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Inactivating hemocyanin from *Oncomelania hupensis* with 4-(chloroacetyl)catechol and its application in snail control

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ABSTRACT

The hemocyanin of *Oncomelania hupensis* (OhH) is essential for the survival of *O. hupensis* and may be an effective target for the development of new molluscicide. 4-(Chloroacetyl)catechol is a substrate analogue of OhH. In this study, we evaluated the toxicity of 4-(chloroacetyl)catechol to *O. hupensis* and Kunming mice. 4-(Chloroacetyl)catechol had strong molluscicidal activities and the molluscicidal activities was time and dose-dependent. With the increase of exposure time, the LC50 values of the 4-(chloroacetyl)catechol decreased from 6.5 mg/L (24 h) to 3.1 mg/L (72 h). The LC90 values decreased from 16.4 mg/L (24 h) to 4.9 mg/L (72 h). In the acute toxicity test of mice, no evident poisoning symptoms and no animal death were detected after 14 days' continuous observation, which indicated that 4-(chloroacetyl)catechol was a low toxic substance for Kunming mice. These results indicated that 4-(chloroacetyl)catechol is potent molluscicides.

1. Introduction

Oncomelania hupensis, a gastropod mollusc, is a unique intermediate host of *Schistosoma japonicum*, which causes schistosomiasis in China. One of the major preventive steps against schistosomiasis is the control of the vector snail population. Sodium pentachlorophenate and niclosamide are the most widely used molluscicides in China at present [1–3]. But they are highly toxic towards nontarget organisms and easily result in the destruction of the ecosystem. Therefore, advent of an environmentally friendly molluscicide would be a significant addition to current methods of the control of the vector snails population.

Hemocyanin is an oxygen transport protein in the hemolymph of *O. hupensis*. As an extracellular oxygen carrier, it is responsible for the precise oxygen delivery from the respiratory organs to tissues. Therefore, the hemocyanin of *O. hupensis* (OhH) is essential for vector snail survival. OhH is biochemically distinct from the oxygen transport protein of mammal and may be an effective target for the development of a new molluscicide [4–6].

Hemocyanins and phenoloxidases serve different physiological functions as oxygen transporters and as enzymes involved in defense response, respectively. However, they are equipped with a structurally similar oxygen-binding active site. The active site contains two copper atoms. Either of them, CuA and CuB, is coordinated by three histidines. The binuclear Cu active site in hemocyanin reversibly binds O_2, whereas the binuclear Cu active site in phenoloxidase activates O_2 for substrate hydroxylation or oxidation [7–10]. Recent results indicate that several hemocyanins can also exhibit phenoloxidase activity [11–15]. We also found that the OhH exhibited phenoloxidase activity which can activate O_2 for oxidation of catechol [16].

Because catechol is a substrate of OhH, which can bind to the oxygen-binding active site of OhH and then be oxidated by the activated O_2, so it is possible that a specific inactivator of OhH can be designed and synthesized based on the chemical structure of catechol, and the structure of the oxygen-binding active site. This specific inactivator can bind to the oxygen-binding active site and form a covalent bond with the protein, thus irreversibly inactivating the snail oxygen transporters and could be used in snail control. In this study, we found that the bands at 340 nm of OhH were observably descended with the addition of 4-(chloroacetyl)catechol, which indicates that 4-(chloroacetyl)catechol can inactivate oxygen-binding active site of OhH. In addition, we evaluated the toxicity of 4-(chloroacetyl)catechol to *O. hupensis* and Kunming mice.

2. Materials and methods

2.1. Mice and snails and reagents

Adult *O. hupensis* snails (9–11 mm in length) were caught from the Poyang Lake, Jiangxi Province, China. The snails were kept in a

Abbreviations: OhH, *Oncomelania hupensis* hemocyanin; *S. japonicum*, *Schistosoma japonicum*.

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laboratory at 20 °C for a week before experiment. Kunming mice, 6 to 8 weeks old, were purchased from the Center of Experimental Animals, Jiangxi University of Traditional Chinese Medicine. 4-(Chloroacetyl)catechol were purchased from Alfa Aesar (CAS: 99-40-1).

2.2. Preparation of OhH protein

The OhH was prepared as previously described by our group [17]. Briefly, Hemolymph of *O. hupensis* was collected from the foot muscles of the snails. Hemocytes and other cells were removed by centrifugation at 8000*g* for 20 min at 4 °C. The hemocyanin was pelleted in an ultracentrifuge at 180,000*g* for 3 h at 4 °C. The blue pellet was resuspended in "stabilizing buffer" consisting of 0.05 M Tris, 5 mM $CaCl_2$, 5 mM $MgCl_2$ and 1 mM phenylmethylsulfonyl fluoride, pH 7.0 and stored at 4 °C.

2.3. Effects of 4-(chloroacetyl)catechol on oxygen binding

In spectroscopic analysis, OhH shows a peak at 340 nm, which is typical for oxygenated hemocyanins and reflects the formation of the copper–oxygen complex. The degree of oxygenation of the protein can be determined from the absorbance at 340 nm [18]. Inactivating oxygen-binding active site will result in the decrease of the absorbance at 340 nm (A_{340}). The changes in A_{340} at a protein concentration of 2 mg/ml of OhH will be recorded with a spectrophotometer in the presence of 50 ppm 4-(chloroacetyl)catechol.

