The facile and sensitive detection of peptides is essential for drug screening, pathogen detection, and protein sequencing. There are still challenges for the real-time single-molecule sensing and detection of peptides due to their versatile shape, structure, and charges brought by amino acids. Nanopore sensing is an emerging technology for sensing of biomolecules including DNA, RNA, and proteins. In this study, the interaction between peptides of different lengths (N6–N10) and charges with an engineered Mycobacterium smegmatis porin A nanopore are systematically studied, and two types blockage events can be identified by quantifying their dwell times and amplitude of blockades. The findings are further applied to the label-free and real-time quantification of protease activity of caseinolytic protease P at nanomolar concentration in 14 min. The protease activity with inhibitor can also be monitored real time by nanopore assay. In summary, this nanopore-based sensing platform shows promising capacity for peptide detection, protease activities assay, and inhibitor screening.

Peptides detection has drawn great research attention for their underlying relationship with expression levels in biological process and diseases including Parkinson’s disease, Alzheimer’s disease, cancer, and viral infections.[1–4] The vast majority of approaches to detect, characterize, and quantify peptides are based on classical fluorescence labeling,[5] immunoassay strategy,[6] and mass spectrometry (MS).[7] However, these traditional methods suffer disadvantages in efficiency or sensitivity. Limitations including high-concentration requirement, time-consuming, and complex sample preparation further constrain their applications.[8] Nanopore sensing technology has emerged as a label-free and amplification-free tool for single molecule analysis.[9–13] The defining feature of confining a single molecule within a nanometer-scale channel makes it a highly sensitive sensor for peptide detection, including characterizing peptides,[14,15] exploring enzymatic kinetics with peptide substrates,[16–18] studying conformation,[19] examining the nature of intermediates,[20] and detecting the aggregation of peptides.[21] Furthermore, nanopores have been used to reveal the dynamics of peptides and even identify differences between single amino acids in peptide chains.[22–24] Mycobacterium smegmatis porin A (MspA) is particularly attractive for peptide detection since it has a short and narrow constriction which can provide improved sensitivity and spatial resolution.[25] MspA self-assembles into a compact homooctamer nanopore with its crystal structure showing a 0.6-nm length and a 1.2-nm diameter at the constriction site.[26] Mutagenesis of MspA has been intentionally used to remove the negatively charged aspartate residue to better facilitate electronegative analyte translocation. Additionally, MspA is among the robust protein nanopores that show quite a strong tolerance to extreme conditions, such as acid-base variation, high temperatures, and protein denaturation,[27] which strengthen the potential of application of the nanopore in diverse and stringent conditions. These characteristics have made MspA to be one of the most commonly used biological nanopore for the detection of nucleic acids.[28] In theory, the ionic current through MspA induced by small polymers is suitable for peptide detection. However, to date, the information on detection of peptides by MspA is still under investigation.

In this study, the engineered MspA is extended to the single-molecule detection of the negatively charged peptides. By modulating the applied potentials and salt gradients, we systematically analyzed two interaction mode between PO4−-TYLYW and the mutant MspA which was further verified via the identification of varying-length (N6–N10) negative peptides. Furthermore, this developed MspA-based nanopore system of peptides detection could be employed to establish a novel, label-free method to quantitatively measure ClpP activity and verify inhibitors by real-time monitoring the proteolytic cleavage of peptide substrates.

