AIP | Applied Physics Letters

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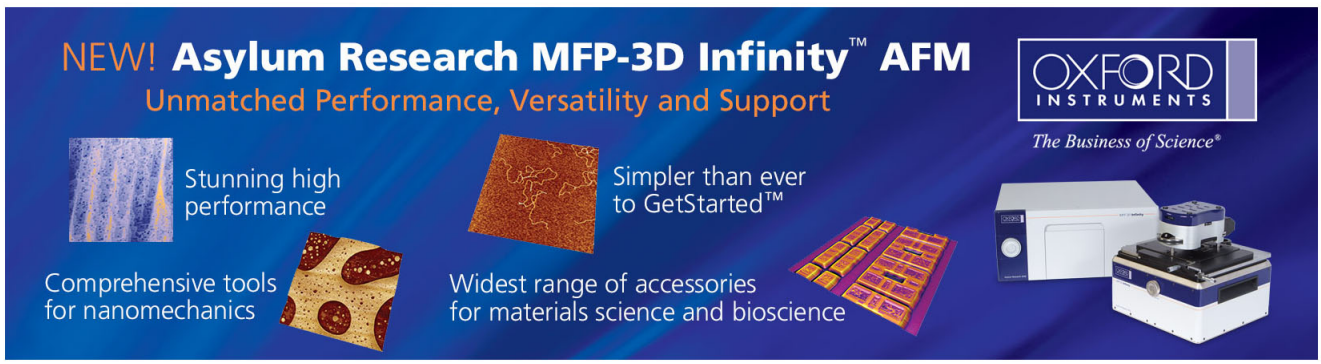
Citation: Applied Physics Letters **104**, 163701 (2014); doi: 10.1063/1.4873676
View online: http://dx.doi.org/10.1063/1.4873676
View Table of Contents: http://scitation.aip.org/content/aip/journal/apl/104/16?ver=pdfcov
Published by the AIP Publishing





# Label-free, real-time detection of the dynamic processes of protein degradation using oblique-incidence reflectivity difference method

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(Received 22 January 2014; accepted 30 March 2014; published online 25 April 2014)

Based on the requirements for studying the dynamic process of proteinase action substrates in life science, we selected six random proteins including 1L-10, SCGB2A2, CENPQ, GST, HK1, KLHL7, as well as five different concentrations of 1L-10 proteins of 1 mg/ml, 0.5 mg/ml, 0.25 mg/ml, 0.125 mg/ml, and 0.0625 mg/ml, and fabricated two types of substrate protein microarrays, respectively. We detected the dynamic processes of proteins degraded by proteinase K using oblique-incidence reflectivity difference (OIRD) method in a label-free and real-time manner. We obtained the relevant degradation velocities and the degradation time. The experimental results demonstrate that OIRD has the ability to study proteinase action substrates which is out of reach of label methods and is expected to offer opportunities to determine protease-substrate relationships on the systems biology level. © *2014 AIP Publishing LLC*. [http://dx.doi.org/10.1063/1.4873676]

Proteases form a large family of important enzymes that are involved in many important aspects of biology in eukaryotes. Malfunction in human proteases are known to cause severe diseases, such as renal insufficiency and osteopsathyrosis.[1,2] To better understand the etiology of protease-related diseases, a crucial step is to determine the molecular mechanisms as how the proteases function in cells. Therefore, identification of their connate substrates on a global scale will serve as a crucial step towards fully understanding of their physiological functions on the systems level. Furthermore, the resulted networks of protease-substrate relationships will provide important clues for diagnosis and prognosis of the diseases, as well as for developing new therapeutics. By definition, proteases remove parts of or even degrade the entire proteins, an opposite action of protein modification enzymes, such as protein kinases, acetyltransferases, and protein methylases.[3,4] Traditionally, labels, either in the form of epitope tags or radioisotopes, are almost always required to biochemically characterize enzymatic activity of a protein of interest. Although there have been numerous successful studies utilizing labels to study the biochemical properties of enzymes that add a chemical moiety to a protein substrate, it has always been challenging to determine substrates of enzymes that remove parts of a protein substrate, such as proteases, deubiquitinases, and deacetylases, due to such a loss-of-signal mode of action. Furthermore, label-dependent detection methods are mostly used for end-point detection, not amenable to monitor the kinetics of the degradation process of proteases.[5,6] A real-time, label-free method would better serve the purpose.

