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Interaction of mercury and copper on papain and their combined inhibitive determination

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ABSTRACT

Influence and interaction of mercury ion (Hg^{2+}) and copper ion (Cu^{2+}) on papain activity in casein hydrolysis were investigated. Single Hg^{2+} or Cu^{2+} at low concentrations induced an increase in papain activity, but decreased it at high concentrations, confirming a typical hormesis phenomenon. The interaction of Hg^{2+} and Cu^{2+} at various concentration combinations showed that the binary interaction of 10^{-8} mol/L Cu^{2+} and 10^{-6} mol/L Hg^{2+} (Binary union S) buffer was of synergistic nature, while 10^{-4} mol/L Cu^{2+} and 10^{-4} mol/L Hg^{2+} (Binary union I) buffer was of competitive inhibition. The conformational changes in papain structure due to the interaction of binary metal ions were studied by ATR-FTIR, UV–vis and intrinsic fluorescence spectroscopies, also the changes of papain catalytic behavior were studied through kinetic analysis. Decreasing of α-helix content with increasing in intermolecular β-sheet aggregates content in Binary union I buffer resulted in an inactivation of papain activity by 57.2% and lower affinity for casein. On the contrary, papain activity increased with α-helix content increasing and intermolecular β-sheet aggregates content decreasing in Binary union S buffer. The competitive interaction between Cu^{2+} and Hg^{2+} on papain activity was found at higher concentrations ($\geq 10^{-4}$ mol/L), and the inhibition of the binary metal ions on papain was of a noncompetitive type.

1. Introduction

Due to incessant industrial development, heavy metal contaminants have been introduced into water environments and become an increasingly serious social problem. Owing to their bioaccumulation and non-degradability, heavy metals pose a serious pollution hazard to the aqueous environment [3,4,7,20]. Certain metal ions are highly toxic, and the determination of trace toxic heavy metals in water environments has become highly important. Among all of them, Hg^{2+} has attracted the most attention due to its strong toxicity and increasing level of its extended use in industrial processes [12]. In order to evaluate the toxicity, bioavailability, bioaccumulation and transport of the heavy metal elements more readily, sensitive analytical procedures are required for detection [2]. Numerous enzymes have been utilized for heavy metal detection, for enzyme activity is an indicator of the toxicity of heavy metals and other pollutants [1,11,15,22]. The aforementioned studies were mostly concentrated on the effect of a single heavy metal ion. However, contaminated systems usually contain various heavy metals rather than a single one in the real environment. As a consequence, enzymes are exposed to multiple metal ions system. Still, most enzyme biosensors detect heavy metals in contaminated water and derive single-substance toxicity data from single-substance criteria, but neglecting the interaction effects among the mixture. So, the detecting methods for the presence of two or more ions are thus urgently needed [5].

Papain is a highly stable enzyme, one of the proteolytic enzymes of papaya latex [16]. Papain has been widely used as biosensors for its wide pH span for optimum activity, high sensitivity, temperature stability, low price, and short response time. But few influence assays on mixed heavy metal ions based on papain have been reported, particularly on inhibition [20]. Metal ions such as Cu^{2+}, Hg^{2+}, Zn^{2+}, and Pd^{2+} were found not only binding to papain but also inhibiting its activity partly or completely. The IC_{50} of Hg^{2+}, Ag^{2+}, Pb^{2+} and Zn^{2+} were 0.39, 0.40, 2.16 and 2.11 mg/L, respectively, while for Cu^{2+} and Cd^{2+}, the limit of quantitation were 0.004 and 0.1 mg/L, respectively [20].

In the preliminary stage of our work, we observed that the interactive effect of the two mixed metal ions on papain activity was

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different from that of single Hg^{2+} and Cu^{2+}, while the inactivation of papain activity was about 58% for Cu^{2+} and nearly 100% for Hg^{2+}. The inactivation mechanism affected by the binary ions (Hg^{2+} & Cu^{2+}) was still not clearly understood. The principal aim of this present work was to evaluate the combined effect of Hg^{2+} and Cu^{2+} on papain activity and structure. The ATR-FTIR, UV–vis intrinsic fluorescence spectroscopies and kinetics analysis were used to investigate the structure-function relationship in the presence of the binary ions.

2. Materials and methods

2.1. Enzyme and regents

Papain (EC3.4.22.2, ≥99%), bovine serum albumin (BSA), tyrosine and casein were purchased from Sigma–Aldrich Company Ltd. All other reagents used were of analytical grade and used without further purification. All solutions were prepared with redistilled and ion-free water.

