Supporting Information

Real-Time Label-Free Kinetics Monitoring of Trypsin-Catalyzed Ester Hydrolysis by a Nanopore Sensor

Mingjuan Li, Ayesha Rauf, Yanli Guo* and Xiaofeng Kang*

*Corresponding author: Prof. Yanli Guo and Prof. Xiaofeng Kang

College of Chemistry & Materials Science

Northwest University, Xi'an 710127, P. R. China

Fax: +86-029-81535025;

Tel: +86-029-81535025;

E-mail: guoyl@nwu.edu.cn; kangxf@nwu.edu.cn

EXPERIMENTAL SECTION

Reagents and Materials. The $(M113R)_7$ α-HL proteins were synthesized, expressed and purified as previously reported.[1-2] Trypsin (EC 3.4.21.4, TPCK) from bovine pancreas was purchased from Sigma. Heptakis-(6-deoxy-6-amino)-β-cyclodextrin (am_7β-CD) (> 99 %) was obtained from Cycloab (Budapest, Hungary). Nα-Benzoyl-L-arginine ethyl ester hydrochloride (BAEE), calcium chloride ($CaCl_2$) and Nα-Benzoyl-L-arginine (BA) were from Aladdin. 1, 2-Diphytanoylphosphatidylcholine (DPhPC) lipid was obtained from Avanti Polar Lipids. The sodium chloride (NaCl, ＞ 99.9 %) and Tris (＞ 99.9 %) that made up the electrolyte were purchased from Kermel Chemical Reagents Co., Ltd. (Tianjin, China). All of the solutions were dissolved in ultrapure water. The concentration of trypsin was 10 mg/mL. The $(M113R)_7$ and trypsin solutions were kept at −20 °C before and immediately after use.

Nanopore Experiments. A bilayer of DPhPC was formed using the traditional method of Montal-Mueller[3] on an aperture 100 ~ 150 μm in diameter in a Teflon film that divided a planar bilayer chamber into two compartments, *cis* and *trans*. All of the experiments were performed under a symmetrical buffer solution with 1.5 mL 1 M NaCl and 10 mM Tris·HCl (pH 8.0) at room temperature. After formation of a planar bilayer, the $(M113R)_7$ was added to the *cis* compartment, which was connected to the ground. The final concentration of the $(M113R)_7$ proteins used for the single-channel insertion was 0.05 ~ 0.2 ng·mL^{-1}. In this way, after the successful insertion of a single α-HL pore, the BAEE and $CaCl_2$ were introduced to the *cis* side, and the am_7β-CD

was added to the *trans* side, following by the background signal for at least 10 min in the absence of trypsin. Then, the BAEE hydrolysis experiments were monitored in real-time after the addition of trypsin to the *cis* side with 120 min of recording. The single channel current was detected with Ag/AgCl electrodes and recorded with a patch clamp amplifier (Axopatch 200B, Axon Instruments, Foster city, CA, USA). The signal was filtered with a low-pass Bessel filter at 5 kHz and sampled at 20 kHz by a computer equipped with a Digidata 1440 A/D converter (Molecular Devices).

Data Analysis. Single-channel current traces were analyzed through pClamp 10.3 (Molecular Devices), and the data were processed by Origin 9.1 (Microcal, Northampton, MA) software. The events were detected using the Event Detection feature in pClamp 10.3, in which amplitude (I_B) and dwell time (τ_{off}) histograms were constructed. Origin 9.1 was used for histogram construction, curve fitting and graph presentation. The τ_{off} and τ_{on} (mean inter-event interval) were obtained from the fitted exponential distributions, and the values of the mean current blockades were produced from the fitted Gaussian distributions.

