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A novel assay to evaluate promoting effects of proteins on calcium oxalate crystal invasion through extracellular matrix based on plasminogen/plasmin activity

Wararat Chiangjong, Visith Thongboonkerd *

Medical Proteomics Unit, Office for Research and Development, Faculty of Medicine Siriraj Hospital, and Center for Research in Complex Systems Science, Mahidol University, Bangkok, Thailand

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ABSTRACT

One of the important processes in kidney stone development is crystal invasion through extracellular matrix (ECM). Some proteins in renal tissue or urine have been thought to aggravate crystal invasion. However, this pathogenic mechanism has been previously under-investigated due to a lack of crystal invasion assay. In the present study, we have developed a novel assay for the investigations of calcium oxalate monohydrate (COM) crystal invasion. Matrix gel was loaded into an in-house migration chamber made on a glass slide to simulate the ECM environment. COM crystals were coated with the tested protein, which was then bound with plasminogen. The crystal-protein-(plasminogen) complex and urokinase plasminogen activator (uPA) were placed on-top of the matrix gel. If the tested protein had plasminogen-binding capability, the remaining plasminogen would be activated by uPA to plasmin, which caused crystal migration through the matrix gel. We then applied this novel assay to evaluate effects of some abundant kidney/urine proteins (including purified albumin, carbonic anhydrase, lysozyme and Tamm–Horsfall protein) on COM crystal invasion. The data revealed that albumin, which is the known plasminogen-binding protein, dramatically induced plasmin activity and crystal invasion, whereas other proteins had no significant effects as compared to the control. In summary, we have successfully developed a novel assay for the investigations of crystal invasion based on the plasminogen/plasmin system. This assay is applicable to examine proteins that may serve as potential aggravators of crystal invasion and thus will be very useful for further studies on kidney stone development.

1. Introduction

Calcium oxalate monohydrate (COM) is a major crystalline compound to form kidney stones [1]. Important mechanisms of COM kidney stone formation include crystal growth, adhesion, aggregation and invasion [2,3]. Recent lines of evidence have demonstrated that the deposited crystals can migrate between intracellular and intercellular compartments, and finally translocate into renal interstitium, leading to activation of immune response and accumulation of inflammatory cells (i.e., monocytes and macrophages) [4]. However, the detailed process of crystal invasion through extracellular matrix (ECM) into the renal interstitium remained unclear, while recruitment of the inflammatory cells into this locale has been extensively studied in many disease models [5–7]. This pathogenic mechanism has been previously under-investigated due to a lack of crystal invasion assay.

Plasminogen/plasmin is a well-known system that regulates innate and adaptive immune responses, coagulation/thrombolysis by fibrin and fibrin degradation products, and extracellular proteolysis [8,9]. Plasminogen is primarily synthesized and secreted by hepatocytes and other cells in kidney, adrenal glands, brain, testis, heart, lungs, uterus, spleen, thymus, and intestines [10,11]. Urokinase plasminogen activator (uPA) was initially found in human urine and can convert plasminogen to plasmin, which can degrade fibrin and ECM components [12,13]. Activation of plasminogen on the cell surface can promote fibrinolysis, cell migration and prohormone processing on various cells [13–16]. The cells, which bind to plasminogen, also express cell-surface plasminogen activator receptor. The co-localization of plasminogen and its activator receptor can promote plasminogen activation [17]. For example, monocyte/macrophage cell surface that presents plasminogen receptor can bind to plasminogen, which is then converted to plasmin by plasminogen activation cascade [14,15].

* Corresponding author. Tel.: +66 2 4195500; fax: +66 2 4195503.
E-mail addresses: thongboonkerd@dr.com, vthongbo@yahoo.com (V. Thongboonkerd).

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Unlike monocytes/macrophages and other cells, COM crystals have no plasminogen receptor on their surfaces. However, the crystals are able to invade into ECM. Actually, COM crystals can bind to a number of various kidney/urine proteins [18,19]. We thus hypothesized that these proteins, on the other hand, are able to bind to plasminogen that is also present in kidney/urine. Plasminogen is then converted by uPA to plasmin, which subsequently degrades ECM component, thereby crystal invasion. In the present study, we have developed a novel assay for the investigations of proteins that might be involved in COM crystal invasion based on the reaction of uPA to activate plasminogen to plasmin. We then applied this novel assay to evaluate effects of some abundant kidney/urine proteins (including purified albumin, carbonic anhydrase, lysozyme and Tamm–Horsfall protein) on COM crystal invasion.