2.2. Effect of single metal ion on papain activity

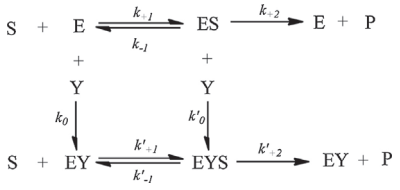
Papain solution (1.0 mg/mL) was obtained by dissolving the enzyme in Tris–HCl buffer (0.1 mol/L, pH 7.0). Stock solution of $HgCl_2$ (0.1 mol/L) and $CuCl_2$ (0.1 mol/L) were prepared in the Tris–HCl buffer and diluted into the concentrations varied from 10^{-10} to 10^{-2} mol/L for papain activity assays. Firstly, papain solution (1 mL) was added into the buffer (1 mL) of different metal ion concentrations at 40 °C, secondly casein solution (3.0 mL, 20.0 mg/mL) was added into the mixture 10 min later. The reaction was carried out at 40 °C for 30 min and then stopped by 2.0 mL trichloroacetic acid (TCA). The activity of papain was determined by a Hitachi U-2001 spectrophotometer at 275 nm. One unit of enzyme activity (U) was defined as 1 μg tyrosine formed per minute at 40 °C and pH 7.0. The relative activity (%) was the ratio of the enzyme activity in the Tris–HCl buffer with different Hg^{2+} or Cu^{2+} concentrations to the corresponding enzyme activity without Hg^{2+} or Cu^{2+}. All the experiments were carried out at least three experiments in each experimental group and the average number was employed as the statistical analysis indicator.

2.3. Effect of the binary ions on papain activity

In order to determine the interactive effect of Hg^{2+} and Cu^{2+} ions on papain activity, the assay was performed by incubating the papain in the binary ions buffer. The buffer was prepared by mixing equal volume of Hg^{2+} and Cu^{2+} buffers at different concentration. Papain activity in the presence of the mixed metal ions was also monitored as described above.

2.4. ATR-FTIR, UV–vis and intrinsic fluorescence spectroscopies

ATR-FTIR spectra of the samples in the ATR cells were recorded on PE Spectrum One B instrument. Background was subtracted using the Opus software. Curve fitting was then performed using Origin 9.0 and PeakFit v4.12 software. The absorbance spectra of the samples were recorded by a Hitachi UV9100 spectrophotometer. The range of wavelength is 190–500 nm. The tryptophan (Trp) fluorescence spectra were recorded by a PE LS55 spectrofluorimeter at 30 °C. The emission spectra were recorded in the range of 300 ~ 410 nm at 500 nm/min, 10 s after excitation, keeping the excitation constant at 288 nm, with slit widths of 5 nm for excitation and emission. Tryptophan ethyl ester was used as internal standard to correct an inner filter effect. The blank spectrum without enzyme was subtracted from the sample spectra.



Scheme 1. The irreversible reaction mechanism of the binary metal ions.

The papain (0.5 mg/mL) was equilibrated in the solutions with the binary ions of 10^{-8} mol/L Cu^{2+} + 10^{-6} mol/L Hg^{2+} (Binary union S) and 10^{-4} mol/L Cu^{2+} + 10^{-4} mol/L Hg^{2+} (Binary union I) under 25 °C for 10 min, respectively, and then centrifuged at 3000 rpm (equal to *g* value 800) for 4 min. The papain without any metal ions treated was used as the control. The supernatant was used for ATR-FTIR, UV–vis and fluorescence spectral measurements. Triplicate samples were analyzed and the data obtained from the triplicate runs were averaged and used as the final result.

2.5. Kinetic measurements

The kinetic model of substrate reaction during irreversible modification of enzyme activity described by Zhao and Tsou was used to study the kinetics of casein hydrolysis by papain with the binary metal ions [24]. The reaction mechanism was considered in Scheme 1, where *E*, *S*, *P* and *Y* represent papain, substrate casein, product tyrosine and the binary ions, respectively. EY, ES and EYS were the respective complexes.

As was usual the case, [*S*] » [E_0] and that the modification reactions were relatively slow compared with the setup of the steady-state of the enzymatic reaction. The product formation can be written as:

$$[P]_t = v't + \frac{v - v'}{A}(1 - e^{-At}) \quad (1)$$

$$A = \frac{k_0 K_m + k_0' [S]}{K_m + [S]} \quad (2)$$

where $[P]_t$ was the concentration of the product formed at time *t*. *A* was the apparent rate constants. [*S*] was the concentration of casein. v and v' were the reaction velocities of reaction in the absence and presence of the binary ions at time *t*, respectively. K_m and K_m' were the Michaelis constants. k_0 and k_0' were the dissociation constants for the modifier with different forms of the enzyme, respectively. V_m and V_m' were maximum reaction velocities. When $v > v'$, the binary metal ions modifier was an activator. When $v < v'$, the modifier binary ions was an inhibitor. When *t* was sufficiently long, the curves become straight lines and the product concentration was written as [P_e]:

$$\frac{1}{[P_e]} = \frac{k_0 \cdot K_m}{V_m} \cdot \frac{1}{[S]} + \frac{k_0'}{V_m} \quad (3)$$

3. Results and discussion

3.1. Effect of single metal ion on papain activity

The effects of different concentrations of Hg^{2+} or Cu^{2+} on papain activity were investigated and typical low-dose stimulation and high-dose inhibition (hormesis) was shown in Fig. 1. Hg^{2+} inhibited papain activity with a relative activity of 6.78% when Hg^{2+} concentration was ≥10^{-4} mol/L, but it was observed that stimulation of papain activity could occur at 10^{-6} mol/L of Hg^{2+} concentration and displayed the highest relative activity of 111.10%. There was no significant difference in papain activity, exposing to $10^{-10} \sim 10^{-7}$ of Hg^{2+}. At the same time, the maximum of 58.10% inhibition by Cu^{2+} (41.90% relative activity) was at 10^{-4} mol/L, and stimulation with