1. The current response of am$_7$β-CD, trypsin and BAEE.

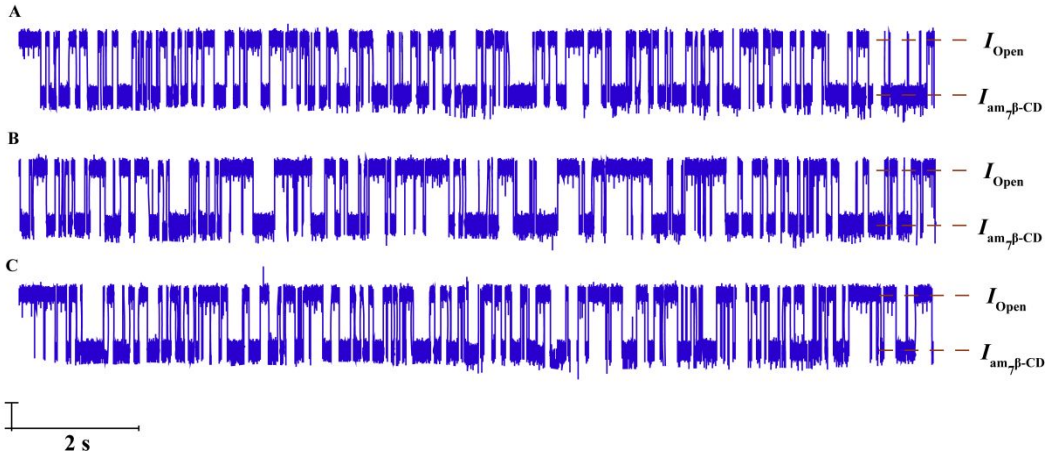

Figure S1 The current signal recordings of (A) am$_7$β-CD, (B) am$_7$β-CD and trypsin (C) am$_7$β-CD and BAEE. These experiments were carried out under 1 M NaCl, 10 mM Tris-HCl and pH 8.0. 40 μM am$_7$β-CD was introduced from *trans* chamber, 0.8 μM trypsin and 66.6 μM BAEE were added from *cis* chamber respectively and the transmembrane voltage was +140 mV.

As shown in Figure S1, neither trypsin nor BAEE would cause current response on the am$_7$β-CD platform.

2. The identification of the product and standard BA.

As shown in Figure S2A, both the product BA (left) and the standard BA (right) could generate current signal on the am$_7$β-CD platform level. The product BA had the same dwell time (τ_{off} = 0.39 ± 0.03 ms) with standard BA (τ_{off} = 0.38 ± 0.02 ms) on am$_7$β-CD platform, and the I_B/I_0 of product BA (I_B/I_0 = 0.73 ± 0.01) was consistent with the standard BA (I_B/I_0 = 0.73 ± 0.02) (Figure S2B, C).