Oblique-incidence reflectivity difference (OIRD) offers a real-time, label-free method for detecting interactions of biomolecules.[7–9] In the past, we and others have demonstrated the use of OIRD method to determine the kinetics of antigen-antibody interactions,[9–12] protein-DNA interactions,[13] DNA hybridization,[14] and protein-protein interactions.[15,16] This method has also been proven to be highly reproducible.[17] The detection sensitivity of protein can reach 14 fg.[15] As described in our previous publications,[8,9] OIRD method measures the fractional difference between the reflectivity of the p- and s-polarized lights from a surface under the condition of oblique incidence, i.e., $\Delta_p - \Delta_s$. We have demonstrated that we were able to obtain the OIRD signals caused by the difference of optical dielectric constant and the mass density in the detected surface. In this study, we report the development and application of OIRD to characterize the enzymatic property of a protease (i.e., Proteinase K) in a real-time, label-free fashion.

We chose Proteinase K for this proof-of-principle study because it is widely used in molecular biology, and because it is a robust protease that degrades most, if not all, of proteins.[18,19] To fabricate the substrate protein microarrays, we selected six random proteins, namely, 1L-10, SCGB2A2, CENPQ, GST, HK1, and KLHL7 (provided by Beijing Institute of Genomics). These proteins were purified to homogeneity (data not shown) and stored in 1 X phosphate buffered saline (1 X PBS) buffer containing 30% glycerol. Before printing the microarray, they were adjusted into final concentrations of 0.5 and 0.25 mg/ml, respectively. We fabricated two types of protein microarrays for the OIRD measurements using a conventional procedure as reported previously.[16] As shown in Fig. 1(a), Microarray I was fabricated by printing the six proteins at the two different concentrations on the epoxy-functionalized glass slides (Telechem, Inc., USA) using a contact spotting robot

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micro-system (CapitalBio Corporation, China). Similar to Microarray I, Microarray II is composed of multiple 1L-10 protein spots at serial dilution (i.e., 1.0, 0.5, 0.25, 0.125, and 0.0625 mg/ml) on an epoxy-functionalized glass slide. None of the protein samples had any labels; each protein sample was spotted in duplicate on the epoxy slides. The resulting protein spots are circular with an average diameter of $\sim 100\,\mu$m and a center-to-center distance of $\sim 400\,\mu$m. The printed slides were cured overnight at 4 °C to allow effective covalent attachment of the substrate proteins on the epoxy surface.

To determine whether OIRD was capable of detecting the degradation process by Proteinase K, protein substrate microarrays were, respectively, installed in a flow-cell chamber in the two-dimensional (2D) scanning stage of the OIRD system. We then measured the OIRD $\mathrm{Im}\{\Delta_p - \Delta_s\}$ signals. First, excess proteins on each printed protein spot were washed off by immersing the microarrays in $ddH_2O$ for 30 min, followed by pumping $1\times$ PBS buffer into the flow-cell at a rate of 3 ml/min for 10 min. Second, the 2D scanning images of OIRD $\mathrm{Im}\{\Delta_p - \Delta_s\}$ intensity of the protein spots on the microarrays were obtained. Next, Proteinase K, diluted to 12.5 $\mu$g/ml in a reaction buffer containing 50 mM Tris-HCl (pH 7.5) and 2 mM calcium acetate, was pumped into the flow-cell chamber. Simultaneously, the scanning program was carried out and the probe light spot scanned along the centerlines of the protein spots on the substrate microarray. The reflective light intensity proportionately corresponding to $\mathrm{Im}\{\Delta_p - \Delta_s\}$ was directly detected to obtain the degradation kinetic curves of the protein substrates. After the $\mathrm{Im}\{\Delta_p - \Delta_s\}$ signals reached saturation, we again obtained of the 2D scanning images of OIRD $\mathrm{Im}\{\Delta_p - \Delta_s\}$ intensities on the substrate microarrays.

