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Comparative Study of Enzyme Activity and Stability of Bovine and Human Plasmins in Electrophoretic Reagents, β-mercaptoethanol, DTT, SDS, Triton X-100, and Urea

Nack-Shick Choi[†,‡], Jeung-Ho Hahm[†], Pil Jae Maeng[‡] and Seung-Ho Kim[†,*]

[†]Proteome Research Laboratory, Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-333, Korea

[‡]Department of Microbiology, Chungnam National University, Daejeon 305-764, Korea

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Effects of common electrophoretic reagents, reducing agents (β-mercaptoethanol [BME] and DTT), denaturants (SDS and urea), and non-ionic detergent (Triton X-100), on the activity and stability of bovine plasmin (b-pln) and human plasmin (h-pln) were compared. In the presence of 0.1% SDS (w/v), all reagents completely inhibited two plns, whereas SDS (1%) and urea (1 M) denatured plns recovered their activities after removal of SDS by treatment of 2.5% Triton X-100 (v/v). However, reducing agents (0.1 M of BME and DTT) treated plns did not restore their activities. Based on a fibrin zymogram gel, five (from b-pln) and four (from h-pln) active fragments were resolved. Two plns exhibited unusual stability in concentrated SDS and Triton X-100 (final 10%) and urea (final 6 M) solutions. Two bands, heavy chain-2 (HC-2) and cleaved heavy chain-2 (CHC-2), of b-pln were completely inhibited in 0.5% SDS or 3 M urea, whereas no significant difference was found in h-pln. Interestingly, 50 kDa (cleaved heavy chain-1, CHC-1) of b-pln and two fragments, 26 kDa (light chain, LC) and 29 kDa (microplasmin, MP), of h-pln were increased by SDS in a concentration dependent manner. We also found that the inhibition of SDS against both plns was reversible.

Key words: Denaturant, Fibrinolysis, Fibrin plate, Plasmin, Zymography

Introduction

Plasmin (about 90 kDa), which formed upon activation of inactive plasminogen through cleavage of a single peptide bond, is a serine protease and plays a central role in the fibrinolytic system in blood. It consists of two polypeptide chains connected by two disulfide bonds. The heavy (A) chain (about 63 kDa, HC) originates from the amino-terminal portion of plasminogen containing its binding sites, and the light (B) chain (about 26 kDa, LC) is derived from the carboxy-terminus containing the active site (Marti *et al.*, 1985; Schaller *et al.*, 1985; Wu *et al.*, 1987a, 1987b).

In general, denaturants or detergents (*e.g.*, SDS, Trition, and Tweens) are routinely used in protein extraction and purification, as well as electrophoretic analyses of proteins. In particular, SDS-polyacrylamide gel electrophoresis has shown wide applicability in the characterization of enzyme activity using zymogram, gels (Choi *et al.*, 2002). However, it is well known that SDS enhances the activities of endothelial cell-type plasminogen activator inhibitor (Erickson *et al.*, 1986; Lambers *et al.*, 1987) and fibrinolytic enzyme from *Eisenia fetida* (Yang and Ru, 1997). In addition, SDS was found to have fibrinolytic activity in a fibrin plate without any enzyme (Chakrabarty, 1989). SDS also inhibits the activity of ribonuclease (Mendelsohn and Young, 1978) and human hemoglobin autoxidation (Reza *et al.*, 2002), and blocks the binding of the adhesion molecule, fibronectin, to collagen and heparan sulfate (Haas and Culp, 1984). Meanwhile, Trition X-100, a nonionic detergent, has been reported to enhance the activation of streptokinase, known as a plasminogen activator (Tang *et al.*, 1981; Kruithof *et al.*, 1982). Therefore, it is necessary to establish the relationship between electrophoretic reagents and enzymes on biological activity. This report is the first detailed study of the effects of commercial electrophoretic detergents on the activity and stability of two plns using a fibrin zymography.

*To whom correspondence should be addressed.
Tel: 82-42-860-4160; Fax: 82-42-879-8593
E-mail: shkim@kribb.re.kr

Materials and Methods

Materials Human fibrinogen, thrombin, human and bovine

plasmins, ultra-pure urea, DTT (dithiothreitol), SDS (sodium dodecylsulfate), and Triton X-100 were purchased from Sigma (St. Louis, USA). BME and pre-stained protein molecular standards were purchased from Bio-Rad (Hercules, USA). Other chemicals were of analytical grade.

