Characterization, solubility and antibacterial activity of inclusion complex of questin with hydroxypropyl- β -cyclodextrin

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Received: 7 January 2019 / Accepted: 27 February 2019 / Published online: 5 March 2019
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Abstract

To increase the water solubility of questin and broaden its application in preventing and treating *Vibrio* diseases in aquaculture, an inclusion complex of questin with 2-hydroxypropyl- β -cyclodextrin (HP- β -CD) was prepared by stirring and coevaporation strategy. The results of thin-layer chromatography and nuclear magnetic resonance spectrum confirmed the inclusion of questin into HP- β -CD. The aqueous solubility of questin in the inclusion complex reached $(62.63 \pm 1.21) \mu\text{g/mL}$, which was 110 times of questin's original solubility. The preliminary agar diffusion method indicated that questin-HP- β -CD inclusion complex showed enhanced antibacterial activity against *Vibrio harveyi* compared with free questin. This finding provided a reliable basis for the further application of questin as aquatic antibacterial agent.

Keywords Questin · 2-Hydroxypropyl- β -cyclodextrin · Inclusion complex · Solubility · Antibacterial activity

Introduction

Marine fungi are a large source of microbial natural product because of their complicated genetic background, various kinds and high yield of secondary metabolites (Gomes et al. 2015; Guo et al. 2016; Sun et al. 2014). Research on the secondary metabolites of marine fungi began in 1945, when a strain of *Cephalosporium acremonium* with antibacterial activity was isolated from seawater near a sewage outlet in Sardinia, Italy. Subsequently, cephalosporium C was isolated from its secondary metabolites, which is the first and by far the most important antibiotic derived from marine fungi. Since then, however, scientists have focused their drug discoveries on marine actinomycetes. Until 1983, cyclosporine A produced by fungus *Tolypocladium inflatum*

has been used as a clinical immunosuppressant. Only then new drug research from marine fungi was reintroduced (Bugni and Ireland 2004). Since the 1990s, more than 2000 novel secondary metabolites have been identified in marine fungi derived from algae, sponge, mangrove ecosystem, marine sediments, marine molluscs, sea squirts, coral fishes and other marine animals and plants. The structure types included alkaloids, polypeptide, polyketone, terpenoids, steroid and halogenated derivatives, mainly nitrogen and polyketone compounds, which possess favourable bioactivities such as antibacterial, anti-tumour and antiviral (Ma et al. 2016; Zhao et al. 2016; Nalini et al. 2018). Questin (1,6-dihydroxy-8-methoxy-3-methylanthracene-9,10-dione, Fig. 1a), an anthraquinone compound, was isolated from marine fungus *Aspergillus flavipes* HN4-13 and exhibited the substantial antibacterial efficacy against *Vibrio harveyi* (Guo and Wang 2017; Guo et al. 2019). However, questin's water solubility is poor, which may limit its pharmaceutical application. Hence, adopting an effective technique to improve the aqueous solubility of questin is very necessary.

2-Hydroxypropyl- β -cyclodextrin (HP- β -CD, Fig. 1b) is the condensation product of β -cyclodextrin (β -CD) and 1,2-epoxypropane. HP- β -CD is a ring oligosaccharide, which has a hydrophilic outer surface and a hydrophobic inner cavity (Hsu et al. 2014). Compared with β -CD, HP- β -CD possesses an excellent aqueous solubility, thermostability, nonhemolytic and non-stimulative properties.

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Fig. 1 The chemical structures of questin (a), HP- β -CD (R=CH₂CH(CH₃)OH) (b), and photographs of HP- β -CD, QHIC, questin, and QHPM in liquid state (c)

HP- β -CD can reduce the toxic and side effects of drugs as a kind of potential pharmaceutical excipient (Gould and Scott 2005; Chen et al. 2018).

In the present study, an inclusion complex of questin with HP- β -CD was prepared by stirring and coevaporation. Thin-layer chromatography (TLC) and nuclear magnetic resonance (NMR) spectroscopy were adopted to confirm the complexation of questin with HP- β -CD. The solubility of questin-HP- β -CD inclusion complex (QHIC) was investigated in distilled water and the antagonistic activity of QHIC against *V. harveyi* was determined by using the agar diffusion method.

Materials and methods

Materials and chemicals

Questin (purity > 94%) was purified and kept in our laboratory (Guo and Wang 2017). HP- β -CD was purchased from Jinsui Biotech Co., Ltd (Shanghai, China). *V. harveyi* (CGMCC 1.8690) were kept in our laboratory. TLC plates (HSGF254, 0.2 mm thickness) were purchased from Yantai Jiangyou Silica Gel Co., Ltd (Yantai, China).

