Supplementary Information

A continuous-flow mass biosensor for the real-time analysis of dynamics of protease inhibition

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1. Experimental Section

1.1. Materials and reagents

Cysteamine hydrochloride and soybean TI (type I-S) were purchased from Sigma (St. Louis, MO, USA). Bovine chymotrypsin, trypsin and porcine pepsin were obtained from Institute of Biochemistry, Chinese Academy of Science (Shanghai, China). Glutaraldehyde and NaCNBH₃ were purchased from Beijing Chemical Reagents Company (Beijing, China). Ethanolamine was obtained from Yili Chemical Co., Ltd. (Beijing, China). All other chemicals were of analytical grade and used without further disposal.

Phosphate buffer (PB, 10 mmol/L) was used as carrier buffer and glycine-HCl solution (100 mmol/L, pH 2.0) was used as regeneration reagent. Unless otherwise mentioned, all sample solutions were prepared using carrier buffer. Triple distilled water was used throughout the study. All the prepared solutions were filtered through a 0.45 μ m filter prior to use.

1.2. Equipment and apparatus

The home-made FIA-QCM system used in this study was described in detail elsewhere¹. Briefly, the FIA-QCM system consisted of a flow-through cell (80 µL internal volume), a frequency oscillator (powered by 3Vdc), a frequency counter (Model EE3386 universal counter, Nanjing Telecommunication Instrument Factory, Nanjing, China), an infusion pump (Model KDS 100, KD Scientific Inc., Holliston, USA), an HPLC injection valve (Rheodyne 7125, California, USA) and a computer with an in-house kinetic analysis software to record the frequency continuously. The AT-cut quartz crystals (12.0 mm diameter, 10 MHz resonant frequency) with gold electrodes (6.0 mm diameter) on both sides were purchased from Beijing Chenjing Radio Electronic Co., Ltd. (Beijing, China).

1.3. Immobilization of TI

The immobilization of TI molecules on the surface of gold electrodes was carried

out as follows: The gold electrodes were first cleaned with Piranha solution $(H_2SO_4:30\% H_2O_2=3:1 v/v)$, and rinsed with water and ethanol, then air-dried. The freshly cleaned gold surfaces were immersed into a 20 mmol/L cysteamine hydrochloride solution for 12 h in dark at room temperature. Then the gold surfaces were washed with ethanol and water several times to remove the excess cysteamine. After a stable self-assembled monolayer of cysteamine was formed on the gold surfaces, glutaraldehyde was used as activating reagent to introduce aldehyde groups. The quartz crystal was immersed into a 2.5% (v/v) glutaraldehyde solution (pH 7.5) at 40 °C for 4 h in an incubator shaker, and stopped by washing the gold surfaces with large amount of water. Then one side of the activated electrode surfaces was exposed to 50 µL of 5 mg/mL TI solution (pH 7.5) at room temperature for 12 h. The crystal was exposed to sodium cyanoborohydride solution (1 mg/mL) for another 2 h. The reaction was stopped by washing the electrode surface with water. Finally, the unreacted aldehyde groups were blocked by applying 1 mol/L ethanolamine hydrochloride (pH 7.5) to the electrode surface. The TI immobilized crystals were kept at 4 °C after the experiments every day.

A control quartz crystal sensor was also prepared following the same steps except the addition of TI.

1.4. Study on the interaction between TI and three proteases

The TI immobilized quartz crystal was mounted in the flow-through system by two silicon rubber O-rings and rinsed with 10 mmol/L phosphate buffer continuously at a flow rate of 40 μ L/min. Prior to the injection of proteases, 600 μ L BSA solution (1 mg/mL, in PB) was introduced into the system, and then the QCM was sufficiently rinsed with PB. After a stable baseline was achieved, 600 μ L of protease (trypsin, chymotrypsin and pepsin, respectively) solutions in various concentrations were injected into the FIA-QCM system via an HPLC injection value. The curves of frequency shifts versus time were recorded and the entire real-time association and dissociation processes were monitored simultaneously on the computer. After each measurement, the quartz crystal was regenerated for next binding by injecting 600 μ L of glycine-HCl solution into the system to dissociate the bound protein.

1.5. Kinetic data analysis

The basic principles and analysis software for dynamic analysis have been described in detail elsewhere¹. In brief, the following equations were used for calculation:

For association,

$$\Delta F = -\frac{k_{ass}C \bullet F_{\max}}{k_{ass}C + k_{diss}} \left[e^{-(k_{ass}C + k_{diss})(t - t_o)} - 1 \right]$$
(1)

For dissociation,

$$\Delta F = -\Delta F_0 e^{-k_{diss}(t-t_0)} \tag{2}$$

For association constant,

$$K_A = \frac{k_{ass}}{k_{diss}} \tag{3}$$

And for dissociation constant,

$$K_D = \frac{k_{diss}}{k_{ass}} \tag{4}$$

Where k_{ass} is association rate constant, k_{diss} is dissociation rate constant, K_A is association constant, K_D is dissociation constant, t is time, t_0 is time at start of reaction, F_0 is the frequency at t_0 and C is sample concentration. F_{max} is the frequency change when the crystal surface of QCM biosensor was completely saturated with targeted molecules.