Fibrin plate assay Enzyme activity was determined using the fibrin plate methods as previously described with minor modifications (Astrup and Müllertz, 1952). In a Petri dish, five ml of 1% (w/v) fibrinogen solution in 50 mM sodium phosphate buffer (pH 7.4) was mixed with the same volume of 2% (w/v) agarose solution along with 0.1 ml of a thrombin solution (10 NIH units/ml). The solution in the Petri dish was left for 1 h at room temperature to form a fibrin clot layer. Twenty microliters of the sample solution was then dropped into holes that had been made in the fibrin plate by a capillary glass tube (5-mm diameter). The plate was then incubated for 12 h at 37°C. The enzyme activity was estimated by measuring the dimension of the clear zone.

SDS-fibrin zymography Fibrin zymography was carried out as described previously (Kim and Choi, 1998; Choi and Kim, 2000). The separating gel solution (12%, w/v) was prepared in the presence of 0.12% fibrinogen (w/v) and 100 ml of thrombin (10 NIH units/ml). Samples were prepared by diluting 5-fold with a zymogram sample buffer (0.5 M Tris/HCl, pH 6.8, 10% SDS, 20% glycerol, and 0.3% bromophenol blue). After electrophoresis in a cold room (at 10 mA constantly), the gel was incubated for 30 min at room temperature on a rotary shaker in 50 mM Tris/HCl (pH 7.4), which contains 2.5% Triton X-100. The gel was washed with distilled water for 30 min to remove Triton X-100, and the gel was then incubated in the zymogram reaction buffer (30 mM Tris/HCl, pH 7.4, and NaN_3) for 12 h at 37°C. The gel was stained with Coomassie blue for 1 h and destained. The digested band was visualized as a non-stained region of the fibrin gel.

Results

Fibrinolytic properties of electrophoretic reagents Fibrinolytic properties of electrophoretic reagents (BME, DTT, SDS, Triton X-100, and urea) were assessed in an *in vitro* fibrin plate assay. Of the five reagents tested, only SDS was found to be fibrinolytic (Fig. 1).

Effects of electrophoretic reagents on the activity of two plns Effects of five reagents on the activity of two plns were tested. Various amounts of reagents, as indicated in the Figure Legend, were mixed with each pln and incubated for 1 h at 37°C. The mixtures were then subjected to a fibrin plate. Activities of the two plns were not affected by urea and Triton X-100 (Fig. 2), whereas reducing agents (DTT and BME) completely inhibited the activity (Fig. 2). The effect of SDS, however, could not be determined (Fig. 2).

Zymogram analysis of the effect of electrophoretic reagents on the activity of two plns We assayed the influences of four electrophoretic reagents (SDS, urea, BME, and DTT) on two plns by using fibrin zymography. Based on the zymogram gel, three major active fragments, heavy chain (HC), cleaved heavy chain (CHC), and light chain (LC) of plns, as illustrated in Fig. 3B, were separated using SDS-fibrin zymography (Fig. 3). In the presence of SDS (0.1%,

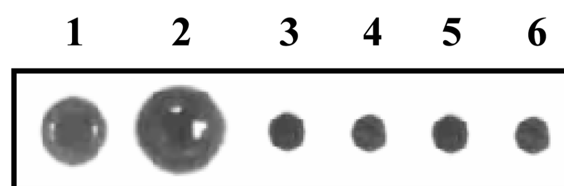

Fig. 1. Fibrinolytic properties of five electrophoretic reagents on a fibrin agarose plate. 1, plasmin (1 NIH unit/ml); 2, SDS (1%); 3, DTT (0.1 mM); 4, BME (0.1 mM); 5, Triton X-100 (1%); 6, urea (1 M). The plate was incubated for 12 h at 37°C.