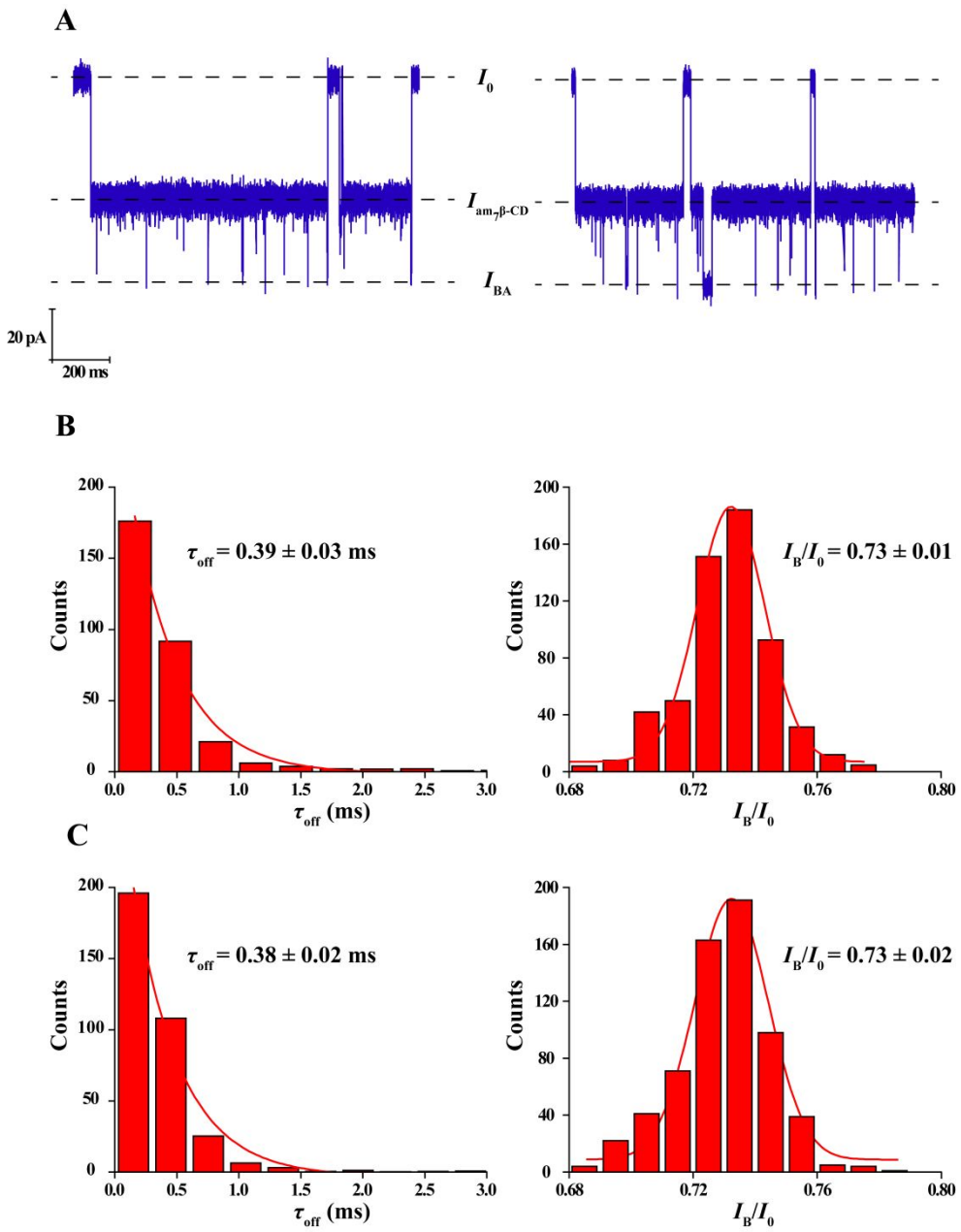

Figure S2 The identification of trypsin-catalyzed product BA and standard BA. (A) The representative single-channel current recordings of cleaved product BA (left) and standard BA (right) interacting with the nanopore sensor. (B) Histograms of the dwell time and I_B/I_0 of the trypsin-catalyzed product BA; (C) Histograms of the dwell time and I_B/I_0 of standard BA. Experiments were conducted in 1 M NaCl, 10 mM Tris-HCl at pH 8.0, 50 μM standard BA was added from *cis* chamber, 40 μM am_7β-CD was introduced from *trans* chamber and the transmembrane voltage was +140 mV.

3. The identification of hydrolysate ethanol.

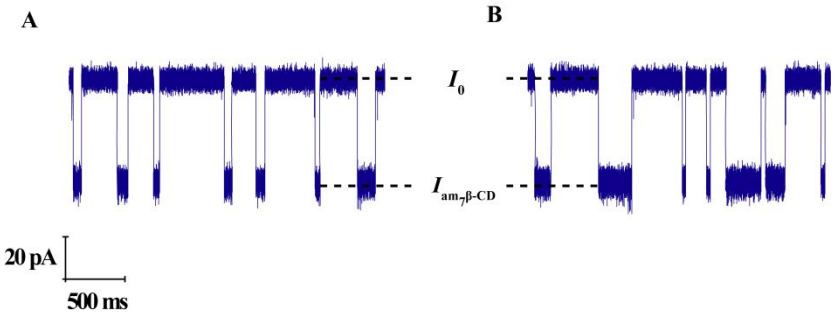

Figure S3 The single-channel current recordings (A) in the absence and (B) presence of standard ethanol. Experiments were conducted in 1 M NaCl, 10 mM Tris-HCl at pH 8.0, and 40 μM am_7β-CD was added from *trans* chamber, and 100 μM standard ethanol added from *cis* or *trans* side respectively, and the transmembrane voltage was +140 mV.

Whether ethanol was added from the *cis* or *trans* chamber of the nanopore, it cannot produce a current response on the am_7β-CD sensing platform (Figure S3). This might be due to the mismatch between ethanol and am_7β-CD - nanopore system.

4. The interaction between BA and $(M113R)_7$ equipped with am_7β-CD.

It has been reported that β-cyclodextrins demonstrate different orientations in distinct mutant α-hemolysin nanopores.[4] In terms of $(M113R)_7$, the wider entrance (secondary hydroxyls) of am_7β-CD faces to the *cis* side of the nanopore.[5] In our experiments, we found that there was no signal when both the standard BA and am_7β-CD were in the *trans* chamber. However, when the standard BA was introduced to the *cis* chamber, a new current signal was emerged in the presence of am_7β-CD added to the *trans* chamber. Therefore, we considered that BA interacted with the broad opening of the am_7β-CD. Then, we investigated the kinetics of the interaction of BA with am_7β-CD. As shown in Figure S4, the event frequency ($f = 1/\tau_{on}$) increased approximately linearly with the BA concentrations, which was consistent with a bimolecular association interaction; while the dissociation rate constants ($k_{off} = 1/\tau_{off}$) were not dependent on the concentrations of BA, which corresponded to a unimolecular dissociation mechanism.[6-7]

