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Short communication

A Method for Direct Application of Human Plasmin on a Dithiothreitol-containing Agarose Stacking Gel System

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A new simplified procedure for identifying human plasmin was developed using a DTT copolymerized agarose stacking gel (ASG) system. Agarose (1%) was used for the stacking gel because DTT inhibits the polymerization of acrylamide. Human plasmin showed the lowest activity at pH 9.0. There was a similar catalytically active pattern observed under acidic conditions (pH 3.0) to that observed under alkaline conditions (pH 10.0 or 11.0). Using the ASG system, the primary structure of the heavy chain could be established at pH 3.0. This protein was found to consist of three fragments, 45 kDa, 23 kDa, and 13 kDa. These results showed that the heavy chain has a similar structure to the autolysed plasmin (Wu *et al.*, 1987b) but there is a different start amino acid sequence of the N-termini.

Keywords: Diagonal electrophoresis, Disulphide, Dithiothreitol, Human plasmin, Zymography

Introduction

Dithiothreitol (DTT) is commonly used as a protective agent in biochemical studies to prevent the oxidation of SH (thiol) groups and to reduce disulfides to dithiols. Generally, a two-dimensional sequential non-reducing/reducing SDS-PAGE (*diagonal electrophoresis*) is used to examine the formation of intermolecular protein disulfide bonds (Brennan *et al.*, 2004). First, a protein sample in the SDS sample buffer without reducing agents was resolved on an SDS-polyacrylamide gel (1.0 mm thickness). After electrophoresis, the gel lanes containing the separated proteins were removed and immersed in a SDS sample buffer containing 50 mM DTT for 10 min (reduction step). Each gel strip was then applied horizontally to another gel (1.5 mm thickness), and electrophoresis was performed in the second dimension. In this study, a new DTT copolymerizsed ASG system was developed to identify human plasmin. Using this method, the primary structure of human plasmin could be established under acidic conditions.

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Materials and methods

Reagents The human fibrinogen, thrombin, plasmin, Triton X-100, and DTT were purchased from Sigma (St. Louis, USA). The other chemicals were of analytical grade.

Zymography The fibrin zymogram gel was used as described previously (Kim *et al.*, 1998; Kim and Choi, 1999; Choi and Kim, 2000). The separating gel solution (12%, w/v) was prepared in the presence of fibrinogen (0.12%, w/v) and 100 ml of thrombin (10 NIH units/ml). The samples were diluted 5 times with a SDS sample buffer with or without DTT (500 mM). Electrophoresis was carried out in a cold room (at 10 mA constantly). Subsequently, the gel was incubated for 30 min at room temperature on a rotary shaker in 50 mM Tris (pH 7.4) containing 2.5% Triton X-100. The gel was washed with distilled water for 30 min to remove the Triton X-100, and incubated in a zymogram reaction buffer (30 mM Tris, pH 7.4, and NaN_3) at 37°C for 12 h. The gels were stained with Coomassie blue for 1 h and then destained. The active bands were visualized as non-stained regions of the zymogram gels.

Sequential two-dimensional non-reducing/reducing SDS-PAGE The protein samples (in the SDS sample buffer without any reducing agents) were resolved on a 12% SDS-polyacrylamide gel [Bio-Rad (Hercules, USA), mini protean III system, 1.0 mm thickness]. After electrophoresis, the gel lanes containing the separated proteins were removed and immersed in a SDS sample buffer containing 50 mM DTT for 20 min. Following a brief wash with an electrophoresis running buffer for 10 min, the gel slice was then applied horizontally to another gel (1.5 mm thickness).