2. Materials and methods

2.1. COM crystal preparation

The stock solutions of 10 mM calcium chloride and 1 mM sodium oxalate were prepared in a buffer containing 90 mM Tris–HCl (pH 7.4) and 10 mM NaCl. The two solutions were gently mixed (1:1) and then incubated at room temperature (RT) overnight. The mixture was centrifuged at 12,000 rpm at 25 °C for 5 min and then COM crystal pellet was collected. The harvested COM crystals were washed with absolute methanol three times and were then dried at 37 °C overnight. Typical COM crystal morphology was observed under a light microscope before use.

2.2. Preparation of a migration chamber containing matrix gel

Materials for making a migration chamber included glass slide (Sail Boat Lab Co., Ltd.; Zhejiang, China), Menzel–Glaser cover slip (Glasbearbeitungswerk GmbH; Braunschweig, Germany), used X-ray film (Kodak, Carestream Health, Inc.; Rochester, NY), two-sided adhesive tape (Siam Armstrong Co., Ltd.; Bangkok, Thailand) and sealing material used for slide mounting (e.g., nail polish) (Fig. 1A). The methods for making a migration chamber are detailed in Fig. 1B. First, the used X-ray film was cut into U-shape (approximately to a size of the cover slip) and then cleaned with 70% alcohol. Three pieces of the U-shape X-ray film were adhered together by two-sided adhesive tape strips. Actually, the number of the U-shape X-ray film, which was used as a spacer, could be modified based on the desired thickness of the matrix gel inside the migration chamber. This spacer was then adhered to a glass slide and, on the other side, to a cover slip using two-sided adhesive tape strips. This chamber was then sealed with nail polish and was decontaminated by UV irradiation for 30 min before use. BD Matrigel™ Basement Membrane Matrix (BD Bioscience; Franklin Lakes, NJ) (containing collagen types I–VI, laminin, fibronectin, osteopontin, and vitronectin) was dissolved in a pre-chilled Eagle's minimum essential medium (MEM) (Gibco; Grand Island, NY) on ice to make 4.5–6 mg/ml final working solution of the matrix gel. Approximately 200 μl of the matrix gel was added into the migration chamber and left at 37 °C for at least 4 h (Fig. 1B).

2.3. COM crystal-protein binding assay and SDS-PAGE

COM crystals (5 mg) were incubated with 1 μg of each of the proteins to be tested in 1 ml of PBS. These tested proteins included purified bovine serum albumin (BSA), carbonic anhydrase (Sigma), lysozyme (Sigma), and Tamm–Horsfall protein (all were from Sigma; St. Louis, MO). The crystal–protein mixture was gently and continuously rotated at 4 °C overnight. The mixture was then centrifuged at 15,000 rpm and 4 °C for 5 min, and the supernatant was discarded. The pellet was extensively washed with PBS three times, 4 mM EDTA three times, and again with PBS three times using high-speed vortex (to make sure that weak or non-specific bindings were completely eliminated). The bound protein was eluted by 1 X Laemmli's buffer, resolved in 12% SDS-PAGE gel using SE260 mini-Vertical Electrophoresis Unit (GE Healthcare; Uppsala, Sweden) at 150 V for approximately 2 h, and then stained with Deep Purple fluorescence dye (GE Healthcare). The gel image was captured by a Typhoon laser

