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Continuous protease assays using liquid crystal as a reporter

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ABSTRACT

Abnormal protease activities are associated with cancers, vascular diseases and Alzheimer diseases. Therefore, detection of protease activities has become increasingly important in recent years. Herein, we report a semi-quantitative, liquid crystal (LC)-based protease assay for naked-eye detection of protease activity. In this assay, casein molecules are cleaved by proteases into small peptide fragments, which can be quantified either by using Lowry's method or using LC. In the latter, peptide fragments adsorb on a solid surface and disrupt LC to produce a bright spot for naked-eye detection. The bright spot is observed only when the surface-adsorbed peptide density exceeds a critical value. In the assay, a major challenge is how to separate undigested casein and remaining protease from peptide fragments to prevent their interferences with LC. To overcome this issue, trichloroacetic acid (TCA) is added to precipitate casein and proteases. By using the assay, we are able to detect 10 ng/mL of protease (activity 7.23 U/mg protease, $R^2 = 0.991$) or 6.5 μg/mL of peptide fragments. Finally, a continuous protease assay is developed to minimize manual sampling and reduce errors in kinetic studies.

1. Introduction

Proteases are enzymes which are able to catalyze the hydrolysis of peptide bonds. They play important roles in food digestion, healing of wounds and blood coagulation [1–3]. Proteases attract much attention in recent years because they are associated with cancers [4,5], vascular diseases [6] and Alzheimer diseases [7]. Therefore, development of facile yet sensitive protease assays for the detection of protease activities is highly important. Traditionally, protease activities can be determined after incubation of proteases with their substrates, such as proteins or peptides. After the incubation period, peptide fragments released from the substrates (due to protease activity) are detected by using analytical instrumentation such as high performance liquid chromatography coupled with mass spectroscopy (HPLC–MS), liquid nitrogen assisted spray ionization mass spectrometry (LNASI-MS), matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and surface assisted laser desorption/ionization time-of-flight mass spectrometry (SALDI-TOF-MS) [8–11]. Based on peptide mass fingerprints and peak heights, one can determine protease cleavage sites and protease activities. This method is accurate, label-free and highly specific, but it relies on expensive instrumentation and is time-consuming [12,13].

Recently, many simple and portable protease assays have been developed [14–20]. These assays can be classified as specific and non-specific assays depending on the substrates used. For specific protease assays, custom-synthesized peptides with a well-defined sequence are used as a substrate for a particular protease. On the other hands, non-specific protease assays use low-cost proteins such as casein as a substrate, which can be digested by proteases into peptide fragments [21]. After precipitation of undigested casein and remaining proteases, the peptide fragments can be quantified by using Lowry's method with Folin–Ciocalteu (FC) reagent, which reacts with tyrosine residue in the peptide fragments and gives a blue color. Although this method is simple and quantitative, the limit of detection (LOD) is only 0.1 U/mL [22]. To address this issue, many researchers used labeled caseins as substrates to lower LODs. For example, Manicourt and Lefebvre used [^{3}H] and [^{125}I]-labeled casein to detect matrix metalloproteinases (MMPs) and plasmin with LODs of 2.5 ng/mL and 5 μg/mL, respectively [23,24]. However, labeling of casein with radioisotopes is tedious, time-consuming, expensive and requires special handling of radioactive materials. Alternatively, casein can be labeled with fluorescent dyes for fluorescence detection. For example, Schade et al. and Mancini et al. employed 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene (BODIPY) and fluorescein-labeled caseins to detect protease activities. They were able to achieve LODs of 50 ng/mL and 170 nM, respectively [25–27]. Although fluorescence technique is highly sensitive, fluorescein becomes non-fluorescent in acidic solutions. Alternatively, some researchers derivatized

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casein with a chromogenic agent 4-(dimethylamino) azobenzene-4′-sulfonyl chloride (DABS-C1) for detecting protease activities. For trypsin and α-chymotrypsin, LODs can go down to as low as 64.16 ng and 89.86 ng, respectively [28]. However, the selectivity of the reaction is poor as the chromophores can bind to any amino acids, peptides and protein molecules. Finally, protease assays based on electrophoretic methods and charge-changing substrates were also reported in the literature. In these assays, LODs could reach 0.3 ng/mL and 500 ng/mL for trypsin and α-chymotrypsin, respectively [29,30]. However, they require custom-synthesized substrates in the assays.

Recently, liquid crystals (LCs) were exploited by many researchers to develop label-free bioassays with naked-eye detection capability [31–35]. LC-based bioassays are portable, easy-to-use, and can be used to detect a wide variety of biomolecules with good sensitivity. For example, Yang and co-workers used LC to detect peptides of different lengths [36–39]. In this assay, LC was directly applied to a surface functionalized with peptides. When long peptides were immobilized on the surface, they disrupted orientations of the LC and gave a bright color. On the other hand, when short peptides were immobilized on the surface, they did not disrupt the orientations of the LC and the LC remained dark. The capability of using LC to differentiate long and short peptides was further exploited to develop a heterogeneous protease assay. In the presence of proteases, surface-immobilized long peptides were shortened by protease activity, and the LC gave a dark spot [40]. However, in the heterogeneous protease assay, the substrates are surface-immobilized peptides which are different from free peptides in buffer solutions. Therefore, there is a need to develop a LC-based, homogeneous protease assays in which free protein molecules can be used as substrates.