Preparation of questin-HP- β -CD inclusion complex

Questin-HP- β -CD inclusion complex (QHIC) was prepared by evaluating the efficacy of various mass ratios

(questin:HP- β -CD = 1:1, 1:4 and 1:9) (Hsu et al. 2013). The inclusion complex at the mass ratio of questin:HP- β -CD = 1:9 has the best solubility, thus the questin-HP- β -CD inclusion complex at this ratio was adopted for the following preparation. 90 mg HP- β -CD was dissolved in 20 mL ethanol and held at 50 °C with moderate stirring by using 85-2 digital thermostatic magnetic stirrer (Jintan, China) for 30 min. Then 10 mg questin dissolved in 5 mL absolute ethanol was slowly added into the solution and agitated continuously for another 6 h. The inclusion solutions were evaporated and dried in vacuum to produce the QHIC in solid state. Meanwhile, questin-HP- β -CD physical mixture (QHPM) was prepared by thoroughly mixing questin and HP- β -CD (1:9, mass ratio) in a grinding bowl for 20 min (Qiu et al. 2016).

Solubility test

A solubility experiment was conducted in accordance with the method of Hsu et al. (2014) and minor modification. Then, 1 mg questin, 10 mg QHIC, and 10 mg QHPM were dissolved in 2 mL distilled water at 37 °C and kept at 120 rpm/min in a HYG-IIa thermostatic shaker (Shanghai Yancheng, China) for 24 h, severally. Next all specimens were centrifuged at 10,000 rpm/min for 10 min to eliminate insoluble substances. The supernatant solution containing soluble questin, QHIC or QHPM were gathered and the absorbance at 425 nm was measured. The standard curve of concentration and absorbance was used to calculate the concentration of questin.

Thin-layer chromatography

A TLC experiment was carried out according to the method (Hsu et al. 2014) with minor modifications. Sample solutions of HP- β -CD, questin and QHIC were produced by dissolving 9 mg HP- β -CD, 1 mg questin and 10 mg QHIC in 5 mL methanol, respectively. Then, 1 mg questin and 9 mg HP- β -CD dissolved in 5 mL methanol were employed as the solution of QHPM. Afterward, 2–5 μ L each sample was pointed onto a TLC plate and spread in a mixed developer (ethyl alcohol/ethyl acetate = 5/1, v/v). After expansion, the solvent was blown dry, and the plate was irradiated using a dark box UV analyser to track the above samples. Then the dried plate was sprayed by using an ethanol solution containing 10% sulfuric acid and then heated at 100 °C for 5 min to form burnt spots.

¹H-NMR spectrum

¹H-NMR spectra were attained by using Avance III HD 500 MHz NMR spectrometer (Bruker, Switzerland). Free questin, HP- β -CD and QHIC were dissolved in deuterated methanol (CD₃OD). The chemical shift (δ) was expressed as ppm, and tetramethylsilane (TMS) was used as the internal standard.

Antibacterial activity assay

The antagonistic activities towards *V. harveyi* of questin and QHIC were evaluated by the agar diffusion assay according to the reference (Guo et al. 2017, 2019). Questin was dissolved in methanol, and QHIC and HP- β -CD were dissolved in sterilized distilled water. Three samples were diluted by the double dilution method to the same molar concentration. Then, 200 μ L of different concentrations of questin and QHIC were added in agar diffusions and cultivated at 37 °C for 12 h. The lowest concentration of samples capable of producing the zone of inhibition was defined as the minimum inhibitory concentration (MIC).

Results and discussion

Solubility

Within the range of 350–600 nm, the maximal absorption peak of the QHIC was at 425 nm, similar to that of questin. However, HP- β -CD, methanol, and distilled water were not absorbed at 425 nm. This observation indicates that the maximum absorption wavelength of questin did not deviate, and it did not interfere with the determination of questin. Therefore, 425 nm was chosen as the detection wavelength for questin. The absorbance of questin at different

concentrations was measured by a Synergy HT absorbance microplate reader, and the linear relationship between the absorbance value and questin concentration was obtained. The following linear regression equation was obtained: $Y = 0.0149X + 0.0442$ ($R^2 = 0.9998$), where, Y is the absorbance at 425 nm and X is the concentration of questin.

Figure 1c presents the images of HP- β -CD, QHIC, questin, and QHPM in liquid states, respectively. As presented in Fig. 1c, QHIC and HP- β -CD are water soluble, while questin and QHPM are partially soluble in water. These results indicate that questin was included into HP- β -CD, and hence, questin's water solubility was improved considerably. In Table 1, the solubility of questin in QHIC is (62.63 ± 1.21) μ g/mL, which is 110 times greater than that of questin without inclusion (0.57 ± 0.10 μ g/mL).

TLC analysis

TLC is an adsorption chromatography adopted to isolate the mixtures. Given the different appeals to the fixed phase and the solubility in the mobile phase, different compounds move at different migration rates. Thus by changing the solvent, the constituents in the sample mixture can be separated. Figure 2 shows the TLC results of HP- β -CD, QHIC, questin and QHPM under UV light and sulfuric acid/heat treatment.