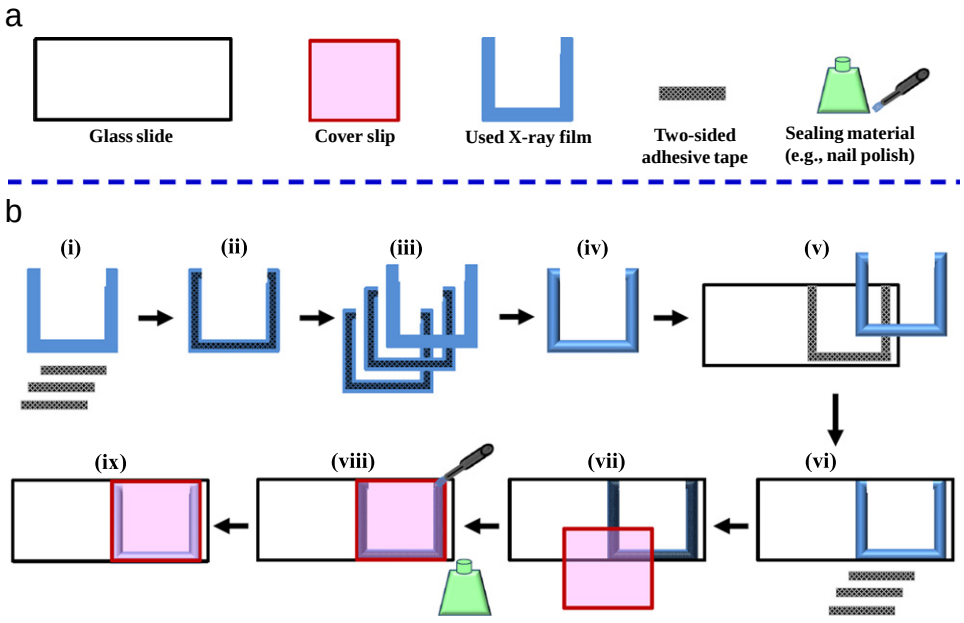

Fig. 1. Preparation of a migration chamber containing matrix gel. All the materials used for making the migration chamber are indicated in panel (A), whereas stepwise methods are detailed in panel (B). (i) The used X-ray film was cut into U-shape (approximately to a size of a cover slip), whereas two-sided adhesive tape was cut into strips. (ii) U-shape X-ray film was adhered with two-sided adhesive tape strips. (iii)–(iv) Three pieces of the U-shape X-ray film were adhered together by two-sided adhesive tape strips to make a spacer. (v) The U-shape spacer was then adhered to a glass slide. (vi)–(vii) A cover slip was adhered on the U-shape spacer using two-sided adhesive tape strips. (viii) This chamber was then sealed with a sealing material (e.g., nail polish). (ix) The migration chamber was then dried and decontaminated by UV irradiation before use.

scanner (GE Healthcare). Intensity of bands appeared in each whole lane was quantitated by Image Master densitometer (GE Healthcare).

2.4. Plasmin activity assay

One microgram of a protein (BSA, carbonic anhydrase, lysozyme, or Tamm–Horsfall protein) in 100 μl of 50 mM $NaHCO_3$ was coated onto each of 96-well plate at 4 °C overnight. Thereafter, the uncoated protein was removed and the well was washed with PBS three times. Thereafter, 200 μl of 6 nM Lys-plasminogen (Fitzgerald Industries International; Acton, MA) in PBS was added into the well and was further incubated at 37 °C for 4 h. The unbound plasminogen was discarded and the well was washed with PBS five times. Thereafter, 200 μl of 1 nM uPA (Fitzgerald Industries International) in PBS and 0.15 mM chromogenic substrate S-2251 (Chromogenix, Instrumentation Laboratory Company; Lexington, MA) were added and incubated with the samples at 37 °C for 24 h. The control was decided using only 100 μl of 50 mM $NaHCO_3$ to coat the well without protein, but with the presence of plasminogen, uPA and chromogenic substrate at later steps. The absorbance of each sample at 0 h and 24-h post-incubation with uPA was measured at λ405 nm using Anthos HTII Microplate Reader (Anthos Labtec Instruments; Salzburg, Austria). The plasmin activity was obtained by subtracting the 24 h absorbance with that of 0 h absorbance of each individual sample.