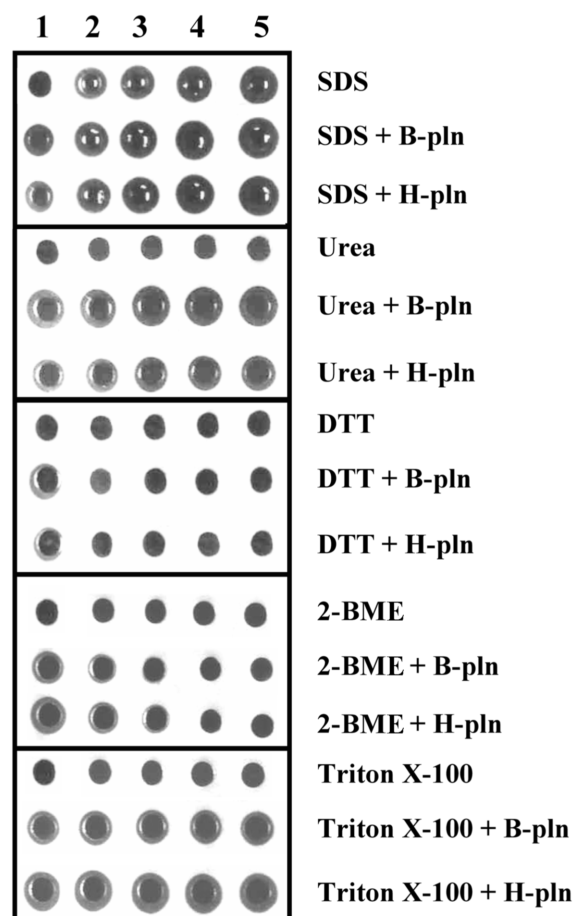

Fig. 2. Effects of five electrophoretic reagents on the activities of two plns using fibrin plates. Equal volume of pln (1 NIH unit/ml) and each detergent were mixed and incubated for 1 h at 37°C. The mixtures were then subjected to fibrin plates. Numbers 1-5 represent the final concentrations of reagents [SDS (0, 0.1, 0.5, 1, and 2%), urea (0, 0.5, 1, 2, and 5 M), DTT (0, 0.1, 0.5, 1, and 2 mM), BME (0, 0.1, 0.5, 1, and 2 mM), and Triton X-100 (0, 0.5, 1, 2, and 5%)].

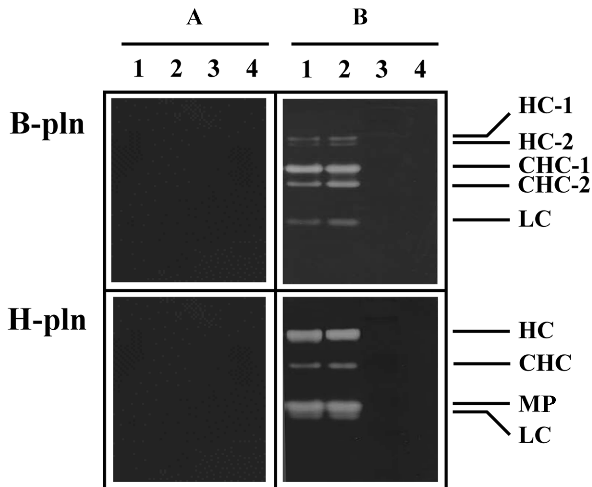

Fig. 3. Effects of four electrophoretic reagents (SDS, urea, BME, and DTT) on the activities of two plns by using fibrin zymography. After electrophoresis, one gel was directly incubated in a zymogram reaction buffer (A) while another gel was incubated for 30 min in 2.5% Triton X-100 (50 mM Tris/HCl, pH 7.4) to remove SDS from the gel (B). The gel was washed with distilled water and incubated in a zymogram reaction buffer for 12 h at 37ºC. Numbers 1-4 represent SDS (0.1%), urea (1 M), BME (0.1 mM), and DTT (0.1 mM), respectively.

