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Effectiveness of cysteine proteases on protein/pigment film removal

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ABSTRACT

Objective: Theaflavin (TF) from the black tea can react to human salivary proline-rich proteins (PRPs) to form stains on exposed dental surfaces. Here, we employed a model of protein/pigment film using TF and dephosphorylated bovine β-casein (Dβ-CN), which has an extended conformation, similar to that of salivary PRPs, on a sensor surface to assess the efficacy of cysteine proteases (CPs) including papain, stem bromelain, and ficin, on removing TF bound to Dβ-CN and the control TF readsorption on the residual substrate surfaces was also measured.

Methods: The protein/pigment complex film was built by using a quartz crystal microbalance with dissipation (QCM-D). The efficacies of CPs were assessed by Boltzman equation model. The surface details were detected by grazing angle infrared spectroscopy spectra, atomic force microscopy images, and contact angles.

Results: The efficacy order of CPs on hydrolyzing protein/pigment complex film is ficin > papain > bromelain. The results from grazing angle infrared spectroscopy spectra, atomic force microscopy images, and contact angles demonstrated that TF bound on the Dβ-CN was effectively removed by the CPs, and the amount of TF readsorption on both the residual film of the Dβ-CN/TF and the Dβ-CN was markedly decreased after hydrolysis.

Conclusion: This study indicates the potential application of the CPs for tooth stain removal and suggests that these enzymes are worthy of further investigation for use in oral healthcare.

1. Introduction

Tooth surface stains develop due to an interaction and a subsequent precipitation reaction between human salivary proteins and dietary pigments [1–4]. The major protein constituent of saliva is a group of proteins that consists of multiple repeats of an unusual amino acid sequence containing a large number of proline residues that are commonly referred to as salivary proline-rich proteins (PRPs). PRPs have

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high binding affinity for phenolic and polyphenols pigments, which are widely distributed in plant-based foods and beverages [5,6]. Extrinsic discolouration is outside the tooth substance and lies on the tooth surface or in the acquired pellicle. The natural phenolic pigments can be also incorporated into the pellicle and produce a stain at the tooth surface [7].

Tooth stains can be removed by a number of methods, including whitening toothpastes, professional cleaning and polishing, bleaching, and enamel microabrasion with abrasives and acid [7,8]. Cysteine proteases (CPs) including papain, stem bromelain, and ficin are enzymes with high proteolytic activity that can hydrolyze casein and other proteins [9,10]. Therefore, the hypothesis of the study is that CPs may help the degradation of protein/pigment film and subsequently remove the proteinaceous stains on tooth.

Using a molecular model of phenolic pigment reaction with human whole saliva [11,12], we previously investigated papain hydrolysis of human whole salivary film and bovine β-casein [13,14]. However, there are no existing reports on CP-mediated hydrolysis of complex protein/pigment films. In the current study we therefore investigated the ability of different CPs to catalyze complex protein/pigment films by using a quartz crystal microbalance with dissipation (QCM-D). The staining film's degradation and prevention by CPs were assessed by measuring QCM-D shifts in resonant frequency. Grazing angle infrared spectroscopy (GA-FTIR) spectra, atomic force microscopy (AFM) images, and contact angles of various films before and after hydrolysis were investigated to assess the utility of CPs for stain removal and readsorption on different films.

2. Materials and methods

2.1. Materials

Papain (EC 3.4.22.2), stem bromelain (EC 3.4.22.32), ficin (EC 3.4.22.3), and 11-mercaptoundecanoic acid (11-MUA) were obtained from Sigma–Aldrich (St. Louis, MO, USA). N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) were both purchased from Thermo Scientific (Waltham, MA, USA). Dβ-CN was kindly provided by the Institute of Life Sciences, Xiamen University, China. Ammonium hydroxide (NH_4OH), hydrogen peroxide (H_2O_2), sodium chloride (NaCl), and absolute ethanol (Sigma–Aldrich) were all used as received. $\geq$ 98% TF from the extraction of the black tea were obtained from Woko pure chemical industries, Ltd., Osaka, Japan. All chemicals were analytical grade.

2.2. Enzyme activity assay

Enzymes were activated before treatments with 1 ml 0.01 M phosphate-buffered saline (PBS), pH 7.0, containing 2 mM EDTA and 5 mM L-cysteine, which were thermally treated at 30 °C for 10 min [10]. Enzyme proteolytic activity was determined using the previously published method of Lei et al. [17]. One unit of enzyme activity was defined as the tyrosine content formed per minute at 30 °C and pH 7.0. All experiments were repeated at least three times.

2.3. Dβ-CN/TF complex film formation

Protein molecule immobilization on the sensor surface was carried out as previously described [11–14]. A solution of 0.25 mg/ml Dβ-CN in 5 mM PBS at pH 7.0, ionic strength 0.01 M was used to incubate the activated surfaces for at least 120 min at 30 °C until the changes in frequency reached steady-state conditions. After rinsing with PBS, the changes of frequency decreased to 137 ± 2 Hz, which was equivalent to 808 ± 9 ng/cm^2 mass and 9.4 ± 0.1 nm thickness of the Dβ-CN film on the sensor surface. At this point, 12 μM TF solution was exposed to the Dβ-CN-modified sensor surfaces, and TF adsorption was simultaneously monitored as a function of time by recording both the shifts in frequency (ΔF) and mass (Δm) at the fundamental resonant frequency along with different overtones until a steady state of adsorption was reached within 60 min. The entire experiment was repeated three times, and the means and standard deviations were calculated.

2.4. Dβ-CN/TF complex film hydrolysis

After the Dβ-CN/TF complex film was established on the QCM-D sensor surface, the control solution (blank), contained PBS, L-Cys, and EDTA except the enzymes, was introduced into the QCM-D cell in an identical fashion, but no significant changes in frequency were observed. At this point, time was reset (zeroing Δt), and the mass acquisition (808 ng/cm^2) was recorded as a baseline to monitor further changes in enzyme hydrolysis. A 1 μM solution of activated CPs (papain, stem bromelain, and ficin), pH 7.0, ionic strength 0.01 M, 30 °C was continuously introduced into the QCM cell using a peristaltic pump at a flow rate of 0.2 ml/min until the changes of mass reached a steady-state condition, typically within 20 min. Enzyme incubation was terminated when no appreciable changes in frequency were observed (ΔF less than 1 Hz min^{-1}). The thickness of various films was determined by modelling the experimental data of ΔF at different overtones using custom written QCM impedance analysis software (version 3.11, KSV Instruments, Helsinki, Finland). The film mass (m) with units of ng/cm^2 was deduced from the Sauerbrey equation [18,19]:

$$\Delta m = -\left(\frac{W}{n}\right)\Delta F \quad (1)$$

where ΔF represents frequency change (Hz), W is the mass sensitivity constant (17.7 ng/cm^2/Hz), and n (overtone number) equals 3 in the Sauerbrey equation.

An empirical model was used to interpret changes in mass when the Dβ-CN/TF complex film was hydrolyzed by various enzymes. The Boltzmann equation and the significance of the respective fitting parameters were explained in full detail in previous publications [18,19]. The Boltzmann equation is, therefore, expressed as:

$$\Delta m = A + \frac{B - A}{(1 + e^{V_{50}-(t/C)})} \quad (2)$$

where Δm and t represent the mass (ng/cm^2) and experimental time (min) variations, respectively. The parameter B represents the minimum mass value in ng/cm^2, corresponding to the lowland region (amount of residual Dβ-CN/TF complex