2.5. COM crystal invasion assay

A total of 20 μg COM crystals was added into 200 μl of MEM. Then, 1 μg of the tested protein (BSA, carbonic anhydrase, lysozyme, or Tamm–Horsfall protein) was added and the mixture was incubated at 4 °C overnight with gently continuous agitation (Fig. 2A). The unbound protein was discarded by a centrifugation at 15,000 rpm and 4 °C for 5 min, and the crystal-protein complex was washed with PBS. Thereafter, 200 μl of 0.3 pM Lys-plasminogen in PBS was mixed and incubated with the crystal-protein complex at 37 °C for 1 h (Fig. 2B). The unbound plasminogen was discarded by a centrifugation at 15,000 rpm for 5 min and the pellet was washed with PBS once. Then, 100 μl of 0.15 pM uPA in PBS was mixed with the crystal-protein-(plasminogen) complex (Fig. 2C) (Note: If the tested protein had plasminogen-binding capability, the remaining plasminogen would be activated by uPA to plasmin, which caused crystal migration. In contrast, if the tested protein did not bind to plasminogen, there would not be plasmin as the final product.). The mixture was then added on-top of the matrix gel inside the migration chamber and incubated at 37 °C for 24 h (Fig. 2D). After 24 h incubation, the solution remained on the upper part of the migration chamber was removed by absorption using a gauze or tissue paper. The invaded COM crystals inside the matrix gel were then imaged using a light microscope with differential interference contrast (DIC) mode (Nikon H600L, Nikon Corp.; Tokyo, Japan) (Fig. 2D). The crystal invasion distance was measured and averaged from at least 50 crystals/field in 15 different fields within the same chamber using Image Frame Work software version 0.9.6 (Tarosoft®; Nonthaburi, Thailand). For the control, COM crystals were processed as aforementioned without binding protein, but with the presence of plasminogen and uPA at later steps.

2.6. Statistical analysis

All the experiments were performed in triplicate independently. The quantitative data are reported as mean ± SEM. Statistical analysis was performed using unpaired Student's *t*-test to compare plasmin activity and COM crystal invasion to the controls. P values less than 0.05 were considered statistically significant.

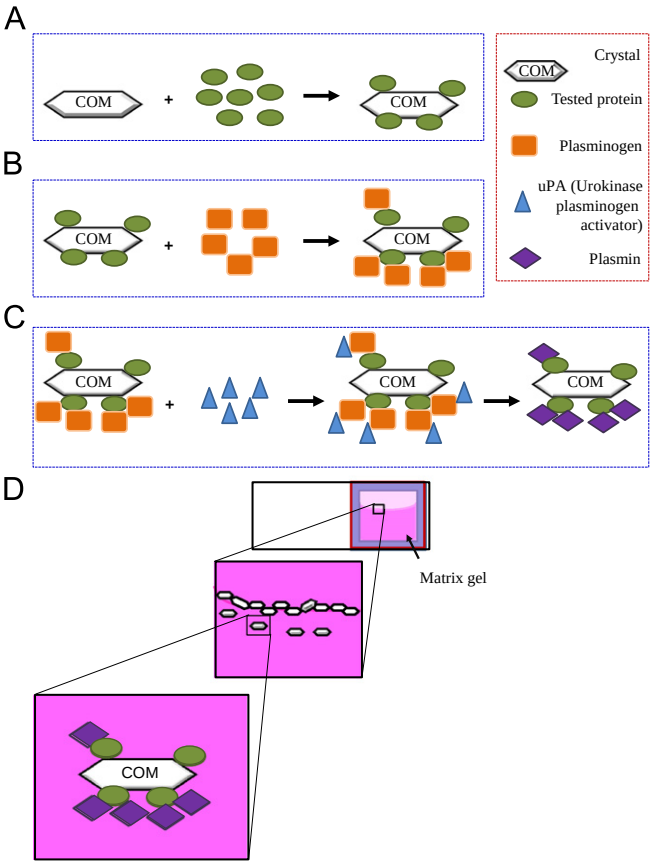

Fig. 2. Methodology of COM crystal invasion assay. (A) *Step 1* COM crystals were incubated with a tested protein at 4 °C overnight. (B) *Step 2* Lys-plasminogen was incubated with the crystal-protein complex at 37 °C for 1 h. (C) *Step 3* uPA in PBS was mixed with the crystal-protein-(plasminogen) complex. If the tested protein had plasminogen-binding capability, the remaining plasminogen would be activated by uPA to plasmin, which caused crystal migration. In contrast, if the tested protein did not bind to plasminogen, there would not be plasmin as the final product. (D) *Step 4* The mixture of crystal-protein-(plasminogen) complex and uPA was added on-top of the matrix gel inside the migration chamber and incubated at 37 °C for 24 h. The invaded COM crystals inside the matrix gel were then imaged using a light microscope with differential interference contrast (DIC) mode.