First, the mutant MspA[29] (Figure S1, Supporting Information) was inserted in the lipid bilayer membrane to form single channel and ultimately utilized for identification of peptides. The
residual current and dwell time were used to characterize the peptides, and the residual current of the blockage events could be obtained by $I_r = I_o - I_b$, where $I_r$ represented the residual current when the pore was blocked, $I_b$ was the blocked current, and $I_o$ was the open pore current. Then, we carried out the translocation experiments utilizing a neutral peptide TYLYW. Unfortunately, few distinct signals were observed. Although previous research on nanopores indicated that electroosmosis could drive uncharged peptides across a nanometer-long channel\cite{31, 32}, it was also reported that neutral peptides passing through protein nanopores remained experimentally challenging\cite{33}. Hence, a new peptide\cite{34} was designed by modifying TYLYW with a phosphate group; the resulting negatively charged peptide was designed to be readily transported through the mutant MspA (Figure 1a) by the electric field force. In contrast to the blank control group (Figure S2, Supporting Information), the addition of $\text{PO}_4^{2-}$-TYLYW in the cis-chamber gave rise to a large number of transient current blockage events (Figure 1a). Interestingly, two distinct current blockages labeled as type 1 and type 2 events were observed with centered at $\approx +25$ pA and $+80$ pA (Figure 1b), and the corresponding $\tau_{\text{off}}$ were $1.92 \pm 0.23$ ms and $0.57 \pm 0.10$ ms (Figure S3, Supporting Information). As the applied voltage increased from 50 to 150 mV, the kinetics of type 1 events displayed remarkable voltage dependence, with $\tau_{\text{off}}$ (mean dwell time) decreasing from $10.25 \pm 1.06$ to $1.10 \pm 0.11$ ms (Figure 1c) (see Figure S4, Supporting Information, for raw data analysis) and $\tau_{\text{on}}$ (inter-event interval) decreasing from $5.00 \pm 0.33$ to $0.10 \pm 0.02$ s (Table S1, Supporting Information). In contrast, although for the type 2 events the $\tau_{\text{on}}$ decreased greatly with increase of applied voltage, $\tau_{\text{off}}$ showed a weak correlation and dependence with the applied voltage (Figure S5, Supporting Information). On the other hand, increasing the concentration of peptides ranging from 0.1 to $1 \times 10^{-6}$ M resulted in an increase number of the type 1 events and the corresponding calibration plots showed a good linear correlation between the number of blockage events and the peptide concentration in this wide range (Figure 1d). Although an increase of the type 2 events was observed, the change in the number of blockage events did not show a linear relationship with the peptide concentration (Figure S6, Supporting Information). From these results, we speculated that the residual current blockage of type 1 could be produced by the effective translocation through MspA, while the blockage of type 2 could be primarily caused

Figure 1. Nanopore MspA sensing of $1 \times 10^{-6}$ M $\text{PO}_4^{2-}$-TYLYW in symmetrical 1 M NaCl electrolyte buffer, and at +100 mV. a) The representative current trace of $\text{PO}_4^{2-}$-TYLYW. b) The schematic mechanism of peptides interaction with nanopore and the corresponding heat map of two current levels. c) $\tau_{\text{off}}$ of type 1 events dramatically decrease with increasing bias voltage. d) Linear correlation between the type 1 events frequency and the peptide concentration. e) The frequency of type 1 and type 2 events under different voltage. f) Signal-to-noise ($f_{\text{type1}}/f_{\text{type2}}$) under different voltages. g) The frequency of type 1 and type 2 events under different ratio of $[\text{NaCl}]_{\text{trans}}/[\text{NaCl}]_{\text{cis}}$. h) Signal-to-noise ($f_{\text{type1}}/f_{\text{type2}}$) under different ratio of $[\text{NaCl}]_{\text{trans}}/[\text{NaCl}]_{\text{cis}}$ ($n = 3$ for each data points from independent trials).
by the instantaneous collision of peptides with the aperture of the pore.