Figures 1 and 2 illustrate the results obtained from the above reactions performed on Microarray I, including all of the processes of the protein degradation detected by OIRD. Figs. 1(a), 1(b), and 1(e) display the 2D scanning images of $\mathrm{Im}\{\Delta_p - \Delta_s\}$ signal intensity after printing, washing and protease reaction, respectively. The proteins in the left and right sample columns were at spotted at an initial concentration of 0.5 and 0.25 mg/ml, respectively. Figs. 1(c), 1(d), 1(f), and 1(g) are the intensity profiles taken along the lines shown in Figs. 1(b) and 1(e). As illustrated in Figs. 1(b)–1(d), the gray scales in the images and $\mathrm{Im}\{\Delta_p - \Delta_s\}$ signal intensity in the intensity profiles of the protein spots are different from proteins to proteins, even though these proteins were printed at the same initial concentrations, indicating that after immobilization these proteins had differences in optical dielectric constant and mass density. As expected, the gray scales and $\mathrm{Im}\{\Delta_p - \Delta_s\}$ signal intensities for the same protein at two concentrations are different, indicating that the same protein with different concentrations have the different occupancy rate at the sample spots. The experimental results are in accordance with our previous results described in Refs. 15 and 16 indicating that the OIRD method is not dependent on labels to detect different proteins in a wide range of concentrations. By comparing the 2D images between Figs. 1(b) and 1(e), the intensity profiles between Figs. 1(c) and 1(f) and between Figs. 1(d) and 1(g), it becomes apparent that the gray scales of protein spots in Fig. 1(e) are obviously smaller than those in Fig. 1(b), and that $\mathrm{Im}\{\Delta_p - \Delta_s\}$ signal amplitudes in Figs. 1(f) and 1(g) are drastically lower than those in Figs. 1(c) and 1(d), indicating that all six proteins on Microarray I were effectively degraded by Proteinase K.

