International Journal of Biological Macromolecules 112 (2018) 405–412

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: https://www.journals.elsevier.com/ijbiomac

# Mechanistic insights into the inhibition of quercetin on xanthine oxidase

Cen Zhang [a,1], Rui Wang [a,1], Guowen Zhang [a,*], Deming Gong [a,b]

[a] State Key Laboratory of Food Science and Technology, Nanchang University, Nanchang 330047, China
[b] New Zealand Institute of Natural Medicine Research, 8 Ha Crescent, Auckland 2104, New Zealand

ARTICLE INFO

*Article history:*
Received 21 August 2017
Received in revised form 26 January 2018
Accepted 29 January 2018
Available online 31 January 2018

*Keywords:*
Quercetin
Xanthine oxidase
Superoxide anion

ABSTRACT

Quercetin, one of the most abundant flavonoid in the daily diet, was found to reversibly inhibit the generation of uric acid and superoxide radicals ($O_2^-$)catalyzed by xanthine oxidase (XOD) in a mixed-type manner with $IC_{50}$ values of $(2.74 \pm 0.04) \times 10^{-6}$ and $(2.90 \pm 0.03) \times 10^{-6}$ mol $L^{-1}$, respectively, and the inhibition of quercetin on $O_2^-$ generation may be ascribed to the reduced form of XOD by a ping-pong mechanism. XOD had one high affinity binding site for quercetin with a binding constant of $4.28 \times 10^4$ L $mol^{-1}$ at 298 K, and the binding process was predominately driven by van der Waals forces and hydrogen bonds on account of the negative enthalpy and entropy changes. Moreover, molecular docking confirmed that the binding site for quercetin located in the isoalloxazine ring of the flavin adenine dinucleotide (FAD) domain of XOD, then the diffusion of $O_2^-$ out of the FAD site was blocked in favor of another electron transferred from $FADH_2$ to $O_2^-$ to form hydrogen peroxide ($H_2O_2$). This study may clarify the role of quercetin on inhibiting XOD catalysis and provide a potential nutritional supplement for preventing gout and peroxidative damage.



## 1. Introduction

Xanthine oxidase (XOD) is a homodimer with a molecular mass of 290 kDa. The two monomers have symmetry related domains, and each of them contains a C-terminal molybdopterin (Mo) domain including four redox centers, a central flavin adenine dinucleotide (FAD) cofactor and an N-terminal domain with two iron sulfur centers [1]. The catalysis of XOD on xanthine and oxygen is a double substrates enzymatic reaction. When the oxidation of xanthine takes place at the Mo center of XOD, the reduction of the substrate oxygen occurs at the FAD center with transferred electrons, generating superoxide anion ($O_2^-$) or hydrogen peroxide ($H_2O_2$) [2]. The hyperuricemia caused by overproduction or underexcretion of uric acid is a key contributor to gout [3], and excessive amounts of $O_2^-$ result in many pathological processes including inflammation, hypertension and atherosclerosis [4]. Although therapeutic agent allopurinol has been identified as effective treatments for gout and other disorders in clinic, side effects of these traditional agents, such as bone marrow depression, allergic reactions, renal and gastrointestinal toxicities, should not be overlooked [5]. Hence, the exploitation of efficient and less toxic XOD inhibitors is necessary for nutraceutical and pharmaceutical applications.