To make a better distinction of type 1 and type 2 events, the effect of the applied voltages and salt gradient on the assay were investigated. Along with increasing voltage from 70 to 150 mV, both type 1 and type 2 events displayed remarkable frequency increase with increasing voltage (Figure 1c). The frequency of type 1 events increased 1.76-fold from 33.23 ± 1.41 min⁻¹ (70 mV) to 58.87 ± 2.38 min⁻¹ (150 mV), and from 192.95 ± 6.34 to 371.68 ± 2.48 min⁻¹ for the type 2 events. However, the highest ratio of \( f_{\text{type 1}}/f_{\text{type 2}} \) was present at 100 mV which could greatly distinguish type 1 and type 2 events (Figure 1f). We also evaluated the effect of the NaCl gradients on the identification of PO₄²⁻→TYLYW by varying the concentration of NaCl in cis and trans chambers ranging from 0.3 to 3 M. We first observed that the asymmetric salt gradient resulted in increased events frequency and decreased \( \tau_{\text{off}} \) for type 1 events compared to the symmetric salt condition (Figure S7 and Table S2, Supporting Information). For instance, under the +100 mV transmembrane potential, events frequency \( [3 \, \text{m NaCl}/0.3 \, \text{m NaCl}, \text{trans/cis}] \) showed an ≈1.3-fold increase over events frequency \( [3 \, \text{m NaCl}/3 \, \text{m NaCl}, \text{trans/cis}] \) (65.88 ± 3.31 min⁻¹ vs 49.02 ± 1.54 min⁻¹) (Figure 1g), while the \( \tau_{\text{off}} \) \( [3 \, \text{m NaCl}/0.3 \, \text{m NaCl}, \text{trans/cis}] \) decreased to ≈8.8% of the \( \tau_{\text{off}} \) \( [3 \, \text{m NaCl}/3 \, \text{m NaCl}, \text{trans/cis}] \) (0.25 ± 0.05 ms vs 2.84 ± 0.47 ms). Apparently, the \( \text{trans/cis} \) salt gradient resulted in a shorter capture time and dwell time of the substrate peptide through the MspA pore. These results were consistent with the report of a salt gradient effect on DNA translocation which was more significant through nanopores.[35] Here, the NaCl gradients are thought to contribute to the regional electric funneling field in the cis side as a result of cation accumulation on the trans side, which satisfy the requirement for a constant current flow.[36,37] Therefore, the negatively charged peptides in the cis side of the pore experienced an electrophoretic force, allowing them to be easily captured and rapidly translocated through the pore. However, with the great increase of type 1 events frequency, the frequency of type 2 events also increased by 57% (from 252.63 ± 6.74 to 396.25 ± 5.46 min⁻¹). As a result, we found that the maximum signal-to-noise ratio (SNR) \( f_{\text{type 1}}/f_{\text{type 2}} \) was obtained under the symmetric salt condition (Figure 1h).

The reports on short polynucleotides (ACTG) driven through aerolysin and pent-arginine translocated through the heterooligomeric channel of *Nocardia farcinica*,[38,39] proposed that the two types of current events were attributed to the interaction between peptides and the protein nanopore, which were also observed in this experiment of negatively charged peptides with engineered MspA. Considering the principle of nanopore sensing and for the verification of our speculation, we utilized the aforementioned condition (100 mV and 1 M NaCl symmetric salt condition) to further investigate the signal type of other eight peptides with different lengths and charges consisting of 6 (N6, Figure 2a), 7 (N7, Figure 2b), 8 (N8, Figure 2c), 9 (N9, Figure 2d), 10 (N10, Figure 2e) residues with negative charge and 5 (P5, Figure S8a, Supporting Information), 7 (P7, Figure S8b, Supporting Information), 9 (P9, Figure S8c, Supporting Information) residues with positive charge. As shown in Figure 2a–c and Figures S9 and S10 (Supporting Information), in comparison with other control groups two distinct types of current blockages were observed for the negatively charged peptides which was similar to peptide PO₄²⁻→TYLYW. The Gaussian fit to the residual current histogram for type 1 events of N6, N7, N8 gave the peak value of 2789 ± 0.59, 23.76 ± 0.62, 18.72 ± 0.62 pA, and to the log₁₀ dwell time gave the peak value of −0.250 ± 0.036, 0.061 ± 0.027, 0.036 ± 0.019 ms, respectively. Obviously, for the type 1 events, the longer peptides would induce a bigger current blockage and a longer dwell time when interaction with a nanopore. On the other hand, as increasing the length of peptides to 9 (N9) and 10 (N10) residues, the number of type 1 events greatly decreased and only type 2 events were observed (Figure 2d,e). All the results demonstrated that the residual current blockage of type 1 was produced by the effective translocation through MspA, while the blockage of type 2 was primarily caused by the instantaneous collision of peptides with the aperture of the pore. As a result, for peptide N9 and N10, they were sterically impeded by the pore which caused the significant decrease of type 1 events.

