to the other gels (Fig. 4B). The fibrin gel showed the same time course for the releasing of unbounded dye, but the amount of unbounded dye was the lowest among the three gels.

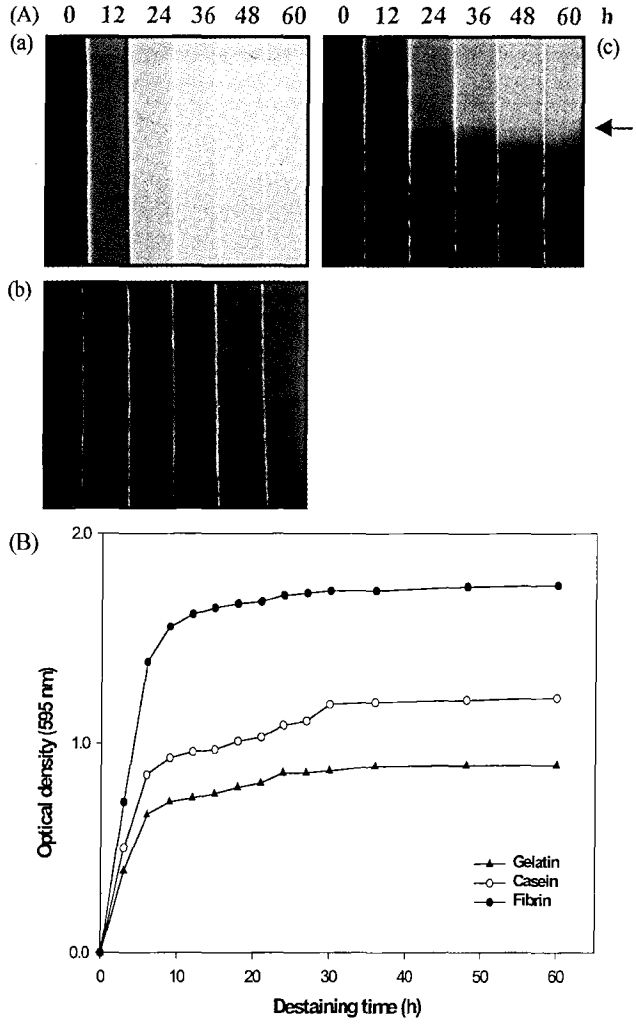

Fig. 4. Comparison of the intensity of stained three zymographic gels after running without sample. Dye stained gel strips were destained for limited periods and kept in distilled water (A). The optical density of the destaining solution of each gel was measured at 595 nm every 3 h (B). (a), (b), and (c) represent gelatin, casein, and fibrin gels, respectively. An arrow indicates the trace described in the results (appeared around 40-50 kDa).

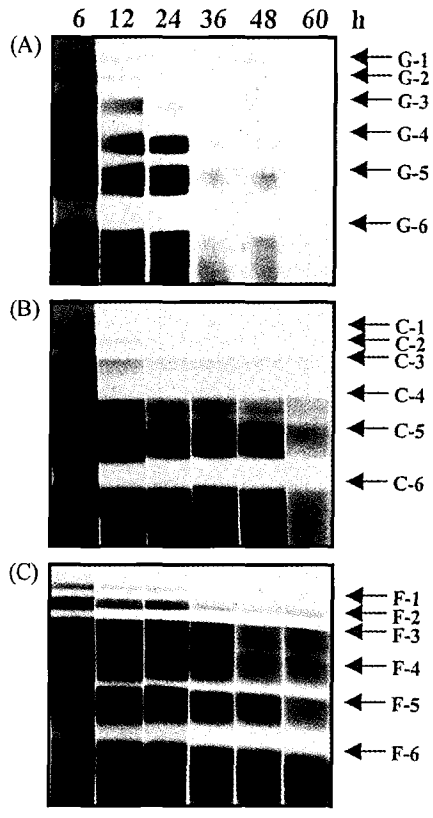

Fig. 5. Zymography of each gel loaded with the culture supernatants of *Bacillus* sp. DJ-3 screened from Doen-Jang. A, B, and C are gelatin, casein, and fibrin gels, respectively. G1-G6, C1-C6, and F1-F6 mean the proteolytic bands of each gel.