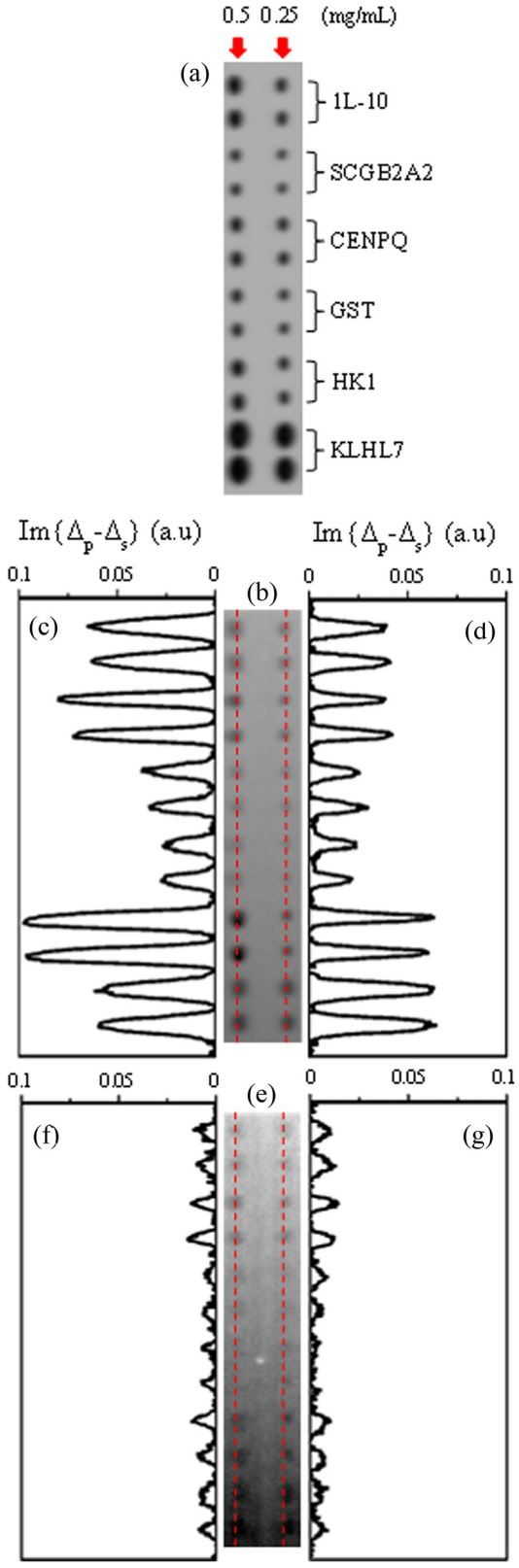


FIG. 1. The measurement results of Microarray I using the OIRD. (a) A 2D scanning image after printing; (b) 2D image after washing; (c) Intensity profile taken along the left line in image (b); (d) Intensity profile taken along the right line in image (b); (e) 2D scanning image after Proteinase K reaction; (f) Intensity profile taken along the left line in image (e); (g) Intensity profile taken along the right line in image (e).

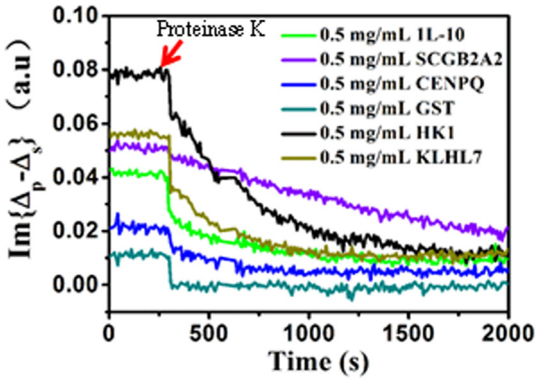


FIG. 2. The degradation time course of six proteins at 0.5 mg/ml by Proteinase K.

Figure 2 shows the degradation kinetics of the six proteins starting at an initial concentration of 0.5 mg/ml. The red arrow indicates the starting point when Proteinase K reaction buffer was pumped into the flow-cell chamber. The values of $\mathrm{Im}\{\Delta_p - \Delta_s\}$ intensity in Fig. 2 represent the average values of the two same sample spots on Microarray I. Differences in the shape of curves showed in Fig. 2 suggested that although the initial concentrations of these protein substrates were the same, the reaction rates were quite different from each other. For example, 1L-10 (18 kD), CENPQ (30 kD), GST (27 kD), KLHL7 (60 kD), and HK1 (115 kD) were rapidly degraded as shown in an exponential degradation curve; whereas SCGB2A2 (13 kD) was a much worse substrate for Proteinase K, as the shape of its degradation curve is close to linear. Therefore, it is obvious that Proteinase K showed somewhat substrate preference based on the observed differences in the kinetics analysis, though it usually considered as a generic protease without any obvious substrate preference.