In order to investigate the minimum length or charge of peptides that can be captured by mutant MspA protein pore, we performed additional experiments of peptides with different lengths (D2: DD, D3: DDD, D4: DDDD) and charges (−3: DVDVD, −2: DDDV, −1: DVV) by mutant MspA. As shown in Figure S11a (Supporting Information), the D4 peptide induced significant amount of blockage signals similar to N5–N8, while the D3 decreased significantly and D2 showed no signals. Therefore, it can be concluded that dipeptide was the minimum length that could be captured by the mutant MspA. In addition, the pentapeptides with only −1 charge could also be captured and detected by mutant MspA (Figure S11b, Supporting Information).

Further experiments at voltage of +120, +130, +140, +150, +160, and +180 mV for N9 and N10 were performed, as shown in Figure S12 (Supporting Information). In comparison with N6, N7, and N8 which required a lower voltage to be translocated (type 1 events), N9 and N10 needed a higher voltage (140 mV). This indicated that the energy barrier for nanopore translocation is higher for longer peptides.[19] It is interesting to observed that the \( \tau_{\text{off}} \) of type 1 events for N9 was higher than that of N10. This could be explained by the possible stronger electrostatic interaction of N9 (−9) with the positively charged inside lumen of MspA nanopore than N10 (−2). From panels (c) and (d) in Figure S12 (Supporting Information), N9 showed a higher events frequency than N10. This could be attributed to the higher capture rate for its stronger charge. In summary, the capture rate of peptides relied on the net charge of the peptide, while energy barrier for translocation was affected by both length and net charge of the peptide.

We also further investigated the interaction between positively charged peptides and mutant MspA, the result showed that very few signals were observed for all the peptides P5, P7, and P9 (Figure S8, Supporting Information). From the electrical properties of the mutant MspA protein residues composing, both mutations D134R and E139K suppressed positive-charged peptides entry into the vestibule but enhanced the capture of negative peptides, and other mutant sites near the constriction of lumen including D118R, D90N, D91N, and D93N provide predominantly electrostatic interaction (Figure S1b, Supporting Information) with negatively charged molecule but show none assistance with positively charged peptides.
Subsequently, we applied the MspA nanopore sensor to measure the enzymatic degradation of peptides by ClpP protease. The ClpP protease plays an important role in proteostasis and affects the bacterial pathogenesis and various human diseases. Thus far, the ClpP activity evaluation assay is mainly explored on the basis of fluorescence changes resulted from the cleavage of the fluorogenic substrates,[40] and it becomes necessary to develop a low-cost, label-free, and more efficient method for the evaluation of ClpP activity. As the ClpP protease degrades small peptides with six or fewer amino acids[41] and the mutant MspA was sensitive to negatively charged peptide,[42] we utilized PO$_4^{2-}$-TYLYW as substrate which was envisioned to be hydrolyzed by ClpP protease. To identify the hydrolysis reaction via nanopore assay, we added the mixture containing PO$_4^{2-}$-TYLYW and 40 × 10$^{-9}$ M ClpP in the cis chamber and recorded the signal under the aforementioned optimized condition (Figure 3a). A significant reduction of the translocation blockage events was observed due to the decrease of substrates (Figure 3b). The substrates PO$_4^{2-}$-TYLYW were cleaved into two fragments, PO$_4^{2-}$-TYL and YW in the presence of ClpP (Figure S13, Supporting Information). However, the fragments were less sensitive toward MspA and were too small to be detected (Figure S14, Supporting Information). In addition, ClpP could not transport through MspA (pore lumen diameter of 8.8 nm, Figure S15, Supporting Information) due to its much larger molecular diameter ($11 \times 10 \times 10$ nm) than the pore constriction. Different from prevailing methods used for detecting protease activity by cleavage-product-induced ionic current modulations,[16,43] we adopted the strategy of analyzing blockage events of the remaining substrate to measure the protease activity, which was straightforward and involved less variations of different cleavage fragments. For better determination of hydrolysis reaction with single molecule method, we first examined the reaction mixture at time points of 5 min, 30 min, 1 h, 3 h, and 6 h. The cleavage curve of PO$_4^{2-}$-TYLYW was developed by the percentage of processivity (%) and the mixture with only substrate was defined as 100% processivity. The hydrolysis of PO$_4^{2-}$-TYLYW proceeded almost linearly in the first 1.5 h and reached saturation after 3 h (Figure 3c). Subsequently, to eliminate the effect of monitoring time on