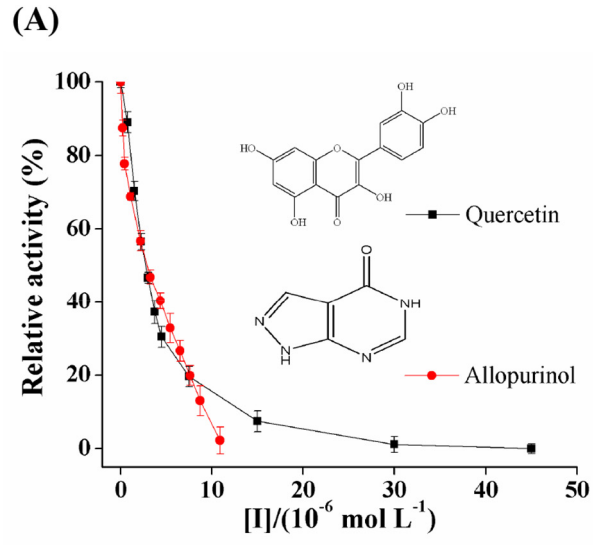
Flavonoid compounds, a group of natural products, are abundant in various foods and plants such as vegetables, fruits and tea [6], many of which have been investigated as potential XOD inhibitors in recent years. Dong et al. reported that pinobanksin and galangin inhibited XOD activity with respect to xanthine in a dose-dependent manner with $IC_{50}$ values of $1.37 \times 10^{-4}$ and $1.63 \times 10^{-4}$ mol $L^{-1}$, respectively [7]. Souza et al. found that the flavonoid hesperetin was a stronger competitive XOD inhibitor with an $IC_{50}$ value of $5.30 \times 10^{-5}$ mol $L^{-1}$ compared to the glycosylate derivatives, and the inhibition constant ($K_i$) value was $1.73 \times 10^{-5}$ mol $L^{-1}$ [8]. Moreover, baicalein was found to inhibit the formation of uric acid with an $IC_{50}$ value of $(2.79 \pm 0.01) \times 10^{-6}$ mol $L^{-1}$ and the generation of $O_2^-$ with catalysis by XOD with an $IC_{50}$ value of $(2.72 \pm 0.02) \times 10^{-6}$ mol $L^{-1}$ [9].

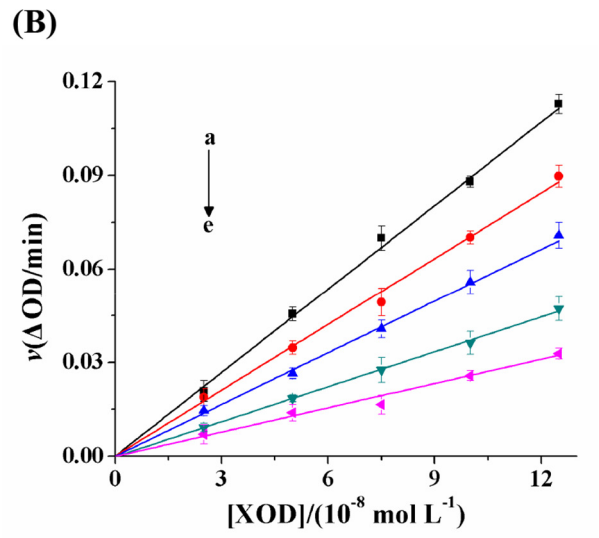
Quercetin (structure shown in Fig. 1A) is a major flavonol in fruits and vegetables, such as apples, grapes, berries, red onions, broccoli, etc. Quercetin is commonly used as an effective antioxidant agent, and other bioactivities of quercetin include anticancer, anti-inflammatory activities and prevention of retinal degeneration [10,11]. Although some research into the inhibition of quercetin on XOD activity has already been done [9], the lack of understanding of the underlying mechanism between quercetin and XOD has seriously limited the application of quercetin and affected the discovery of new natural XOD inhibitors. Moreover, it is very interesting and significant to reveal the inner relation between inhibiting uric acid formation and $O_2^-$ generation by quercetin during enzymatic reaction.

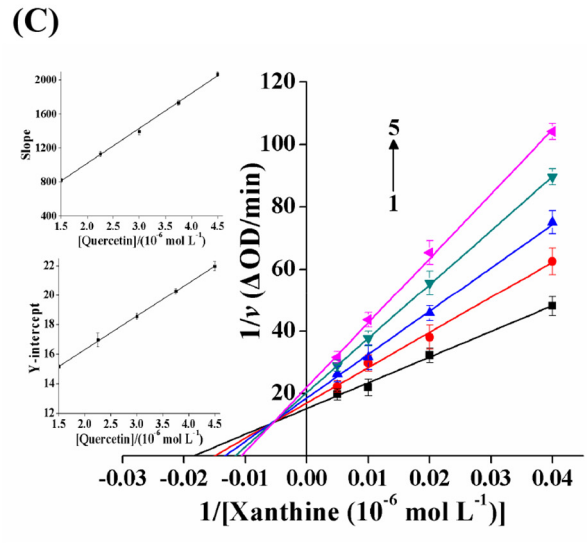
The aim of the current study was to explore the two-substrate kinetic and inhibitory mechanism of quercetin on XOD by a combination of kinetic analysis, molecular modeling technique and multispectroscopic methods including UV–vis absorption, fluorescence and circular dichroism (CD) spectroscopy. In addition, the binding

* Corresponding author.
*E-mail address:* gwzhang@ncu.edu.cn (G. Zhang).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.ijbiomac.2018.01.190
0141-8130/




characteristics and the conformational changes of XOD induced by quercetin were evaluated.