As displayed in Fig. 2a, no spot was noted in the UV light because HP- β -CD does not possess a fluorescent group. However, QHIC, questin, and QHPM possess fluorescent spots that are derived from questin. Questin and QHPM migrated at similar rates that are much faster than that of the QHIC. The analogical migration rates of questin and QHPM indicated that the questin in QHPM is not clathrated by HP- β -CD. The different transmission ratios of questin with QHIC revealed that questin is involved in the HP- β -CD cavity. The interactions between questin and the fixed phase and developer in the QHIC were different from that of the unpacked questin.

Figure 2b displays that after sulfuric acid and heat treatment, HP- β -CD became visible and formed a carbonized imprint. The physical mixture displayed two points: one was questin, and the other was HP- β -CD. HP- β -CD had a lower migration rate than that of questin because of the former's greater molecular polarity. QHIC

Table 1 Solubility of questin, QHPM, and QHIC in distilled water at 37 °C

Sample	Solubility ($X \pm SD$, $n = 3$, μ g/mL)	Folds
Questin	0.57 ± 0.10	
QHPM	1.84 ± 0.17	3
QHIC	62.63 ± 1.21	110

Fig. 2 Thin-layer chromatography results of HP- β -CD, QHIC, questin and QHPM: **a** under an ultraviolet light and **b** after treatment with sulfuric acid and heat

also showed two spots, which were produced by the mixing solvents to decompose QHIC during development. The HP- β -CD in the QHIC displayed a lower propagation rate than those of pure HP- β -CD and QHPM. This result proved that the questin in the QHIC is contained by HP- β -CD, which slows down the migration rate due to increased polarity. The questin in the QHIC complex had a lower travel rate than that of the uncontained questin, similar to that in TLC under UV light. These results are consistent with reports (Hsu et al. 2013, 2014).

^1H -NMR spectral analysis

^1H -NMR spectral analysis can provide some powerful evidence for determining the chemical components of organic compounds (Hsu et al. 2013; Qiu et al. 2016). Figure 3a shows the ^1H -NMR spectrum of HP- β -CD, the chemical shifts of hydrogen atoms from HP- β -CD were in the range of 3.43–5.00 ppm according to previous literature (Table 2) (Kim et al. 2004). The ^1H -NMR spectrum of questin is shown in Fig. 3b, the chemical displacements within the range of 6.75–7.75 ppm indicated that questin contains an aromatic skeleton (Table 2) (Wang et al. 2013). Figure 3c

Fig. 3 Nuclear magnetic resonance (^1H -NMR) spectrum of **a** HP- β -CD, **b** questin, and **c** QHIC

Table 2 Chemical shift (δ , ppm) change values relating to the signals of HP- β -CD and questin in the free and the complexed states

Protons	δ (ppm)	
	HP- β -CD	QHIC
H-1	4.993	4.997
H-2	3.533	3.547
H-3	3.983	3.993
H-4	3.430	3.443
H-5	3.851	3.850
H-6	3.750	3.746
Protons	Questin	
	Questin	QHIC
H-2	7.275	7.306
H-4	7.522	7.539
H-5	7.087	7.106
H-7	6.750	6.857

shows the ^1H -NMR spectrum of QHIC. As can be seen from Fig. 3c and Table 2, QHIC was composed of questin and HP- β -CD compared with the ^1H -NMR spectra of questin and HP- β -CD.

Antibacterial activity

The antagonistic activities of questin and QHIC against *V. harveyi* were appraised by the agar diffusion assay. Questin and QHIC had antagonistic activities against *V. harveyi* with the MIC values of 0.10 μM and 0.05 μM , respectively. Meanwhile, HP- β -CD exhibited no bacteriostatic activity, which indicates that the formation of QHIC enhanced the antagonistic activity of questin towards *V. harveyi*.

Conclusion

In conclusion, questin was successfully complexed with HP- β -CD by stirring and coevaporation to form an inclusion complex. The QHIC manifested a higher aqueous solubility than that of questin without inclusion, that is, 110 times that of questin's original solubility. TLC and ^1H -NMR spectral results indicated the successful inclusion of questin into HP- β -CD. Meanwhile, the QHIC exhibited enhanced antibacterial activity against *V. harveyi* compared with free questin. This finding provided a reliable basis for the further application of questin as aquatic antibacterial agent.

Acknowledgements This work financially supported by the Priority Academic Program Development of Jiangsu Higher Education Institutions (CXKT20180216), Postgraduate Research & Practice Innovation Program of Jiangsu Province (SJCX18_0942), Natural Science Foundation of Jiangsu Province (BK20151283), Technical Plan Project of Lianyungang (CG1612) and 521 Talented Project of Lianyungang (KKC17001).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards This article does not contain any studies with human participants or animals performed by any of the authors.

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