3. Results and discussion

Multi-step process of intratubular kidney stone formation is initiated with supersaturation of calcium and oxalate ions, followed by crystal nucleation, crystal growth and aggregation [20]. Additionally, the crystals can be internalized into the cells by endocytosis and then migrate through basement membrane and ECM [21,22]. In another theory of kidney stone development, the stone is initiated primarily in the tubulointerstitial compartment with the well-known Randall's plaque formation [23]. The plague is essential to develop the stone, particularly at the basement membrane of the thin Henle's loop [24]. Both intratubular and interstitial pathogenic mechanisms of kidney stone disease have the common crucial processes required for the stone development, including crystal retention, crystal invasion, and tissue inflammation [25]. While crystal retention and tissue inflammation have been extensively investigated in association with kidney stone disease, crystal invasion has been previously under-investigated because there was no available tool or assay to address such important pathogenic mechanism.

In the present study, we aimed to (i) develop a novel assay to investigate COM crystal invasion; and (ii) apply such novel assay to evaluate effects of some abundant kidney/urine proteins on COM crystal invasion. We first made an in-house migration chamber by using common materials and reagents available in almost all life-science laboratories (Fig. 1A). These included common glass slide for microscopy, cover slip, used X-ray film, two-sided adhesive tape, and sealing material used for slide mounting (e.g., nail polish). Stepwise methodology for making the migration chamber is detailed in Section 2 (Fig. 1**B**). Thereafter, the matrix gel (containing collagen types I–VI, laminin, fibronectin, osteopontin, and vitronectin) was added into this chamber. Crystal-protein-(plasminogen) complex was prepared as detailed step-by-step in Section 2 (see also Fig. 2). It should be noted that this crystal invasion assay is based on plasminogen/plasmin system. If the tested protein had plasminogen-binding capability, the remaining plasminogen would be activated by uPA to plasmin, which caused crystal migration. In contrast, if the tested protein did not bind to plasminogen, there would not be plasmin as the final product and the crystals could not migrate (Fig. 2).

We then confirmed that all the tested proteins, which are abundant kidney/urine proteins, could bind to COM crystals (in *Step 1* of the COM crystal invasion assay described in Fig. 2A) for reasonable application in subsequent COM crystal invasion assay. The data obtained from COM crystal-protein binding assay and SDS-PAGE using equal amount of each purified protein confirmed that all the tested proteins, including purified albumin, carbonic anhydrase, lysozyme, and Tamm–Horsfall protein were bound to the crystals, as demonstrated by their bands at respective sizes (Fig. 3A). To determine relative amounts of all proteins bound to COM crystals, intensity of protein bands appeared in each whole lane was measured. The data showed that albumin, carbonic anhydrase, and lysozyme had comparable relative amount of the bound fraction, whereas Tamm–Horsfall protein had the greatest amount of the bound fraction (Fig. 3B).

To also confirm that *Steps 2 and 3* of the COM crystal invasion assay (as described in Fig. 2B,C) worked well, plasmin activity was measured in individual samples. This experiment was also to prove that once the protein was bound to the crystals, if it had a plasminogen-binding capability, plasminogen would remain in the crystal-protein-(plasminogen) complex and thus could be activated by uPA to plasmin. Plasmin could then cut the chromogenic substrate (H-D-Val-Leu-Lys-pNA) to amino acid residues (H-D-Val-Leu-Lys) and chromophore (pNA), which presented the yellow color that was measurable at λ405 nm. The data confirmed that some the COM crystal-binding proteins (i.e., albumin) could retain plasminogen in the crystal-protein-(plasminogen) complex and thus could activate the plasmin activity, whereas other COM crystal-binding proteins had no effects on plasmin activity (Fig. 4). This data was in agreement with the findings reported previously that albumin serves as a carrier protein for plasminogen and thus could bind to plasminogen for subsequent activation to plasmin [26,27]. It should be noted that the plasmin activity was obtained by subtracting the absorbance of the sample at 24 h post-incubation with uPA to that of 0 h. Unlike other conventional enzymatic assays, e.g. ELISA, in which non-specific bindings (or another word, bindings of proteins onto the plate surface) are