ASG two-dimensional non-reducing/reducing SDS-PAGE After non-reducing electrophoresis (12% acrylamide, 1.0 mm thickness),

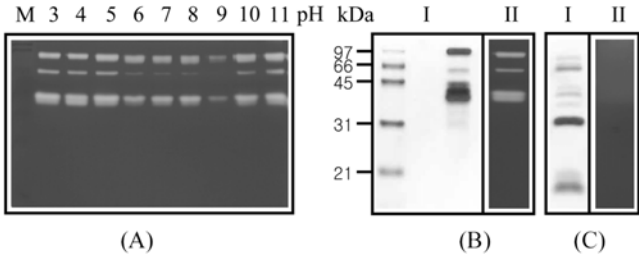

Fig. 1. SDS-PAGE and fibrin zymography of human plasmin. After incubating 10 μl of plasmin (1.0 NIH unit/ml) under various pH conditions (pH 3-11) for 2 h, the samples (non-reducing) were applied to the zymogram gel (A). The plasmin incubated at pH 10 were diluted 5 times with the SDS sample buffer containing DTT (C) or no DTT (B). The samples were then applied to the SDS and zymogram gels. The SDS (I) and zymogram (II) gels were visualized using silver and Coomassie blue staining, respectively.

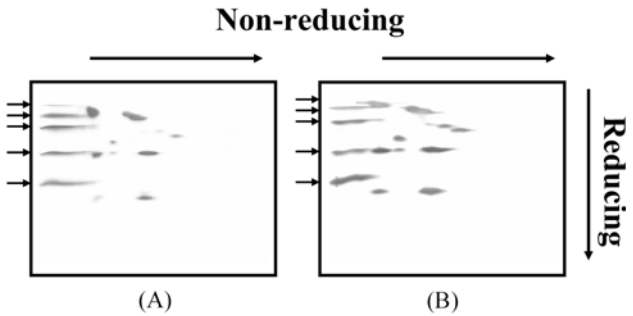

Fig. 2. Comparison of the ASG 2-DE gel (A) with sequential gel system (B). After non-reducing electrophoresis with human plasmin, the second gels were used as described in Materials and Methods. The gels were visualized by silver staining. The arrows indicate five standard molecular masses; 97, 66, 45, 31, and 21 kDa.

the gel slice containing the separated proteins was loaded directly on the DTT containing ASG. The DTT containing ASG (1.0 mm thickness) was prepared by adding 100 μl of 1 M DTT in 0.01 M sodium acetate (pH 5.2) to a pre-warmed agarose solution [1.0% (w/v) in 0.5 M Tris buffer, pH 6.8] at 55-60°C.

N-terminal amino acid sequencing After SDS-PAGE was carried out, the protein on the gel was transferred to a polyvinylidene difluoride (PVDF; Bio-Rad) membrane by electroblotting (Matsudaira, 1987). The membrane was then stained with Coomassie blue. The stained portion was excised and used directly for N-terminal sequencing using a Gas-phase protein sequencer (model Procise 491, ABI, USA) according to the automated Edman degradation method.

Results and discussion

A fibrin zymographic technique that detects plasmin-like fibrinolytic enzymes in nanogram quantities was previously described (Kim *et al.*, 1998; Kim and Choi, 1999; Choi and Kim, 2000). The enzyme activity and stability of bovine and human plasmin in electrophoretic reagents (*e.g.* β-mercaptoethanol, DTT, SDS, Triton X-100, and urea) was also reported (Choi *et al.*, 2005).

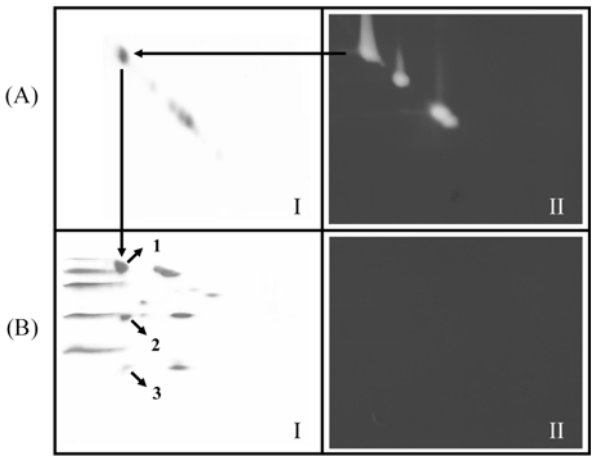

Fig. 3. Separation of human plasmin by two-dimensional SDS-PAGE and zymography. After non-reducing electrophoresis with the same condition sample described in Fig. 1 (B), the gel slices containing the separated proteins were loaded on the second gel (A, not contained DTT) and DTT containing ASG (B). The SDS (I) and zymogram (II) gels were visualized using silver and Coomassie blue staining, respectively.