1.6 Circular dichroism (CD) measurements

Pepsin solutions were prepared with phosphate buffers of pH 2.0 and pH 7.5 respectively (5 μ M). The CD spectra were recorded at 25 °C with a Jasco-815 spectrophotometer (JASCO, Tokyo). The spectra were measured from 190 to 250 nm for 3 times.

1.7. Structural analysis of proteases

Ribbon drawing of trypsin (PDB ID 4I8H), chymotrypsin (PDB ID 1YPH) and pepsin (PDB ID 4PEP) were generated and analyzed with RASTOP software. And protease structures with surfaces were generated by RSCB-Protein Workshop.^{2,3}

2. Preparation of the TI-immobilized quartz crystal surface

As a monomeric protein containing 181 amino acids, trypsin inhibitor (20.1 kDa) can be assembled on the bare gold surface of the sensor via its amino groups. However, such direct attachment may cause severe steric hindrance which will prevent sufficient contact of TI with proteases during interaction. Therefore, the gold surface was firstly functionalized by a self-assembling monolayer of cysteamine through the strong Au–S bonds. To provide TI with flexible spatial orientation, different bi-functional molecules have been examined as linkers to conjugate TI. 1,4-butanediol diglycidyl ether was firstly used to immobilize TI, the frequency decrease on this quartz crystal was 42.2 Hz after trypsin injection. In comparison, the quartz crystal employing glutaraldehyde as spacer responded with 53.9 Hz. These results indicate that glutaraldehyde can provide TI with flexible spatial orientation and is more suitable spacer for TI immobilization.

For the control sensor which was prepared by the same procedure except the immobilization of TI, negligible frequency changes were detected after injection of three proteases. These results verify the effectiveness of ethanolamine in blocking the non-specific adsorption from the residue aldehyde groups. Thus, the frequency shifts

on TI sensor was clearly caused by the binding towards TI.

3. Regeneration and reproducibility

Four conventional regeneration reagents including glycine-HCl buffer (100 mM, pH 2.0), 20 mM phosphate buffer containing 0.5 M NaCl (pH 7.4), HCl solution (10 mM) and NaOH solution (10 mM) were examined respectively to remove the bound proteases from the TI immobilized QCM sensor. As shown in Figure S1, the mild glycine-HCl buffer showed the best elution efficiency for all the three proteases. The frequency can return to the baseline within 20 min after injection of glycine-HCl buffer. Neither high ionic strength medium (NaCl-containing phosphate buffer) nor strong acidic/basic solutions can acquire such rapid and complete elution effect. Moreover, the mild glycine-HCl can well maintain the natural activity of the immobilized TI. On contrary, strong acid and base are destructive and would shorten the work life of the sensor. Hence, glycine-HCl buffer was chosen as the optimal regeneration reagent for the following studies.



Figure S1. The binding-dissociation process of trypsin on TI-immobilized QCM sensor. Mobile phase, 10 mM phosphate buffer; regeneration solution, glycine-HCl solution (pH 2.0).

Reproducibility of the biosensor was evaluated by measuring the frequency responses after 30 rounds of binding-dissociation process on one sensor. As shown in

Figure S2, even used for thirty times, the sensor showed no significant change in frequency response to trypsin. The loss of the signal was calculated to be 3.3% at the 30th cycle. Moreover, the inter-sensor reproducibility was also estimated by comparing the frequency changes from three TI-immobilized sensors. The inter-senor variation (RSD) were 4.6% and 4.3% by responding to 0.1 mg/mL trypsin and chymotrypsin respectively, suggesting good stability and reproducibility of the biosensor.



Figure S2. Frequency response of the TI-immobilized sensor to the 1st and 30th injections of trypsin solution (0.1 mg/mL).

4. Quantitative sensing of trypsin and chymotrypsin





5. Circular dichroism (CD) measurements

Considering pepsin is most active at pH 2.0, its structural stability under pH 7.5 was examined by circular dichroism spectroscopy (CD). No significant difference was observed in the spectra of pepsin under these two pH values (Figure S4), suggesting pepsin is stable and remained its natural conformation at pH 7.5. These results also agree with previous studies on pH effect on pepsin activity.⁴



Figure S4. Circular dichroism assay of pepsin in phosphate buffers (PB) of pH 2.0 and pH 7.5.

6. Structural analysis of pepsin



Figure S5. Structural analysis of pepsin. The Ser-Ser sequences (residue 35-36, 46-47, 61-62, 156-157, 172-173, 250-251, 294-295) are shown in red. Ribbon drawing of trypsin, chymotrypsin and pepsin were generated and analyzed with RASTOP software.

Besides the Asp-Ser-Cys sequence, pepsin structure was further examined and compared with the S1 sites of trypsin and chymotrypsin. The same sequence was found both at the bottom of S1 active site of chymotrypsin (Ser-Ser) and in pepsin at seven different sites (residue 35-36, 46-47, 61-62, 156-157, 172-173, 250-251, 294-295) (Figure S5). Different from the particular spatial distribution of these sequences in the S1 pockets of chymotrypsin, these similar elements in pepsin are all located on protein surface. The exposed binding loop of TI may contact with them, which would therefore contribute to the weak interaction between pepsin and TI.

References

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