## 2. Materials and methods

### 2.1. Materials

XOD (Grade I, approximately 10.4 units $mL^{-1}$) from bovine milk and xanthine were purchased from Solarbio Co. (Beijing, China). The stock solutions of XOD ($5.0 \times 10^{-6}$ mol $L^{-1}$) and xanthine ($5.0 \times 10^{-4}$ mol $L^{-1}$) were prepared in 0.05 mol $L^{-1}$ Tris—HCl buffer (pH 7.4). Quercetin (analytical grade) obtained from Aladdin Industrial Co. (Shanghai, China) was dissolved in absolute ethanol as stock solution ($6.0 \times 10^{-3}$ mol $L^{-1}$), and then diluted to different concentrations with Tris—HCl buffer. Both nitroblue tetrazolium (NBT) and phenazine methosulfate (PMS) were purchased from Sinopharm Chemical Reagent Co. (Shanghai, China), and β-NADH (NADH) was supplied by Aladdin Chemistry Co. (Shanghai, China). 2,2 Diphenyl 1 picrylhydrazyl (DPPH) provided by Sigma–Aldrich Co. (St. Louis, MO) was dissolved in ethanol to the concentration of $3.0 \times 10^{-3}$ mol $L^{-1}$. All other reagents and solvents were of analytical reagent grade, and the ultrapure water was used throughout the experiments.

### 2.2. Assay of uric acid generated by XOD

A UV–vis spectrophotometer (Shimadzu UV–2450, Japan) equipped with a 1.0 cm path length cell was used to measure the inhibitory activity of quercetin against XOD at room temperature. An array of reaction mixture was prepared by mixing XOD (final concentration $7.5 \times 10^{-8}$ mol $L^{-1}$), Tris—HCl buffer and various amounts of quercetin. After 30 min incubation at 37 °C, XOD activity was determined by monitoring the formation of uric acid at a wavelength of 290 nm with xanthine as the substrate [12]. Allopurinol, a clinically used XOD inhibitor, was used as a positive control.

### 2.3. Analysis of inhibitory kinetics

The mixed-type inhibition was analyzed by Lineweaver–Burk plots and described by the following equations [13]:

$$\frac{1}{v} = \frac{K_m}{V_{max}}\left(1 + \frac{[I]}{K_i}\right)\frac{1}{[S]} + \frac{1}{V_{max}}\left(1 + \frac{[I]}{\alpha K_i}\right) \quad (1)$$

Secondary plot can be obtained from:

$$\text{Slope} = \frac{K_m}{V_{max}} + \frac{K_m[I]}{V_{max}K_i} \quad (2)$$

and

$$\text{Y-intercept} = \frac{1}{V_{max}^{app}} = \frac{1}{V_{max}} + \frac{1}{\alpha K_i V_{max}}[I] \quad (3)$$

where $K_m$ and $K_i$ are the Michaelis–Menten constant and inhibition constant, respectively. The enzyme reaction rate is expressed as $v$ in the absence and presence of quercetin. [I] and [S] stand for the concentrations of inhibitor and substrate, respectively. α is the apparent coefficient. If the secondary plot of slope and Y-intercept versus [I] were linearly

**Fig. 1.** (A) XOD activity in the absence and presence of quercetin and allopurinol at different concentrations (pH 7.4, $T = 298$ K). $c$(XOD) $= 7.5 \times 10^{-8}$ mol $L^{-1}$, and $c$(xanthine) $= 5.0 \times 10^{-5}$ mol $L^{-1}$. (B) Plots of $v$ vs. [XOD]. $c$(xanthine) $= 5.0 \times 10^{-5}$ mol $L^{-1}$, and $c$(quercetin) $= 0$, 0.75, 1.5, 2.25 and $3.75 \times 10^{-6}$ mol $L^{-1}$ for curves 1 → 5, respectively. (C) Lineweaver–Burk plots. $c$(XOD) $= 7.5 \times 10^{-8}$ mol $L^{-1}$, and $c$(quercetin) $= 1.5$, 2.25, 3.0, 3.75 and $4.5 \times 10^{-6}$ mol $L^{-1}$ for curves 1 → 5, respectively. The secondary plot represented slope and Y-intercept vs. [quercetin] in the insert, respectively.