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# Dissection of the binding of hydrogen peroxide to trypsin using spectroscopic methods and molecular modeling

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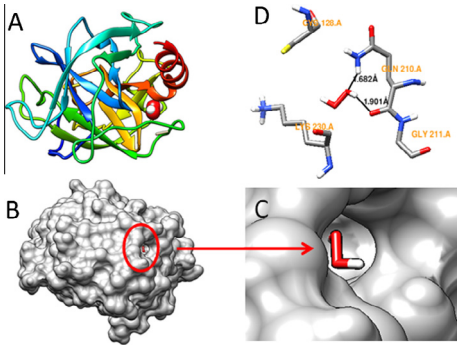
## HIGHLIGHTS

- The fluorescence of trypsin was quenched by hydrogen peroxide in a static quenching.
- Van der Waals forces and hydrogen bonding interactions are main forces during their binding process.
- No distinct activity change of trypsin occurs with the concentration of hydrogen peroxide less than 0.12 M.
- The toxicity of hydrogen peroxide to trypsin was reported at the molecular level.

## GRAPHICAL ABSTRACT

Van der Waals forces and hydrogen bonds play the major role in the interaction of trypsin and hydrogen peroxide. The two hydrogen bonds are 1.901 Å and 1.682 Å formed between hydrogen peroxide and the GLN 210.A residue.

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## ABSTRACT

Studies on the effects of environmental pollutants to protein *in vitro* has become a global attention. Hydrogen peroxide ($H_2O_2$) is used as an effective food preservative and bleacher in industrial production. The toxicity of $H_2O_2$ to trypsin was investigated by multiple spectroscopic techniques and the molecular docking method at the molecular level. The intrinsic fluorescence of trypsin was proved to be quenched in a static process based on the results of fluorescence lifetime experiment. Hydrogen bonds interaction and van der Waals forces were the main force to generate the trypsin-$H_2O_2$ complex on account of the negative $\Delta H^0$ and $\Delta S^0$. The binding of $H_2O_2$ changed the conformational structures and internal microenvironment of trypsin illustrated by UV–vis absorption, fluorescence, synchronous fluorescence, three-dimensional (3D) fluorescence and circular dichroism (CD) results. However, the binding site was far away from the active site of trypsin and the trypsin activity was only slightly affected by $H_2O_2$, which was further explained by molecular docking investigations.



## Introduction

Trypsin, a serine protease, is produced in pancreas and found in many vertebrates, where it hydrolytes proteins [1]. It cleaves peptide chains mainly on the carboxyl side of the amino acids

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lysine or arginine and is used for many biological processes involving digestion, activation of zymogens of chymotrypsin and other enzymes [2–4]. Trypsin is composed of 223 amino acid residues with four tryptophans, ten tyrosines and six phenylalanines that can be used as intrinsic fluorophores [4].

$H_2O_2$ plays an important role in the chemical, industrial, medical, environmental and biological processes [5,6]. Hydrogen peroxide residues in foods can be assimilated by the digestive system, appear in the intestine and contact with trypsin, which may affect the structure and function of trypsin. Besides, monitoring the content of hydrogen peroxide may help to control the biochemical process [7–9].

Studies have found that a certain amount of hydrogen peroxide exists in rainwater, especially in acid rain, which may damage the balance of ecosystem [10]. In recent years, a growing number of scholars are engaged in the study on harms of hydrogen peroxide. For instance, $H_2O_2$ can increase the permeability of cell and induce apoptosis [11], resulting in an increase in malonaldehyde (MDA) of endothelial cell and Lactate dehydrogenase (LDH) as well as a decrease in superoxide dismutase (SOD) activity [12]. From previous assessment of hydrogen peroxide toxicity, we found that the current evaluation system mainly focus on the cellular level. However, molecular level studies are rarely reported.

This article have revealed the toxic effects of $H_2O_2$ to trypsin at a molecular level using the fluorescence, synchronous fluorescence, fluorescence lifetime, 3D fluorescence, UV–vis absorption, enzyme activity test and CD in combination with molecular docking, which is of great help for clarifying the effect of hydrogen peroxide on the trypsin structure and function to perfect the toxicity assessment system of $H_2O_2$.

## Experimental

The trypsin solution (trypsin, bovine pancreas, AMRESCO) was diluted in ultra-pure water to form a $10^{-4}$ mol/L solution as a stock solution.

Hydrogen peroxide solution was purchased from Laiyang Kangde Chemical Co., Ltd. (Laiyang, China). $H_2O_2$ decomposes easily under condition of sunlight or heat, so its concentration has to be determined before using. Take 100 μl of hydrogen peroxide to 100 mL brown volumetric flask and shake well, then measure its absorbance at 240 nm. Calculate the solution concentration according to Beer–Lambert law finally.