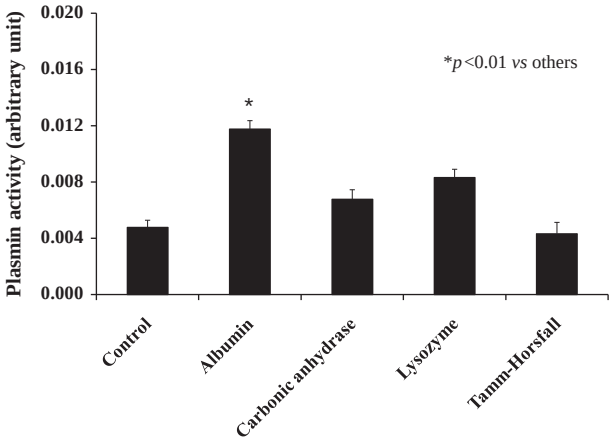

Fig. 4. An assay to confirm that plasminogen could bind to a specific plasminogen-binding protein. The tested protein (purified albumin, carbonic anhydrase, lysozyme, or Tamm–Horsfall protein) was coated onto each of 96-well plate at 4 °C overnight, whereas the controlled well was incubated with 100 μl of 50 mM $NaHCO_3$ without protein. After washing, Lys-plasminogen was added into the well and was further incubated at 37 °C for 4 h. After the final wash, uPA and chromogenic substrate S-2251 were then incubated with the samples at 37 °C for 24 h. The plasmin activity was measured at λ405 nm using a microplate reader. Each bar was derived from three independent experiments and the data are reported as mean ± SEM. $^*p < 0.01$ compared to other groups.

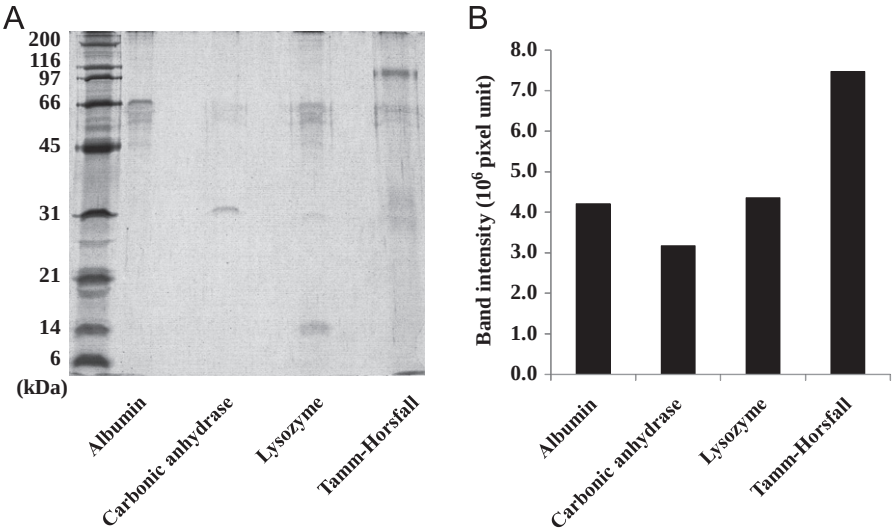

Fig. 3. SDS-PAGE to confirm that the tested protein was bound to COM crystals. COM crystals were incubated with purified albumin (BSA), carbonic anhydrase, lysozyme, or Tamm–Horsfall protein at 4 °C overnight. The bound protein was then eluted by 1 X Laemmli's buffer, resolved in 12% SDS-PAGE gel, and stained with Deep Purple fluorescence dye (A). Intensity of bands appeared in each whole lane was then quantitated using a densitometer (B).