Comparing three zymographic gels after the run with samples Fig. 5 shows the results of the zymography with the culture supernatant of *Bacillus* sp. DJ-3. In the case of the gelatin gel, six bands appeared clearly at first, but they disappeared during the destaining process (Fig. 5A). Especially affected were the bands that are located in upper part of the gel. The casein gel also had six bands at first (like the gelatin gel), but the bands in the upper part of the gel become paler as the destaining progressed (Fig. 5B). The bands in the fibrin gel were clear and sustained during the destaining process (Fig. 5C).

Discussion

Zymography is known as a versatile two-stage technique that involves protein separation by electrophoresis, followed by detection of proteolytic activity in polyacrylamide gels under non-reducing conditions (Heussen and Dowdle, 1980; Raser *et al.*, 1995; Kim *et al.*, 1998; Kim and Choi, 1999; Choi and Kim, 2000). According to the enzyme's property, casein (for common protease) (Raser *et al.*, 1995), collagen (for collagenase or matrix metalloproteinase, MMP), fibrin (fibrinase) (Kim *et al.*, 1998; Choi and Kim, 1999, 2000; 2001; Kim and Choi, 1999, 2000), gelatin (for gelatinase or MMP) (Heussen and Dowdle, 1980; Kleiner and Stetler-Stevenson, 1994; Leber and Balkwill, 1997), and other protein substrates were used in zymogram gels. The *Bacillus* strain is well known for secreting various extracellular proteases (Kim *et al.*, 1996; Park *et al.*, 1999; Kim and Choi, 2000). We applied three *Bacillus* strains (DJ-1, DJ-2, and DJ-3) that were isolated from Doen-Jang, a Korean traditional fermented food, to the three-zymographic gels system (casein, fibrin, and gelatin). There was no significant difference in the pattern of extracellular proteases from the three *Bacillus* strains through the zymogram gels (Figs. 1 and 5). The three gels revealed that the enzyme activity of each protease vary on the different substrates, judged from the difference of the same band on the different gels.

Many reports have demonstrated that zymography is a

powerful technique for the identification of proteases at nanogram quantities (Kleiner and Stetler-Stevenson, 1994; Kim *et al.*, 1998). Through this study, three substrates (casein, fibrin, and gelatin) that contain gels were compared for their sensitivity using a densitometric analysis. As shown in Fig. 2, the gelatin gel showed the highest sensitivity among the three gels, based on the same molar ratio.

The blue color intensity of the destained gels may reflect the difference of the dye binding capacity of the substrate proteins. The capacity is correlated with the basic amino acid (Arginine and Lysine) content of the protein (Compton and Jones, 1985). The destaining result of the gelatin gel could be explained from its amino acid composition. Gelatin is a protein that is composed of a repeating unit of Glycine-X (usually Proline)-Y (usually Hydroxyproline). Therefore, its dye binding capacity may be low since it has a rather low number of basic amino acids. Casein (23.6 kDa) is rather small protein that has 14 basic amino acids, compared to fibrinogen (about 340 kDa) that has 126 basic amino acids. Therefore, a weak blue color of destained gel may result from the difference of strength of the binding site (Congdon *et al.*, 1993). This result suggests that a good protein substrate would be one that has more basic amino acids and strong binding sites. Fibrin, among the three substrates (Fig. 3), showed the highest binding capacity on the Coomassie brilliant blue.