**Figure 2.** Single-molecule analysis of varying-length negative peptides. a–e) The schematic diagram of varying-length negative peptides interaction with the mutant MspA (left), and the representative current traces (middle), with the analysis of their current steps and dwell times (right).
hydrolysis, continuous recordings for 14 min using single nanopore for reaction mixture were analyzed. The results showed that the nanopore assays could accurately detect the hydrolysis reaction in a 14-min monitoring window, and the frequency of translocation increased slowly during the initial monitoring time and became gradually constant after 14 min (Figure 3d). However, decreasing the monitoring time to 3 min (Figure 3c) would lead to a lower detection accuracy compared to high-performance liquid chromatography (HPLC) analysis (Figure S16, Supporting Information). Therefore, the sensitive detection of ClpP activity was able to be accomplished in a 14-min recording at the ClpP concentration of $40 \times 10^{-9}$ m.

To demonstrate the feasibility of using the developed MspA sensing platform as a screening tool for protease inhibitors, cleavage of $\text{PO}_4^{2-}$-TYLYW by ClpP was further investigated in the presence of AV170, a phenyl ester inhibitor of ClpP.\(^{[40]}\) The experimental procedures were similar to the stated above for ClpP activity detection, except the preincubation of ClpP with $0.5 \times 10^{-6}$ m AV170 step. Our experiments showed that in the absence of inhibitors, $\approx 300$ characteristic $\text{PO}_4^{2-}$-TYLYW translocation events were observed in a 14-min single-channel recording (Figure 4a). In contrast, $\approx 574$ $\text{PO}_4^{2-}$-TYLYW translocation events were identified in the presence of $0.5 \times 10^{-6}$ m AV170 (Figure 4b). As shown in Figure 4c, when adding the inhibitor AV170 into the reaction mixture, the processivity of ClpP degrading substrate peptides reduced from $49.5 \pm 1.2\%$ to $19.9 \pm 3.0\%$, and the calculated corresponding inhibition ratio was $59.8\%$, which was consistent with the reported data.\(^{[40]}\)

The inhibitor AV170 and ClpP had no effect on translocation events of decapeptide which could not be cleaved by ClpP with more than six amino acids (Figure 4d,e and Figure S17, Supporting Information), the results verified that the inhibitor specifically targeted to ClpP and prevented the hydrolysis of substrate $\text{PO}_4^{2-}$-TYLYW. For each sample of ClpP substrates, the measurement was repeated at least three times independently, and the average relative standard deviation (RSD) was ranging from $1.2\%$ to $3.0\%$, which indicates that the precision and reproducibility of the method are acceptable. These results demonstrated that the designed strategy identify protease ClpP inhibitors successfully.

In conclusion, single-molecule events of peptides interaction with the engineered MspA nanopore have been studied. Two types of signals derived from peptides interaction with nanopore could be observed, and the type 1 event was correlated with peptides translocation through nanopore while the type 2 event was resulted from random collision. Furthermore, via the identification of different-length peptides with negative charges by mutant MspA nanopore, these two interaction modes were verified. Here, our results provided the evidence about peptides interaction with the mutant MspA which would assist understanding of the interaction mode between peptides and protein nanopores. Based on this, an efficient, label-free, and real-time detection method of peptide degradation was developed, and ClpP protease activity has been successfully quantified by measuring the quantities of remaining substrate. In combination with the unique and robust stability of MspA, the developed sensing technique has considerable potential in protease-target inhibitors screening, diagnostic and prognostic detection of peptide biomarkers, structural characterization of peptides, and eventually protein sequencing.