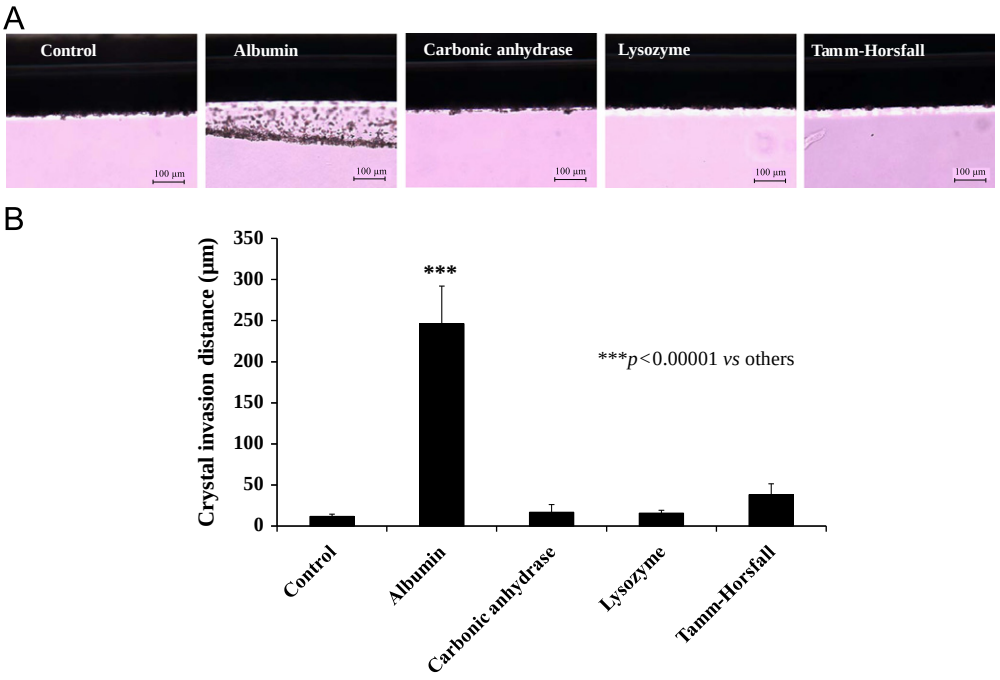

Fig. 5. COM crystal invasion assay to evaluate effects of proteins on crystal invasion. The assay was performed as detailed in Figs. 1 and 2 (A) At the end, the micrographs of the invaded crystals were obtained from individual assays to examine the tested proteins. The bar indicates 100 μm scale. (B) The crystal invasion distance was measured and averaged from at least 50 crystals/field in 15 different fields within the same chamber. For the control, COM crystals were processed as aforementioned in the presence of plasminogen and uPA, but without a binding protein on the crystal surface. Each bar was derived from three independent experiments and the data are reported as mean ± SEM. $^{***}p < 0.00001$ compared to other groups.

normally blocked by BSA in the blocking step, the present study omitted such blocking step to avoid the interference from the blocking protein (BSA) to the plasmin activity. Without any blocking, plasminogen could also bind to the plate surface non-specifically. Therefore, the control sample without any tested protein (but with plasminogen, uPA, and chromogenic substrate) revealed a background of plasminogen activity. In contrast to the other proteins that had comparable background plasmin activity as compared to the control, albumin provided significantly greater plasmin activity (Fig. 4).

The crystal-protein-(plasminogen) complex together with uPA (which was originally found in the urine and also ECM of the kidney) was then used for subsequent COM crystal invasion assay. The crystal invasion data (Fig. 5) were consistent to those of plasmin activity shown in Fig. 4. The findings demonstrated that only the crystals bound with albumin, which was the only protein that could induce plasmin activity, caused the crystal invasion, whereas other proteins had no significant effects as compared to the control (Fig. 5). Considering the relative amounts of individual proteins bound to the crystals (Fig. 3), the selectivity of albumin for induction of COM crystal invasion could be highlighted. Whereas Tamm–Horsfall protein had the greatest amount of the bound fraction and carbonic anhydrase and lysozyme had comparable relative amount of the bound fraction as compared to albumin, none of them showed significant induction of COM crystal invasion.

In summary, we have successfully developed an in vitro assay for the investigations of COM crystal invasion, based on the reaction of plasminogen and uPA, which can activate plasminogen to plasmin that causes crystal invasion through ECM. This novel assay is also applicable to examine the proteins of interest to search for the potential protein modulators that serve as the activators of COM crystal invasion. Thus, it will be very useful for further studies on kidney stone development.

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