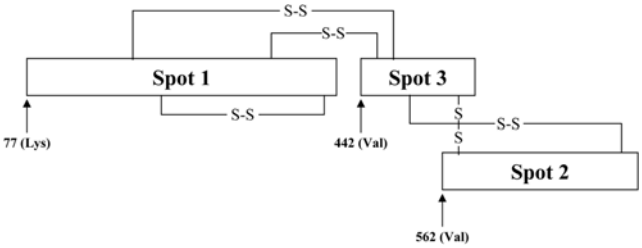

Fig. 4. Structure of human plasmin under acidic condition (pH 3.0). The disulfide bonds have been proposed by Wu *et al.* [8].

First, the activity of human plasmin was identified with zymography under various pH conditions (pH 3-11). The sample was incubated in either a 0.1 M citrate-phosphate buffer (pH 3.0 to 5.0), sodium phosphate buffer (pH 6.0 to 7.0), tris-HCl buffer (pH 8.0 to 9.0), or a glycine-NaOH buffer (pH 10.0 to 11.0) for 2 h. As described previously, the activity of plasmin declined at almost neutral pH (pH 6.0) (Wu *et al.*, 1987a). However, it showed the lowest activity at pH 9.0,. A similar catalytically active pattern was observed under acidic conditions, which is similar to that observed under alkaline conditions (Wu *et al.*, 1987a) (Fig. 1A).

The plasmin activity was next examined under reducing or non-reducing conditions at pH 3.0. As shown in Fig. 1B, three major active bands were identified in the non-reducing gel, whereas no activity was detected in the reducing gel (Fig. 1C). This suggests the presence of intramolecular disulfide bonds in all proteins, which were completely inhibited by the

Table 1. N-terminal amino acid sequences of the spots

Spot No.	Sequence	Position (amino acid)[a]
1	KKVYLSEC	77-441
2	VVGGCVAH	562-791
3	VVAPPPVV	442-561

[a]Accession No. NM 000301.

reducing agent (DTT). It was reported that plasmin consists of two polypeptide chains that are connected by two disulfide bonds. The heavy (A) chain contains the binding sites and the light (B) chain contains the active site (Schaller *et al*., 1985; Wu *et al*., 1987a, 1987b).

The specific fragmentation and enzymatic activity for the structure-function study of plasmin were identified using two methods to determine the sequence of the fragments: a *diagonal electrophoresis* (Brennan *et al*., 2004) and a newly designed DTT copolymerized ASG system. Agarose (1%) was used for the stacking gel because DTT inhibits polymerization of acrylamide (data not shown). Based on SDS and the zymogram gels, the two methods showed similar protein profiles of human plasmin (Fig. 2). The primary structure of heavy chain could be established using the ASG system (Fig. 3 and Table 1). This protein was found to consist of three fragments, 45 kDa (spot 1), 23 kDa (sopt 2), and 13 kDa (spot 3). In addition, its primary structure was also determined based on the report by Wu *et al*. (Wu *et al*., 1987b) (Fig. 4). These results showed a similar structure with the autolysed plasmin, but differed from the start amino acid sequence of the N-termini.

This study developed an improved and simplified procedure for identifying disulfide-linked enzyme using a DTT copolymerized ASG system. The ASG method has two distinct advantages over the *diagonal electrophoresis* method (Brennan *et al*., 2004). First, the ASG method is a timesaving method in that it does not require a reduction step. Second, the first and second gels, having a similar thickness, can be used throughout the 2-DE process. In contrast, when using the diagonal electrophoresis method, the first gel swells during the reduction and washing steps resulting in the need for a thicker second gel. The ASG system allows the non-reducing gel to be applied directly to the second gel, thereby shortening the assaying time whilst yielding similar results. Overall, this method can be used to identify disulfide-linked enzymes from many other organisms.

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