**Experimental Section**

_Materials:_ $\text{PO}_4^{2-}$-TYLYW was modified at the threonine (T) position by a phosphate group ($\text{PO}_4^{2-}$) and was purchased from Sangon Biotech by custom-order. The purity of $\text{PO}_4^{2-}$-TYLYW was assayed by HPLC and
purify was 99.2%. All the other chemicals were obtained from Sigma-Aldrich. MspA porin and ClpP protease were purified as described next and were both kept at -80°C before use. Peptides and other chemicals were dissolved in ddH2O or DMSO, while the peptide stock solution (prepared with electrolyte) was kept at -80°C both before and after use. Other chemicals were kept at -20°C before use. Lipid 1, 2-diphytanoyl-sn-glycero-3-phosphocholine was obtained from Avanti Polar Lipids and redissolved in n-decane after flushing with nitrogen. The sequence of peptides in this study are:

N6: DDDDDD  
N7: DDDDDDD  
N8: DYKDDDDK (Flag)  
N9: DDDDDDDDD  
N10: DMHDFFVGLM (Neurokinin B)  
P5: RLRFD (γ-Bag Cell Factor)  
P7: APRLRFY (α-Bag Cell)  
P9: RRRRRRRRR  

All these peptides were purchased from Sangon Biotech.

Cloning and Protein Purification: A synthetic gene of MspA adjusted for E. coli codon usage was cloned into plasmid Pet28b and expressed in the E. coli strain BL21(DE3) after induction with 1 × 10⁻³ m IPTG. After being lysed by sonication while being constantly cooled on ice, the cell lysis was spun at 47,000 g and the supernatant was loaded on a 5-mL anion exchange column (GE Healthcare). The column was washed with 25 × 10⁻³ m Hepes (pH 7.5), 1 m NaCl. Fractions were then precipitated in saturated ammonium sulfate and subjected to centrifugation at 47,000 g. The precipitate was suspended and then incubated overnight. Finally, the supernatant was purified with a size exclusion column (HiLoad 16/60 Superdex 200 pg (GE Healthcare)) and octamer MspA solution fragments were collected. Tag-free ClpP from S. aureus ATCC 33591 were expressed and purified as described previously[40]. In short, the plasmid was expressed in E. coli BL21(DE3) cells, and purified by Q-Sepharose FF column and HiLoad 16/60 Superdex 200 pg gel filtration column (GE Healthcare). The purity of the obtained ClpP was above 85% by sodium dodecyl sulfate poly-acrylamide gel electrophoresis (SDS-PAGE).

Electrophysiological Recording: In a typical single-channel experiment, two compartments were separated into cis and trans (the cis compartment is connected to the ground) chambers by a planar lipid bilayer. The formation of the bilayer was achieved using a method described previously[44]. Both compartments were filled with a 1 mL electrolyte solution of 1 m NaCl and 10 × 10⁻³ m Hepes (pH 7.5). Single-nanopore insertion was initiated by adding the solution containing mutant porin MspA (final concentration of 0.01–0.05 ng mL⁻¹) into the cis compartment under +180 mV bias voltage. Peptides and peptide-containing samples resulting from the enzymatic digestion were added to the cis side. Unless otherwise mentioned, all analytes were added into the cis side because of the gating properties of porin MspA under negative potential[45]. Subsequently, ionic current through the nanopore was recorded at a holding potential of -100, 50, 70, 80, 100, 120, 130, and 150 mV.

