Chromogenic Substrate Assay for Determining the Activity of Plasma Kallikrein

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[Abstract] The activation of the intrinsic pathway takes place at negatively charged surfaces, such as bacteria, and involves activation of cogulation Factor XII, which then leads to the activation of plasma kallikrein (PK) and coagulation Factor XI. To determine the PK activity on bacterial surfaces, bacteria were pre-incubated with peptides, followed by incubation with plasma, and the effect of peptide was recorded by measuring the PK activity.

Materials and Reagents

1. Specific bacteria [*e.g. Escherichia coli* (*E. coli*), ATCC, catalog number: 25922; *Pseudomonas aeruginosa* (*P. aeruginosa*), ATCC, catalog number: 27853)]
2. 3% Todd-Hewitt (TH) broth
3. 3% TH agar plates
4. Human citrated plasma (fresh)
5. 50 mM Tris/HCl (pH 7.4, sterile)
6. 50 mM $ZnCl_2$ in H_2O (sterile)
7. Chromogenic substrate S-2302 (Chromogenix, catalog number: S820340) (4 mM in H_2O)
8. DAPTTIN TC (Technoclone, catalog number: 5035060)
9. Peptides (*e.g.* antimicrobial peptide LL-37) or other agents to be tested

Equipment

1. Microcentrifuge tube
2. BD vacutainer® plus blood collection tubes (BD, catalog number: 364305)
3. 96-well flat bottom plates
4. Centrifuge for microcentrifuge tubes
5. ELISA reader (A = 405 nm)
6. Incubator 37 °C

Procedure

A. Assay with bacteria

Day 1

1. Grow bacteria overnight in fresh 10 ml 3% TH medium, at 37 °C and shaking with 200 rpm.

Day 2

2. In a fresh 10 ml 3% TH medium add 100 µl of overnight culture bacteria and subculture bacteria to mid-exponential phase (OD_{620} ~ 0.5), at 37 °C and 200 rpm.
3. Centrifuge bacteria (10 min, 5,000 rpm) and remove the supernatant.
4. Wash bacterial pellet twice with 10 ml of 50 mM Tris/HCl (pH 7.4).
5. Resuspend bacterial pellet in 50 mM Tris/HCl (pH 7.4) + 50 µM $ZnCl_2$ to a final concentration of 2 x 10^9 cfu/ml.

   Note: To get 2 x 10^9 cfu/ml, growth curves for the specific bacteria are necessary in order to know how much volume is needed for resuspention, before doing the experiment.

   Take 100 µl of bacteria and incubate with 10 µl of the test agent or buffer (positive control) for 60 sec at RT.

   Notes:

   a. *Negative controls contain only 100 µl bacteria mixed with 100 µl 50 mM Tris/HCl (pH 7.4) instead of plasma.*
   b. *Incubation times and amount of substrate might need adjustements according to the specific bacterium tested* (Papareddy *et al.*, 2013).
6. Add 100 µl citrate plasma to the samples.
7. Incubated 35 min at 37 °C on rotation 200 rpm.
8. Spin down the bacteria (6 min, 5,000 rpm) and wash the bacterial pellets once with 50 mM Tris/HCl (pH 7.4) (100 µl/sample).
9. Resuspend in 100 µl of 50 mM Tris/HCl (pH 7.4) + 50 µM $ZnCl_2$ buffer containing 2 mM of the chromogenic substrate S-2302 (mix well).
10. Incubate samples for 30-60 min at 37 °C (check yellow colour development in positive control, an indication of chromophore cleavage and dissociated from the enzyme), to determine incubation time.
11. Centrifuge samples (6 min, 5,000 rpm) and transfer the supernatants to a 96 well plate.
12. Measure absorbance at 405 nm. Values are presented in relation to the positive control (defined as 100%).

B. Assay without bacteria

1. Incubate 10 µl of your desired agent with 100 µl of Dapttin for 60 sec at RT.

2. Add 100 µl of human citrate plasma and incubate samples for 3 min at room temperature.
3. Centrifuge the pellet (6 min, 5,000 rpm) and wash twice in 50 mM Tris/HCl (pH 7.4) before suspension in 100 µl 50 mM Tris/HCl (pH 7.4) + 50 µM $ZnCl_2$ buffer containing 2 mM of the chromogenic substrate S-2302.
4. Incubate for 30 min at RT.
5. Centrifuge samples.
6. Measure absorbance of the supernatant at 405 nm. Values are presented in relation to the positive control (defined as 100%).

   Notes:

   a. *Regarding negative controls, add 100 µl of 50 mM Tris/HCl (pH 7.4) instead of adding 100 µl of human citrate plasma at step B2. But, everything else is the same from steps B1-6.*
   b. *Incubation time for the cleavge of substrate and subsequent colour development may differ. If the development of colour is to low, concentration of the substrate can be increased.*

Acknowledgments

This protocol is adapted from Papareddy *et al.* (2013).

References

1. Papareddy, P., Kalle, M., Sorensen, O. E., Malmsten, M., Morgelin, M. and Schmidtchen, A. (2013). The TFPI-2 derived peptide EDC34 improves outcome of gram-negative sepsis. *PLoS Pathog* 9(12): e1003803.