To determine whether our OIRD platform can be used to further characterize the biochemical behavior of Proteinase K, we spotted 1L-10 proteins in a dilution series on Microarray II and monitored its degradation kinetics by Proteinase K in a real-time, label-free fashion. As illustrated in Fig. 3(a), after Proteinase K was pumped into the reaction chamber, the OIRD $\mathrm{Im}\{\Delta_p - \Delta_s\}$ signal intensities in a time course of 1500 s clearly showed the differences in the shape of the reaction curves as a function of 1L-10 protein's initial concentrations, ranging from 1 mg/ml to 0.5, 0.25, 0.125, and 0.0625 mg/ml. The red arrow again indicates the starting point when Proteinase K was added. The values of $\mathrm{Im}\{\Delta_p - \Delta_s\}$ intensities in Fig. 3(a) were obtained by taking average values of the two same sample spots in Microarray II. It was evident that the OIRD $\mathrm{Im}\{\Delta_p - \Delta_s\}$ signal intensities changed with the reaction time and showed an exponential behavior, while the changes of $\mathrm{Im}\{\Delta_p - \Delta_s\}$ signal intensities for the various concentrations of 1L-10 proteins were different. The higher initial the 1L-10 protein concentration was, the faster the degradation velocity. However, the time to reach completed degradation of 1L-10 (signal reaches the lower plateaus) remained the same (i.e., ~900 s) for all concentrations tested, suggesting that the degradation time does not correlate with the initial protein concentration.

To better understand the relationship between substrate concentrations and Proteinase K activity, we plotted 1L-10 initial concentrations against the initial degradation velocity in Fig. 3(b). To do so, we used the absolute value of slope from dashed lines of degradation curves shown in Fig. 3(a) at the starting point as the initial velocity of the degradation processes. We noticed that the curves of 0.5 mg/ml and 1 mg/ml in Fig. 3(a) are almost superposed, and that the initial velocities of degradation almost become a constant when the concentration of 1L-10 is higher than 0.5 mg/ml, suggesting that the 1L-10 proteins immobilized on the substrate microarray reached saturation when the initial concentration >0.5 mg/ml.

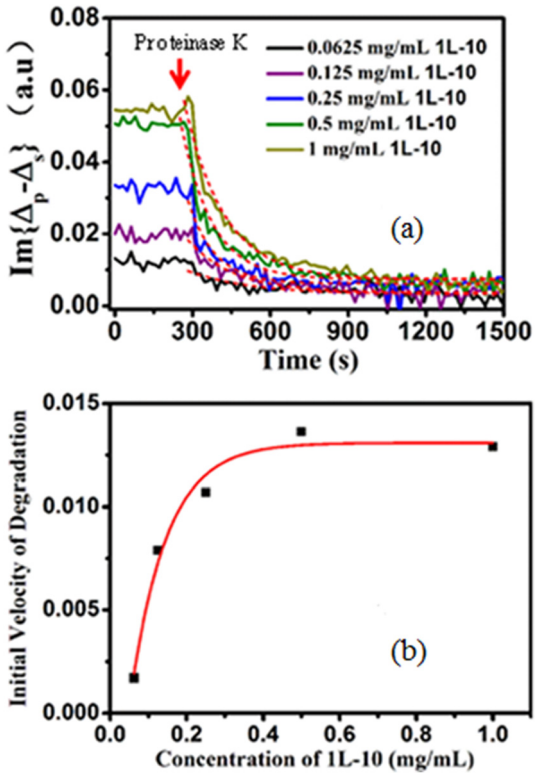


FIG. 3. Relationship between substrate concentrations and Proteinase K activity. (a) OIRD $\mathrm{Im}\{\Delta_p - \Delta_s\}$ signals versus reaction time for IL-10 protein, at the concentrations of 1 mg/ml, 0.5 mg/ml, 0.25 mg/ml, 0.125 mg/ml, and 0.0625 mg/ml, dashed lines are fitted for degradation curves; (b) The relationship between the concentration of IL-10 protein and the initial degradation velocity.

In summary, we have monitored the dynamic processes of protein degradations using OIRD method in a label-free and real-time manner, and obtained the degradation velocities, as well as the degradation time. These experimental results demonstrated that OIRD offers an approach to characterize enzymatic properties in a "loss-of-signal" mode, which has been extremely challenging to achieve using the traditional biochemistry methods. Based on our previous studies of high-throughput detection,[20] further research on protease activities, including screening substrates, studying the dynamic process, and acquiring the kinetic and thermodynamic parameters, are under way.

The work was supported by the National Basic Research Program of China (Grant No. 2007CB935701); and NIH U54 HG006434 to H.Z.

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