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Real-Time Monitoring of Peptide Cleavage Using a Nanopore Probe

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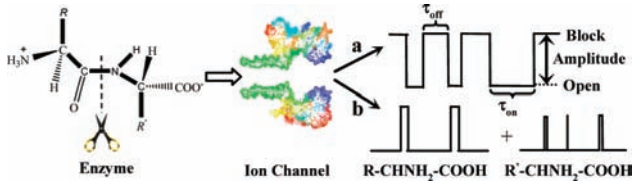
Interactions among biomolecules (such as proteins, peptides, and proteases) are involved in a wide variety of physiological activities within living cells.[1] Evaluating the functional roles of these biologically active molecules and elucidating their interactions will benefit biomedical research.[2] For example, of paramount importance in the clinical assessment of neurodegenerative problems, including Alzheimer's and Parkinson's diseases, is early detection and monitoring of the conformational change of amyloid-β (A-β) peptide, which is attributed to the proteolytic cleavage of the amyloid-based precursors by β- and γ-secretases.[3] Many approaches currently available in proteomics research rely on peptide cleavage for the identification of proteins and other biomarkers. At present, the majority of studies performed in this area are dependent on expensive, time-consuming techniques that frequently require sample labeling.[4] Although several label-free approaches have been utilized in the detection of biomarkers and the study of protease−substrate interactions,[5] there remains a definite need for a more rapid, less costly, and easier-to-use methodology for protein/peptide cleavage studies and enzymatic activity assays.

Herein we demonstrate that use of a protein channel provides a viable new approach for probing peptide cleavage. It has long been established that ion channels play a pivotal role in the regulation of mass and energy transfer in many biological processes. More recently, it has been clearly established that these nanometer-sized channels offer exciting new possibilities for the development of ultrasensitive biosensors, the study of biomolecular folding and unfolding, and the investigation of covalent and noncovalent bonding interactions.[6] In the present work, peptide cleavage was monitored by recording peptide translocation through an ion channel in the absence and the presence of an enzyme using a single-channel recording technique [Figure S1 in the Supporting Information (SI)]. As documented below, our method permits the study of protease kinetics via real-time monitoring of the ionic current modulations arising from the enzyme−peptide interactions. As shown in Scheme 1, in the absence of the enzyme, the current modulations are caused only by the substrate (Scheme 1a). However, after addition of the enzyme to the buffer solution, the substrate is cleaved into two fragments. Thus, new blockage events having residence times (τ_{off}) and/or amplitudes that differ from those of the substrate can be observed (Scheme 1b).

Scheme 1. Probing Peptide−Protease Interactions in an Ion Channel[a,b]



[a] Without the enzyme, the current modulations are caused only by the substrate. [b] With the enzyme, new blockage events having residence times and/or amplitudes that differ from those of the substrate can be observed as a result of cleavage of the substrate.

To demonstrate this concept, an engineered version of the α-hemolysin (αHL) channel, $(M113F)_7$, was used to study the trypsin cleavage of peptide A-β(10−20). The $(M113F)_7$ protein has been shown to provide a significantly enhanced resolution for biomolecule recognition versus the wild-type αHL pore.[7] Trypsin is commonly used as a serine protease to cleave peptide bonds after Arg (R) or Lys (K) amino acid residues.[8] Since the spherical molecular diameter of trypsin (38 Å)[9] is larger than that of the αHL transmembrane domain (20 Å),[10] trypsin cannot enter the pore and hence cannot produce current blockage events that might interfere with the identification of the target peptide(s). Our experimental results show that in the absence of trypsin (Figure 1, t = 0 min), the buffer solution containing peptide A-β(10−20) produced only a single type of current blockage, having a large mean residence time of 0.63 ± 0.06 s (Figure 1a and Figure S2 in the SI) and a small mean residual current of −1.8 ± 0.3 pA (Figure 1b). In sharp contrast, after addition of trypsin (Figure 1, t =10, 20, and 60 min), two new types of current modulation events were clearly observed, both of which had significantly shorter residence times (0.85 ± 0.08 and 0.75 ± 0.05 ms) and larger mean residual blockage currents (−4.4 ± 0.3 and −12.2 ± 0.6 pA, respectively). These shorter-duration blockages are attributed to YEVHHQK (YK-7) and LVFF fragments, the breakdown products of A-β(10−20). Their identities were confirmed by direct measurement of current blockages using both single standards of YK-7 and LVFF peptides

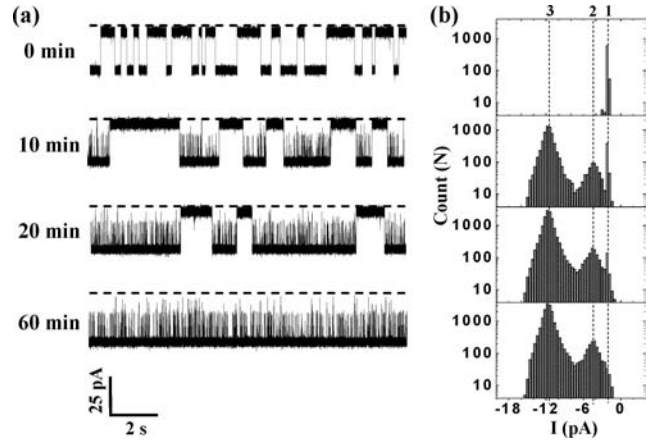


Figure 1. Monitoring of A-β(10−20) cleavage by trypsin. (a) Representative segments of a single-channel recording trace at various times. Dashed lines represent the levels of zero current. (b) Corresponding time-dependent event amplitude histograms. Dashed lines 1, 2, and 3 represent the mean residual current levels for peptides YEVHHQKLVFF, YEVHHQK, and LVFF, respectively. The experiment was performed at −40 mV with 10 μM A-β(10−20) and 0.025 μM trypsin.