Figure 4. Effect of inhibitor on ClpP cleavage of PO₄²⁻-TYLYW, in symmetrical 1 m NaCl electrolyte buffer, and at +100 mV. a,b) Histogram statistics of substrate PO₄²⁻-TYLYW translocation through MspA with and without inhibitor AV170. c) The quantitative analysis of different inhibitive ratios. d,e) Histogram statistics of peptide N10 translocation through MspA with and without inhibitor AV170 in the control experiment. f) The frequency of type 2 events with and without AV170 (n = 3 for each data points from independent trials).
In the salt-mediated peptide translocation experiments, an electrolyte solution containing 0.3 M NaCl, 1 M NaCl, and 3 M NaCl was added into cis and trans side of the chamber, respectively, and peptides were added into cis side chamber.

In the assay of ClpP protease activity and the verification experiments of its inhibitor AV170, an electrolyte solution containing 1 M NaCl, 10 × 10⁻³ M Hepes (pH 7.5) was added into both chambers, and ionic current through the nanopore was recorded at a holding potential of +100 mV. The reaction mixtures contained 1 × 10⁻⁶ M of peptides and 40 × 10⁻⁹ M ClpP (assay of ClpP protease activity) as well as 0.5 × 10⁻⁶ M AV170 (verification experiments of its inhibitor AV170) under nanopore detecting conditions.

Electronic signals were amplified using an Axopatch 200B/700B amplifier (Molecular Devices) and filtered with a built-in four-pore low-pass Bessel filter at 5 kHz. Sampling at 50 kHz was performed using a Digidata 1550B converter (Molecular Devices).

Data Analysis: All events in the nanopore detection data were analyzed by Clampfit 10.3 and Origin 8.5 software. The mean dwell time ($t_{\text{dwell}}$) and interevent interval ($t_{\text{int}}$) were calculated by fitting exponential functions to cumulative distribution. The residual current ($I_o$) and the blockage current ($I_b$) by fitting the distribution to Gaussian functions. In periods of extended recording time, the increased translocation rate was analyzed by applying exponential functions.

The inhibition ratio can be calculated by ($a-b$)/$a$ 100%, where $a$ is the hydrolisys efficiency without inhibitors and $b$ is the hydrolisys efficiency with inhibitors.

Liquid Chromatography-MS (LC-MS) Analysis of Hydrolyzates: The reaction mixtures contained 1 × 10⁻⁸ M of peptides and 40 × 10⁻⁹ M ClpP under nanopore detecting conditions. Samples with the peptide LYLYW and its inhibitor AV170, an electrolyte solution containing 1 M NaCl, 10 × 10⁻³ M Hepes (pH 7.5) were added into both chambers, and ionic current through the nanopore was recorded at a holding potential of +100 mV. The reaction mixtures contained 1 × 10⁻⁶ M of peptides and 40 × 10⁻⁹ M ClpP under nanopore detecting conditions. Samples with the peptide LYLYW and its inhibitor AV170, an electrolyte solution containing 1 M NaCl, 10 × 10⁻³ M Hepes (pH 7.5) was added into both chambers, and ionic current through the nanopore was recorded at a holding potential of +100 mV. The reaction mixtures contained 1 × 10⁻⁶ M of peptides and 40 × 10⁻⁹ M ClpP under nanopore detecting conditions.

Electronic signals were amplified using an Axopatch 200B/700B amplifier (Molecular Devices) and filtered with a built-in four-pore low-pass Bessel filter at 5 kHz. Sampling at 50 kHz was performed using a Digidata 1550B converter (Molecular Devices).

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements
This work was supported by the National Key Research and Development Program of China (Grant No. 2016YFA0201400).

Conflict of Interest
The authors declare no conflict of interest.

Author Contributions
K.S. and Y.J. contributed equally to this work. J.G. and Y.L. designed the experiments and wrote the manuscript; K.S., Y.J., and C.C. performed experiments; K.S., Y.J., C.C., P.Z., and E.S. analyzed data. K.S. prepared the figures for the manuscript. All authors reviewed this manuscript.

Keywords
inhibitor screening, nanopores, peptides sensing, protease activity
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