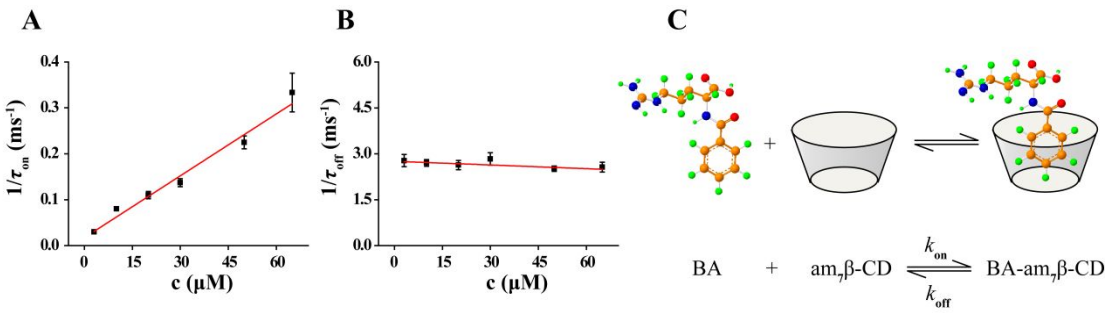

Figure S4 The dependence of reversible current events of the BA interaction with am$_7$β-CD under different standard BA concentrations (3.0 ~ 65.0 μM). (A) The reciprocal of the mean interevent interval (τ_{on}) and (B) Reciprocal of the dwell times (τ_{off}) versus the standard BA concentrations. (C). The kinetic scheme of BA combined with am$_7$-βCD. The measurement was carried out in 1 M NaCl, 10 mM Tris-HCl (pH 8.0), and the transmembrane voltage was +140 mV. The event statistics demonstrated in this part were analyzed based on at least 20 min single-channel recordings.

5. The response of BA on (M113R)$_7$ - β-CD platform.

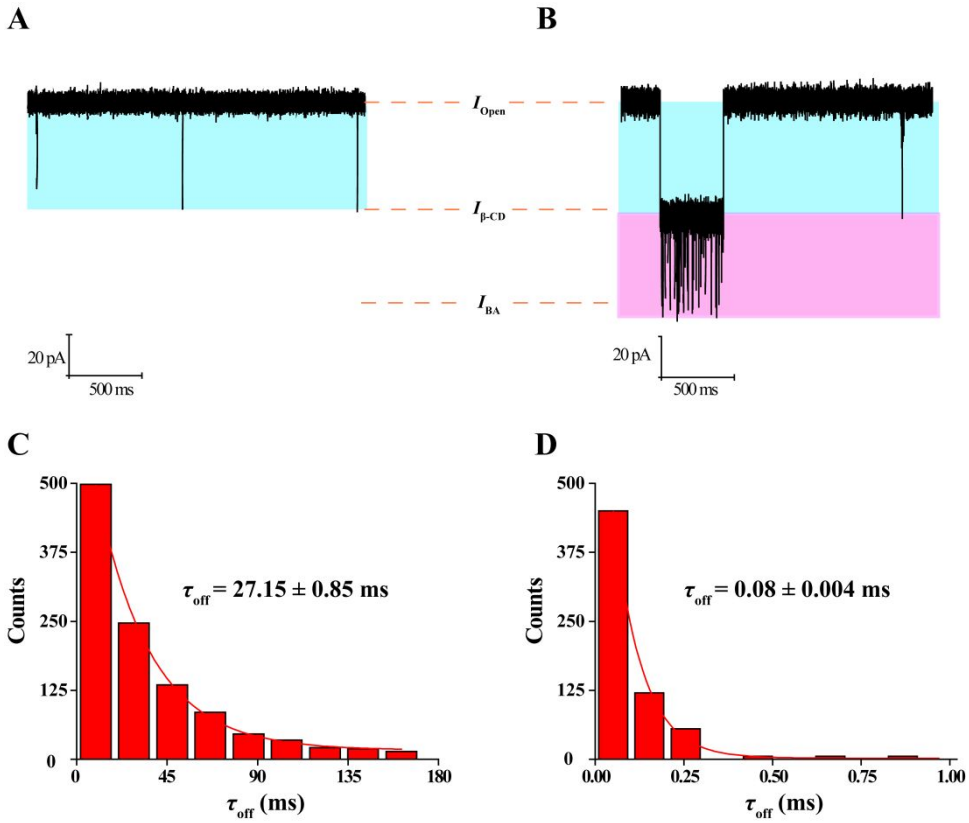

Figure S5 The representative single-channel current recording traces of (A) β-CD interaction with (M113R)$_7$ and (B) BA interaction with (M113R)$_7$-β-CD. (C) The dwell time of the am$_7$β-CD on (M113R)$_7$ was 27.15 ± 0.85 ms; (D) The dwell time of the β-CD on (M113R)$_7$ was 0.08 ± 0.004 ms. Experimental conditions: 1 M NaCl, different β-cyclodextrins in the *trans* compartment, 10 mM Tris-HCl at pH 8.0, +140 mV.