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as well as 1:1 solution mixtures of these two compounds. As shown in Figure S3 in the SI, blockage events with the two peptides produced mean residence times (0.9 ± 0.1 and 0.77 ± 0.07 ms, respectively) and residual currents (−4.5 ± 1.0 and −14 ± 2 pA, respectively) similar to those of the A-β(10−20)/trypsin digestion products. It should be noted that the mean residence time (τ_{off}) and/or amplitude of the events can be used as a signature to identify a peptide, while the event frequency f (=$1/\tau_{on}$) can be used to quantify the remaining substrate or the fragments produced (Figure 1 and Figure S2 in the SI). The time-dependent event frequency provided further evidence that the enzymatic cleavage process was responsible for the appearance of the new blockage events. As the reaction time increased, the frequency of A-β(10−20) events decreased while those of fragment events increased, a clear indication that the substrate was being digested by trypsin. In fact, the large-duration and small-residual-current events disappeared after ∼1 h of digestion, suggesting that all of the A-β(10−20) substrate had been cleaved by that point in time. As an added control, translocation of peptide YYYYYY, which is not a substrate for trypsin, in the $(M113F)_7$ pore was examined. No new types of events or changes in the event τ_{off} or amplitude were observed after addition of trypsin (Figure S4 in the SI), consistent with this analysis.

To determine the enzyme kinetics, quantitative analysis of the above single-channel current recording (Figure 1) for peptide A-β(10−20) digestion by trypsin was performed (see the SI). The time curve of the substrate digestion (Figure 2a) shows that

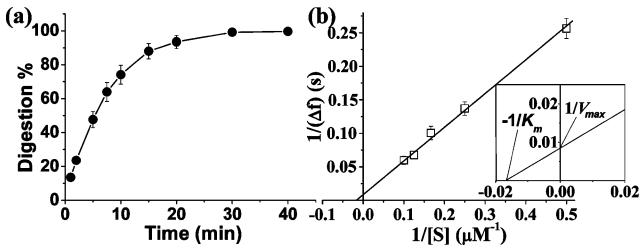

Figure 2. Kinetic profiles for the A-β(10−20)/trypsin interaction. (a) Time curve of the digestion with $[substrate]_0$ = 10.0 μM and [trypsin] = 0.025 μM. (b) Lineweaver−Burk plot for the determination of K_m and k_{cat}. The inset of Figure 2b shows an enlargement of the x- and y- intercept region. The experiments were performed with 0.025 μM trypsin and various concentrations of A-β(10−20).

the rate of A-β(10−20) cleavage decreased rapidly with reaction time. For example, 93.5 ± 3.8% of the substrate was cleaved in the first 20 min, whereas only an additional 6.1 ± 0.7% of the substrate was digested in the second 20 min. Our observation is reasonable considering that the concentration of the substrate is relatively large in the early stage of the trypsin/A-β(10−20) interaction. A Lineweaver−Burk plot (Figure 2b) using the frequency of the cleavage product LVFF events revealed that the Michaelis constant K_m of the reaction is 59.2 μM, whereas k_{cat} (=V_{max}/[trypsin]) is 4.43 s^{-1}. These values agree with those of other studies involving cleavage of A-β peptide carried out under experimental conditions similar to those involved in the present investigation.[11] Notably, the enzymatic activity was influenced by substrate, pH, temperature, and salt concentration.[12]

It should be noted that at the early stage of the enzymatic reaction, the product concentration was very low (nanomolar range). This required a relatively long period of time (e.g., 1 min in this work) to collect the hundreds of individual single-molecule events necessary for the statistical analysis of the event frequency. This limitation could be remedied by replacing the event mean frequency with the number of event occurrences in the analysis of enzyme kinetics. This approach allowed the product concentration to be determined in seconds, and a substrate digestion curve similar to Figure 2a was obtained (Figure S6 in the SI). Furthermore, in principle, the method developed in this work should be compatible with the analysis of enzymatic reactions involving long peptides/proteins and complex mixtures. The former could be achieved by using as the sensing element synthetic nanopores with large pore diameters or engineered protein pores that could enhance biomolecular translocation,[13] and the latter could be done by considering only the cleavage products and focusing on the frequency increase in their events.

In summary, we report a new rapid, label-free method for monitoring peptide cleavage and obtaining quantitative chemical kinetics information on enzymatic processes. In view of the need for such information, such as in disease diagnostics and drug discovery, further development of this technique into miniature nanopore sensing systems, including systems having more automated controls, is currently underway. In addition, given the fact that properly engineered protein pores can differentiate the sequences of short peptides,[7] we believe this peptide/protein cleavage approach offers the potential for further development as a novel rapid, label-free protein-sequencing technique.

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Supporting Information Available: Experimental details, analysis of enzyme kinetics, complete ref 3, and additional figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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