Phosphate buffer solution (0.2 mol/L, a mixture of $Na_2HPO_4{\cdot}12H_2O$ solution and $NaH_2PO_4{\cdot}2H_2O$ solution, pH = 7.6) was used to control pH at 7.6 throughout the whole process. $Na_2HPO_4{\cdot}12H_2O$ and $NaH_2PO_4{\cdot}2H_2O$ were purchased from Tianjin Damao Chemical Reagent Factory (Tianjin, China), pure for analysis.

BAEE (N-benzoyl-L-arginine ethylester, Shanghai Guoyao Chemicals Co., Ltd., Shanghai, China) was dissolved in ultra-pure water to form a 0.02 mol/L solution.

Ultrapure water was used throughout the experiments.

### *Apparatus and methods*

#### *Fluorescence measurements*

Trypsin ($10^{-4}$ mol/L) and a known amount of $H_2O_2$ were added into a 10 mL tube with about 1 mL phosphate buffer in it. Shake well and put them in a dark place for 20 min before a measurement.

Fluorescence experiment was conducted using a spectrofluorimeter (Hitachi, Japan) equipped with a 1.0 cm quartz cell and a 150 W Xenon lamp. The excitation wavelength was 278 nm and the emission wavelength ranged from 290 to 450 nm. The excitation and emission slid width were both set at 5.0 nm. Scanning speed was 1200 nm/min and the voltage was 700 V.

The synchronous fluorescence spectra were measured ($\Delta\lambda$ = 15 nm, $\lambda_{ex}$ = 265–320 nm and $\Delta\lambda$ = 60 nm, $\lambda_{ex}$ = 250–310 nm).

The three–dimensional fluorescence spectra were collected under the following conditions: the emission wavelength at 290–470 nm, the excitation at 200–300 nm, sampling interval 5 nm and the photomultiplier tube voltage 700 V.

Inner filter effect refers to the absorption at fluorescence excitation and emission wavelength of the fluorescer, which can affect its fluorescence spectrum, especially when the concentration of such fluorescer is higher [13]. In order to reduce the filter effects induced by the absorption of small molecule or part of macromolecule, the absorption value at the excitation wavelength and emission wavelength should be measured respectively, and corrected with the formula (1) [14]:

$$F_{cor} = F_{obs} \times 10^{\frac{A_1+A_2}{2}} \quad (1)$$

$F_{cor}$ and $F_{obs}$ represent the corrected and original fluorescence intensity respectively. $A_1$ and $A_2$ mean the absorbance at the emission and excitation wavelength, respectively.

#### *UV–vis absorption measurements*

UV–vis absorption spectra were recorded on UV-2450 spectrometer (Shimadzu, Japan). The quartz cell width was 1 cm and the slit width was 2.0 nm. The absorbance was calibrated to zero with ultrapure water before each measurement. The UV–vis spectra were measured from 190 nm to 450 nm with single trypsin solution for reference.

#### *CD Measurements*

CD Spectrum was measured on JASCO J-810 CD spectrometer. Scanning range was 200–260 nm at a speed of 400 nm/min and the spectra were measured with an interval 1 nm. The measurements were repeated for three times and the average was calculated for later use.

#### *Molecular docking study*

Autodock 4.2 was used to do molecular docking calculation. It applies a semi-flexible docking method, conformational changes of small molecule and evaluates docking results on the basis of binding free energy [15,16]. The default search function is Lamarkian Genetic Algorithm (LGA), a hybrid genetic algorithm with local optimization, which estimates binding energy by a parameterized free-energy scoring function. The three-dimensional structure of trypsin was downloaded from PDB database (PDB ID 2PTN). The structure of $H_2O_2$ was drawn by Gaussian 03 and optimized by Material Studio 4.4.

#### *Trypsin activity determination*

Trypsin activity was determined by ultraviolet absorption spectroscopy employing BAEE as a substrate and recorded on UV-2450(Japan Shimadzu). Trypsin can catalyze hydrolyzation of BAEE into BA (N-benzoyl-L-arginine) whose absorbance is far stronger than that of BAEE at 253 nm, and the total absorbance of the system was increased as a result.

## Results and discussions

### *Fluorescence spectra studies*

#### *Fluorescence measurements and quenching analysis*

Proteins can launch strong endogenous fluorescence due to the presence of tryptophan (Trp), tyrosine (Tyr) and phenylalanine (Phe) residues [17]. In this study, the excitation wavelength was set at 278 nm where the emission fluorescence from phenylalanine