The dwell time of am$_7$β-CD on (M113R)$_7$ is about two orders of magnitude longer than β-CD on (M113R)$_7$. Therefore, in order to more intuitively observed the change

of the product BA with time, we chose $am_7\beta$-CD - $(M113R)_7$ system.

6. The calibration for concentration of hydrolysis product BA.

Table S1 The comparison of product BA concentration calculated at 30 min and substrate concentration.

$[BAEE]_0$	$f_{t=30min}$	[BA]	Er/%
6.60	0.046 ± 0.001	6.43 ± 0.21	-2.58
8.25	0.050 ± 0.003	7.29 ± 0.24	-11.64
13.20	0.066 ± 0.002	12.08 ± 0.20	-8.48
26.47	0.138 ± 0.010	26.99 ± 3.11	1.96
66.60	0.335 ± 0.021	70.81 ± 1.25	7.29

$[BAEE]_0$ indicated the initial concentration of substrate BAEE, $f_{t=30min}$ represented the BA frequency of trypsin hydrolysis of BAEE at 30 min, [BA] showed the calculated concentration of the product BA based on the calibration curve of standard BA and event frequency in Figure S3A, Er/% corresponded the relative error between calculated BA concentration and initial concentration of substrate BAEE. The data listed in the above table was obtained based on 1 M NaCl, 10 mM Tris-HCl at pH 8.0, various concentrations (6.60 ~ 66.6 μM) of BAEE and 0.8 μM trypsin were added to *cis* side, 40 μM $am_7\beta$-CD was introduced from *trans* side, and the voltage was +140 mV.

In order to evaluate the real concentration of the hydrolysis product BA in the solution, the standard curve of frequency of standard BA was set up corresponding to different concentration standard BA (3.0 ~ 65.0 μM). By comparing the frequency of BA generated by the trypsin hydrolysis of BAEE with this calibration curve (Figure S3A), we could obtain the concentration of BA in the enzymatic reaction mixture solution. We checked frequency ($f = 1/\tau_{on}$) of product BA (t = 30 min) at various substrate BAEE concentrations at a certain amount of trypsin (0.8 μM) (Table S1). The results showed the concentrations of calculation BA were approximately consistent with those of substrate BAEE, which could further confirm that the concentration of the substrate BAEE consumed in a given period of time t = 30 min was equal to the product BA produced in the enzymatic hydrolysis reaction. According to the above analysis, the value of the frequency of BA could represent the real concentration of the product BA produced.

References

1. Cheley, S.; Braha, O.; Lu, X.; Conlan, S.; Bayley, H., A functional protein pore with a “retro”

transmembrane domain. *Protein Sci.* **1999,** *8*, 1257-1267.

2. Wang, L.; Han, Y.; Zhou, S.; Wang, G.; Guan, X., Nanopore biosensor for label-free and real-time detection of anthrax lethal factor. *ACS Appl. Mater. Interfaces.* **2014,** *6*, 7334-7339.

3. Montal, M.; Mueller, P., Formation of Bimolecular Membranes from Lipid Monolayers and a Study of Their Electrical Properties. *Proc. Natl. Acad. Sci. U. S. A.* **1972,** *69*, 3561-3566.

4. Banerjee, A.; Mikhailova, E.; Cheley, S.; Gu, L. Q.; Montoya, M.; Nagaoka, Y.; Gouaux, E.; Bayley, H., Molecular bases of cyclodextrin adapter interactions with engineered protein nanopores. *Proc. Natl. Acad. Sci. U. S. A.* **2010,** *107*, 8165-8170.

5. Astier, Y.; Braha, O.; Bayley, H., Toward single molecule DNA sequencing: Direct identification of ribonucleoside and deoxyribonucleoside 5 '-monophosphates by using an engineered protein nanopore equipped with a molecular adapter. *J. Am. Chem. Soc.* **2006,** *128*, 1705-1710.

6. Hammerstein, A. F.; Shin, S.-H.; Bayley, H., Single-Molecule Kinetics of Two-Step Divalent Cation Chelation. *Angew. Chem. Int. Ed.* **2010,** *122*, 5211-5216.

7. Shin, S. H.; Steffensen, M. B.; Claridge, T. D.; Bayley, H., Formation of a chiral center and pyrimidal inversion at the single-molecule level. *Angew. Chem. Int. Ed.* **2007,** *46*, 7412-7416.