2.4. Molluscicidal assay

The molluscicidal effects of 4-(chloroacetyl)catechol against *O. hupensis* were determined by Mao's method [19]. The 4-(chloroacetyl)catechol was first dissolved in ethanol at 100 mg/ml, then diluted by dechlorinated water to 20 mg/L, 10 mg/L, 5 mg/L and 2.5 mg/L, respectively. Snails were collected in a nylon mesh bag (30 snails per bag) and immersed into 1000 mL dechlorinated water solution of a known concentration of 4-(chloroacetyl)catechol and exposed for 24, 48 and 72, respectively. After exposure and 24 h recovery period in only dechlorinated tap water, mortality was checked. No response to a needle probe under dissecting microscope was the evidence of death. The data were subjected to the probit analysis and the half and nearly full-lethal doses LC50 and LC90 were calculated. Ethanol (200 μl/L) controls were added to the test.

2.5. Study on toxicity and safety evaluation of 4-(chloroacetyl)catechol on Kunming mice

The acute toxicities of 4-(chloroacetyl)catechol on Kunming mice were determined according to the Regulatory Guide on the Techniques for Drug Research [20]. Kunming mice were randomly assigned to groups and weighed. 4-(Chloroacetyl)catechol are slightly soluble in water, so suspensions were used for their administration. Study substance was administered intragastrically to mice via a stomach tube. Control mice received the same amount of water. After administration of the study substance, the animals were closely observed during the first 8 h, and occasionally thereafter for 14 days, for the onset of convulsions, toxic signs and symptoms, and death.

2.6. Data analysis

The effect of 4-(chloroacetyl)catechol on *O. hupensis* was expressed by LC50 and LC90 and their 95% confidence limit.

3. Results

3.1. Effects of 4-(chloroacetyl)catechol on oxygen binding

The absorption peak of OhH was observed at 275 nm and 340 nm, which corresponded to aromatic residues and Cu^{2+}–O^{2+}, respectively (Fig. 1I). The bands at 340 nm were observably descended by the addition of 4-(chloroacetyl)catechol (Fig. 1II), which indicates the degree of oxygenation of the protein decline. The bands at 275 nm were also slightly descended by the addition of 4-(chloroacetyl)catechol. In the spectroscopic analysis, 4-(chloroacetyl)catechol shows a peak at 275 nm, which reflects phenyl. It is speculated that 4-(chloroacetyl)catechol can bind to oxygen-binding active site and form a covalent bond with the OhH, then the 4-(chloroacetyl)catechol will be warped by the OhH which results in the slight decline of the absorption peak at 275 nm.

3.2. Molluscicidal activities

Molluscicidal activities of 4-(chloroacetyl)catechol against the snail *O. hupensis* was shown in Table 1. It shows time- and dose-dependent effects. The dead numbers of snails increased with the increase of 4-(chloroacetyl)catechol concentration and exposure time. With the increase of exposure time, the LC50 values of the 4-(chloroacetyl)catechol decreased from 6.5 mg/L (24 h) to 3.1 mg/L (72 h) against *O. hupensis*. The LC90 values of the 4-(chloroacetyl)catechol decreased from 16.4 mg/L (24 h) to 4.9 mg/L (72 h).

3.3. Study on toxicity and safety evaluation of 4-(chloroacetyl)catechol on Kunming mice

Mice were filled into the stomach with three doses of 500 mg/kg, 1000 mg/kg and 2000 mg/kg of 4-(chloroacetyl)catechol. Water was used as the control group. The result showed that no evident poisoning symptoms and no animal death were detected after 14 days' continuous observation.

4. Discussion

The control of snails is currently a main strategy in the Chinese National Schistosomiasis Control Programme, which covers an area

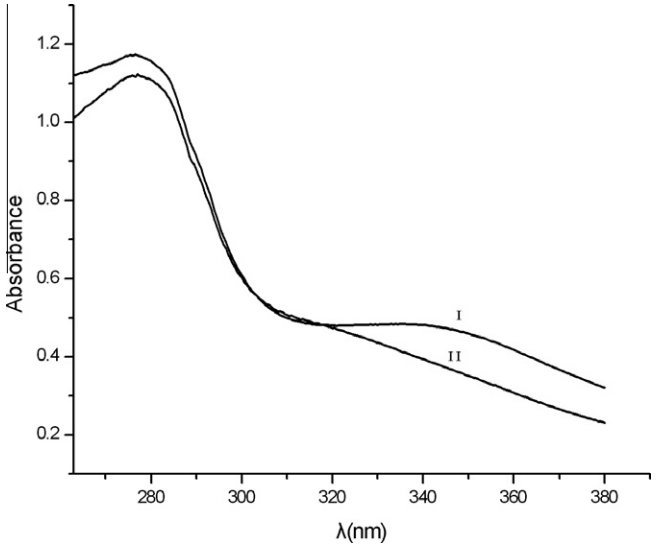

Fig. 1. Absorption spectra of total hemocyanin from *Oncomelania hupensis*. Maximum peaks were at 275 and 340 nm (I), the bands at 340 nm observably descended by the addition of 4-(Chloroacetyl)catechol (II).