In this study, we developed a LC-based protease assay by using casein as a substrate and used it to determine protease activity and perform kinetic studies. Unlike other LC-based bioassays, this assay is a homogenous assay in which proteases and substrates were mixed homogeneously in a buffer solution to release peptide fragments. Thus, the protease activities can be determined more precisely than heterogeneous assays. However, undigested substrates and remaining proteases must be separated from the solution before peptide fragments can be analyzed. In this study, we first studied how to precipitate casein and proteases by using TCA and avoid their interferences with LC. Parameters such as casein and protease concentrations, mixng ratios of TCA to casein and protease were optimized for complete precipitation of casein and protease. Next, LC was used to detect peptide fragments produced by protease activities. Finally, to minimize manual sampling, a continuous protease assay with a syringe pump was developed.

2. Experimental

2.1. Materials

Polytetrafluoroethylene (PTFE) tubing (ID 0.8 mm × OD 2.4 mm) and 96-well microplates were purchased from Cole Palmer (Singapore). A T-shape polypropylene connector was purchased from Kartell (Italy). *N*, *N*-dimethyl-*n*-octadecyl-3aminopropyltrimethoxy- silyl chloride (DMOAP), protease from bovine pancreas type I (≥5 U/mg solid), sodium acetate, potassium phosphate buffer, calcium acetate, casein from bovine pancreas, trichloroacetic acid (TCA), Folin & Ciocalteu's phenol reagent (FC reagent), sodium carbonate, Tween 20 and L-tyrosine were purchased from Sigma Aldrich (Singapore) and used as received. Liquid crystal 4-cyano-4-pentylbiphenyl (5CB) was purchased from Merck (Singapore), Glass slides were purchased from Fisher Scientific (U.S.A).

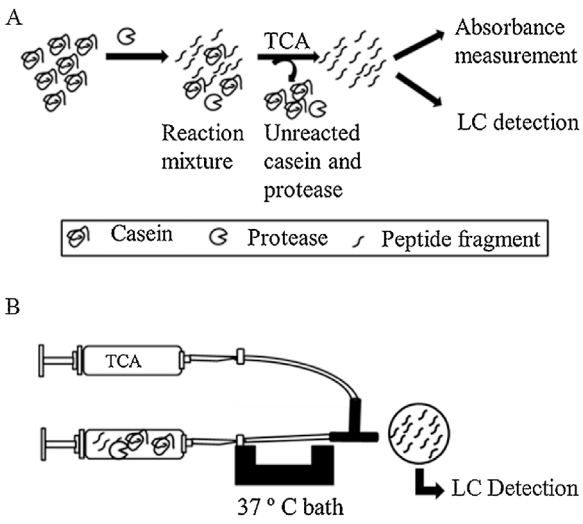

Fig. 1. (A) Working principle of a nonspecific protease assay. Casein is used as a substrate for protease and TCA is used to precipitate remaining casein and proteases. Detection of peptide fragments is accomplished by using absorbance measurement or LC. (B) A continuous protease assay in which reaction products are mixed with TCA at a T-junction. Peptide fragments emerging from the millifluidic device were further analyzed by using LC.

2.2. Measurement of protease activity

Initially, casein (6.5 mg/mL) was dissolved in potassium phosphate buffer (50 mM, pH 7.5) and used as a substrate solution [22]. After that, protease solutions of different concentrations were prepared in sodium acetate (10 mM, pH 7.5) containing 5 mM of calcium acetate (protease diluent). To measure protease activity, approximately 400 μL of the casein solution was incubated at 37 °C for 5 min under constant shaking (100 rpm). Then, 100 μL of a protease solution was added to the casein solution and incubated for another 10 min. Subsequently, 600 μL of TCA solution (110 mM) was added to the reaction mixture to stop the reaction. The mixture was incubated for another 30 min to precipitate all casein and protease. At the end of the incubation, the reaction mixture was centrifuged for 1 min. A clear supernatant (200 μL) was transferred into a vial with 500 μL of sodium carbonate (500 mM) and 100 μL of FC reagent (500 mM). FC reagent was used to form blue color complex with tyrosine of peptide fragments in the supernatant [22] .The solution was incubated for another 30 min for color development. For absorbance measurement, 200 μL of the solution was transferred into a 96-well microplate. Absorbance was measured by using microplate reader at 660 nm and 20 °C. Fig. 1A shows the mechanism of protease assay.

2.3. Surface modification

Glass slides were washed with deionized (DI) water twice. Subsequently, the glass slides were immersed in a 5% Decon 90 solution overnight for cleaning. After that, the glass slides were sonicated and rinsed rigorously with DI water. Afterwards, the glass slides were sonicated twice for 15 min in DI water. Then, the glass slides were immersed in an aqueous solution containing 0.1% of DMOAP for 5 min and washed with DI water. DMOAP-coated glass slides were heated in a vacuum oven at 100 °C for 15 min to allow cross-linking of DMOAP [38].

2.4. LC-based protease assays

To detect protease activity by using LC, we collected 0.5 μL of a sample solution after TCA was added and incubated for 30 min.