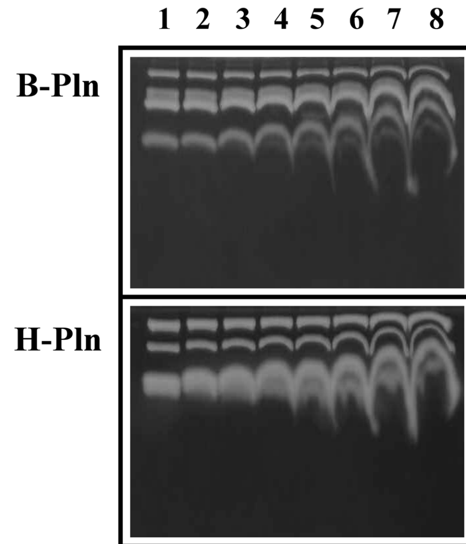

Fig. 4. Effect of Triton X-100 on the activities of two plns by using fibrin zymography. Equal volume of pln (1 NIH unit/ml) and various concentration (0-20%) of Triton X-100 were mixed and incubated for 1 h at 37ºC. The mixtures were then subjected to fibrin zymography. After electrophoresis, the gel was washed with distilled water and incubated in a zymogram reaction buffer. Numbers 1-8 represent the final concentration of Triton X-100 (0, 1, 2, 3, 5, 7, 9, and 10%).

electrophoretic concentration) on the gel, all reagents completely inhibited both plns (Fig. 3A). However, after removing SDS from the gel by treatment of Triton X-100, the activity was recovered on two denaturants, SDS and urea (Fig. 3B, lanes 1 and 2), whereas the activity on the reducing agents (DTT and BME) was not restored (Fig. 3B, lanes 3 and 4).

Effect of Triton X-100 on the activity of two plns The effect of Triton X-100 on the activity of two plns was studied. Various concentrations of Triton X-100 (0-20%, v/v) were mixed with two plns and incubated for 1 h at 37ºC. The mixtures were then subjected to the fibrin zymogram gel. Activities of the two plns were not influenced by Triton X-100, which only caused migration of the plns (Fig. 4).

Effects of urea and SDS on the activity of two plns We also investigated the effects of urea and SDS on two plns. After incubation of two denaturants and the plns for 1 h at 37ºC, the samples were then loaded on the fibrin zymogram gel. After electrophoresis, the gel was treated with Triton X-100. As shown in Fig. 5, both plns exhibited unusual stability in the concentrated SDS (final 10%) and urea (final 6 M) solutions. However, two bands, heavy chain-2 (HC-2) and cleaved heavy chain-2 (CHC-2), of b-pln disappeared in 0.5% SDS and in 3 M urea, whereas no significant difference was found in h-pln. Interestingly, 50 kDa (CHC-1) of b-pln and two fragments, 26 kDa (LC) and 29 kDa (MP), of h-pln were increased by SDS in a concentration dependent manner. Finally, we studied the relationship between SDS and plns by repeating addition and removal of SDS and Triton X-100. As a result, we found that the inhibition of SDS against both plns was reversible (Fig. 6).

Discussion

This report describes a comparative study of the activity and stability of two plns, bovine and human, in electrophoretic reagents (*e.g.* BME, DTT, SDS, Triton X-100, and urea). Plns consist of two polypeptide chains connected by two disulfide bonds. The heavy (A) chain (about 63 kDa, HC) contains the binding sites and the light (B) chain (about 26 kDa, LC) contains the active site. After autolysis, various active forms (*e.g.* HC, CHC, MP, and LC) from pln were produced (Marti *et al.*, 1985; Schaller *et al.*, 1985; Wu *et al.*, 1987a, 1987b). These forms were completely inhibited by reducing agents (BME and DTT) (Fig. 3). This indicates that intramolecular disulfide bonds exist in all proteins. Two plns, as shown in Fig. 3, showed the same pattern in reducing agents. Also, a nonionic detergent, Triton X-100, did not influence on the activity, only resulting in the migration of the proteins of plns (Fig. 4). In common, an average polypeptide binds about 1.4 ± 0.3 g of SDS per gram of protein. And, nonionic detergent was used to effectively displace SDS from proteins (Bischoff *et al.*, 1998). Thus, it is proposed that Triton X-100 influences SDS to bind the plns, which caused migration on the SDS gel.