The color intensity of the destained gels after the run may reflect the difference in mobility of proteins in SDS-PAGE. Casein (23.6 kDa) is a rather small protein that moves fast in the electric field, compared with fibrin (about 340 kDa). Therefore, the expected result would be a weak blue color of stained gel. The difference between the casein and fibrin gel might originate from the size and overall structure of the two proteins. Gelatin is a mixture of collagen that has different molecular weights. Therefore, the weak blue color of the gel after the running might be the result of a low dye binding capacity. When contrasted to Fig. 3, the peak points of the unbounded dye releasing time from the zymogram gel before and after the run were at 24 and 12 h, respectively. This result means that the electric current affects the binding capacity between the substrate and dye in the zymogram gel (Figs. 3 and 4). On the other hand, the samples of the zymographic gel procedures showed a similar result on the dye binding capacity in the destaining process (data not shown).

The high molecular weight of fibrin (about 340 kDa) allows a higher binding capacity of Coomassie brilliant blue dye than those of casein or gelatin. Also, the fibrous structure of fibrin permits it to be compactly packed into the polyacrylamide matrix. These factors allow fibrin to remain as a substrate in its initial co-polymerized gel with acrylamide, even after electrophoresis. It also contributes to a good resolution of high molecular weights proteases (F1-F4, Fig. 5).

As a result, we summarized the comparison of the three substrates (casein, fibrin, and gelatin) in the zymographic gel. In the three gels, there was no significant difference in the pattern of extracellular enzymes from the *Bacillus* strain. The gelatin and fibrin gels showed the highest enzymatic sensitivity, as well as the highest dye binding capacity, respectively. Therefore, the exceptional stability of the Commassie brilliant blue stained fibrin gel in the destaining solution allowed the gel to be stored a long time. Whereas, in the case of the casein gel, a trace, which could be mistaken as a proteolytic band, appeared by the electric current around 40-50 kDa. Further study will be required to understand the interaction between the substrates, loading protein, and staining dye.

References

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.

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 gel. *Anal. Biochem.* **281**, 236-238.

Choi, N. S. and Kim, S. H. (2001) The effect of sodium chloride on the serine-type fibrinolytic enzymes and the thermostability of extracellular protease from *Bacillus amyloliquefaciens* DJ-4. *J. Biochem. Mol. Biol.* **34**, 134-138.

Compton, S. J. and Jones, C. G. (1985) Mechanism of dye response and interference in the Bradford protein assay. *Anal. Biochem.* **151**, 369-374.

Congdon, R. W., Muth, G. W. and Splittgerber, A. G. (1993) The binding interaction of Coomassie blue with proteins. *Anal. Biochem.* **213**, 407-413.

Granelli-Piperno, A. and Reich, E. (1978) A study of proteases and protease-inhibitor complexes in biological fluids. *J. Exp. Med.* **148**, 223-234.

Heussen, C. and Dowdle, E. B. (1980) Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulfate and copolymerized substrates. *Anal. Biochem.* **102**, 196-202.

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.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Leber, T. M. and Balkwill, F. R. (1997) Zymography: a single-step staining method for quantitation of proteolytic activity on substrate gels. *Anal. Biochem.* **249**, 24-28.

Loskutoff, D. J., van Mourik, J. A., Erickson, L. A. and Lawrence, D. (1983) Detection of an unusually stable fibrinolytic inhibitor produced by bovine endothelial cells. *Proc. Natl. Acad. Sci. USA* **80**, 2956-2960.

Paech, C., Christianson, T. and Maurer, K. H. (1993) Zymogram of proteases made with developed film from nondenaturing polyacrylamide gels after electrophoresis. *Anal. Biochem.* **208**, 249-254.

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.

Raser, K. J., Posner, A. and Wang, K. K. W. (1995) Casein zymography: a method to study mu-calpain, m-calpain, and their inhibitory agents. *Arch. Biochem. Biophys.* **319**, 211-216.

Sprengers, E. D., Verheijen, J. U., van Hinshergh, V. W. M. and Emeis, J. J. (1984) Evidence for the presence of two different fibrinolytic inhibitors in human endothelial cell conditioned medium. *Biochim. Biophys. Acta* **801**, 163-170.