Denaturants such as SDS and urea are routinely used in biochemical laboratories in processes such as solubilizing

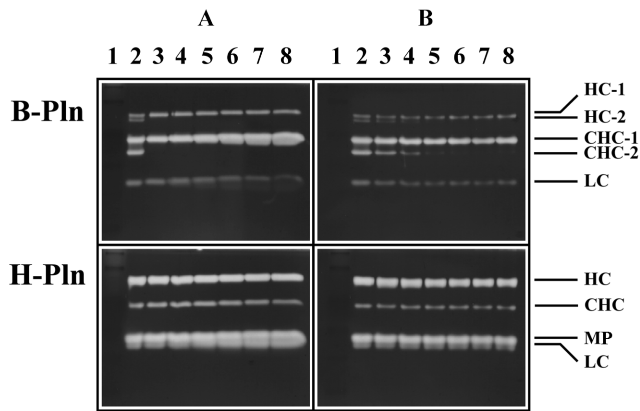

Fig. 5. Effects of SDS (A) and urea (B) on the activities of two plns by using fibrin zymography. After incubation of two detergents and plns for 1 h at 37ºC, the mixtures were then loaded on the fibrin zymogram gel. After electrophoresis, the gels were treated with Triton X-100: (1) protein marker; (2-8) the final concentrations of SDS (0, 0.5, 1, 3, 5, 7, and 10%) and urea (0, 1, 2, 3, 4, 5, and 6 M).

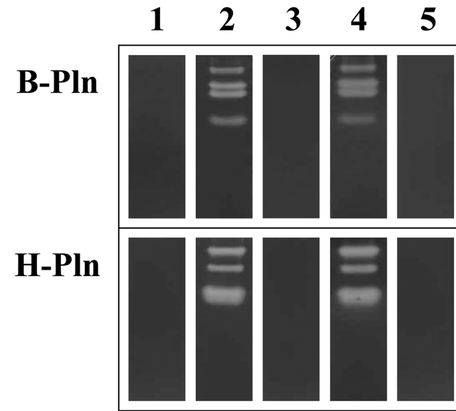

Fig. 6. Inhibition study of SDS against two plns by using fibrin zymography. After electrophoresis, the gels were repeated addition and removal of SDS: (1) without any treatment, (2) Triton X-100 for 30 min, (3) Triton X-100 for 30 minSDS (1%) for 30 min, (4) Triton X-100SDSTriton X-100, and (5) Triton X-100SDSTriton X-100SDS.

cellular proteins and electrophoretic analyses. In particular, SDS is an extremely effective denaturant for proteins; in its presence most proteins lose their functions either completely or partially with a disruption of tertiary and quaternary structure (Lantz and Ciborowski, 1994; Bischoff *et al.*, 1998). Most enzymes are denatured by treatment with SDS and are inactive when complexed with this denaturant. However, most proteases do renature following removal of SDS from the gel (Lantz and Ciborowski, 1994; Han and Damodaran, 1997; Bischoff *et al.*, 1998). As shown in Fig. 3, after treatment with Triton X-100, two plns recovered their activities, which were denatured by SDS (0.1%, electrophoretic concentration). Interestingly, two bands (HC-2 and CHC-2) of b-pln did not recover their activities in 0.5% SDS, as shown in collagen type to gelatin, which is irreversibly denatured by SDS (Lantz and Ciborowski, 1994) (Fig. 5). It has been reported that bovine and human plasmin (ogen) contain the same structure and functional domains (Schaller *et al.*, 1985) and the major difference is their ability to be activated to plasmin by a plasminogen activator, streptokinase (Wulf and Mertz, 1969). However, through this report, we newly found that b-pln and h-pln show different enzymatic pattern in SDS solution (Fig. 5). In addition, 50 kDa of b-pln and two bands, 26 and 29 kDa, of h-pln were increased by SDS in a concentration dependent manner (Fig. 5A). Meanwhile, urea did not affect the h-pln, whereas it inhibited the HC-2 and CHC-2 of b-pln (Fig. 5B), as occurred in SDS (Fig. 5A).

Based on our results, we found that two plns were very sensitive to SDS and the inhibition of SDS against both plns is shown to be reversible (Fig. 6). In addition, we newly found that b-pln and h-pln exhibited some different enzymatic properties in SDS and urea solutions, although their protein structure and functional domains are very similar. In conclusion, the work described here will allow further structure-function study regarding SDS-sensitive or -stimulated fragments.

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