

## KINETICS OF QUERCETIN NITRATION BY HORSERADISH PEROXIDASE

Andrija Šmelcerović<sup>1</sup>, Nataša Sokolov<sup>1</sup>, Živomir Petronijević<sup>2</sup>

In this study we investigated the kinetics of the nitration of quercetin by horseradish peroxidase. Quercetin nitration reaction was followed by recording the spectral changes over the time at 380 nm. The reaction rate increases with increasing of the quercetin concentration and follows the Michaelis-Menten type kinetics. Kinetic parameters of the studied enzymatic reaction were determined. *Acta Medica Medianae* 2013;52(2):5-9.

**Key words:** quercetin, nitration, horseradish peroxidase

University of Niš, Faculty of Medicine, Niš, Serbia<sup>1</sup>  
University of Niš, Faculty of Technology, Leskovac, Serbia<sup>2</sup>

Contact: Andrija Šmelcerović  
Department of Chemistry, Faculty of Medicine  
University of Niš, Bulevar Dr Zorana Đinđića 81  
18000 Niš, Serbia  
E-mail: a.smelcerovic@yahoo.com

## Introduction

Flavonoids are the major class of plant polyphenolics which comprise several thousand compounds sharing a common phenylchromane skeleton. This basic structure allows a variety of substitution patterns leading to different flavonoid subclasses, such as flavonols, flavones, flavanones, flavanols, anthocyanins, dihydroflavonols, isoflavones and chalcones (1). Flavonoids are extensively distributed in the medicinal plants, vegetables, fruit juices, and a variety of beverages (tea, coffee, wines, and fruit drinks) (2). Biological effects of flavonoids are linked to their potential cytotoxicity and their capacity to interact with enzymes through protein complexation. Furthermore, flavonoids act as scavengers of free radicals (3). It has been reported that consuming food which contains flavonols is related to the decrease in risks to arteriosclerosis (4). They also have antimicrobial properties (3) and might have some antidepressant activity (5). Havsteen (6) published a detailed review on the biochemistry and medical significance of the flavonoids.

Flavonoids, and particularly quercetin derivatives, have received more attention as dietary constituents during the last few years (2). Quercetin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxychromen-4-one, Figure 1) is one of the major polyphenols in many plant foods (1). He has shown to be an effective inhibitor of xanthine oxidase and lipoxygenase, the enzymes involved in processes such as inflammation, atherosclerosis, cancer and ageing (7, 8).

Enzymatic methods have become increasingly important in chemical synthesis in the past three decades. Crude and purified enzymes have been used on both laboratory and industrial scale to synthesize value-added compounds which may be difficult to produce using traditional chemical

methods. The field of enzymatic synthesis is currently dominated by the use of hydrolases such as lipases, proteases, and glycosidases, mainly due to their ease of use, high stability and activity under synthetic conditions, and their independence from expensive cofactors. The use of oxidative enzymes in synthesis, however, has been more challenging because these enzymes are often less stable and typically require cofactors. Nonetheless, interest in developing processes using oxidative enzymes is increasing (9). The oxidation of tyrosinase on quercetin is well described (10). Nitrate is secreted into the oral cavity as a salivary component. Bacteria in the cavity can reduce nitrate to nitrite. On the other hand, vegetables and fruits contain quercetin glycosides. When foods that contain quercetin glycosides are ingested, the glycosides are hydrolyzed to quercetin, and the quercetin can be oxidized by peroxidase-dependent reactions in the oral cavity (4, 11). Budde et al. (9) have shown that soybean peroxidase is an effective catalyst for the nitration of phenols in the presence of various organic co-solvents at concentrations up to 40% (v/v). The mild reaction conditions (ambient temperatures and neutral pH) make this approach the most appropriate for complex compounds (both natural and synthetic) with limited aqueous solubility and with labile functional groups.

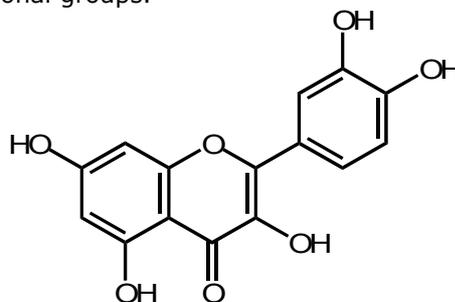


Figure 1. Chemical structure of quercetin

The aim of this work was to investigate the nitration of quercetin by horseradish peroxidase. Kinetic parameters of the studied enzymatic reaction were determined.

## Material and methods

### Chemicals

Horseradish peroxidase (298 U/mg) and quercetin were purchased from Sigma. Hydrogen peroxide, dimethyl sulfoxide (DMSO) and sodium nitrite (Alkaloid) were p.a. quality.

### Spectrophotometric assays

Kinetic assays were carried out by measuring the appearance of the product in the reaction medium on VARIAN Cary-100 Spectrophotometer and controlled by using a VARIAN Cary-100 UV-Winlab software. Firstly, the reaction mixture was scanned at wavelengths of 200-800 nm, and then the quercetin nitration reaction was followed by recording of spectral changes over the time at 380 nm.

Enzymatic reaction was performed in such a way that phosphate buffer and water were always first added in a tube. Then, sodium nitrite solution and quercetin solution were added. Afterwards, the solution of horseradish peroxidase was added and finally the reaction began by adding solution of hydrogen peroxide.

The reaction medium contained 2500  $\mu\text{l}$  of 50 mM phosphate buffer pH 7, about 2000  $\mu\text{l}$  of distilled water, 500  $\mu\text{l}$  of 50 mM sodium nitrite solution, 30, 80 or 120  $\mu\text{l}$  of 10 mM solution of quercetin, 25  $\mu\text{l}$  of peroxidase solution (0.34 mg of peroxidase dissolved in 50 ml of cold phosphate buffer), and 100  $\mu\text{l}$  of 10 mM solution of hydrogen peroxide.

Determination of kinetic parameters of enzymatic reaction

Kinetic parameters (Michaelis constant,  $K_m$ , and maximal rate,  $V_{max}$ ) were determined from the graphic of linear Michaelis-Menten equation. Catalytic constant,  $k_{+2}$ , represents the number of

moles of the product formed per time unit with one mole of pure enzyme, which is saturated with substrate. The efficiency of the enzyme activity was calculated as the ratio  $k_{+2}/K_m$ .

## Results and discussion

The progress of the studied enzymatic reaction at quercetin concentrations of 0.06 mM, 0.16 mM and 0.24 mM are given in Figures 2-4, respectively. In all studied reactions a decrease of absorbance at 380 nm in the function of reaction time was observed, which indicates a decrease in the concentration of quercetin in the mixture due to the occurrence of the nitration reaction products.

The slopes of the graphics obtained (Figures 2-4) are directly proportional to the rates of enzymatic reaction at different quercetin concentrations. On the basis of the quercetin nitration rate dependence upon its concentration (Figure 5), it can be concluded that reaction rate increases with increasing of the substrate concentration and follows Michaelis-Menten type kinetics.

From the Lineweaver-Burk plot of enzymatic nitration at different quercetin concentrations (Figure 6) the kinetic constants  $K_m$  (0.0833 mM) and  $V_{max}$  (0.02629  $\Delta A_{380}/\text{mM}$ ) were obtained. The ratio of the maximum reaction rate and Michaelis's constant ( $V_{max}/K_m$ ) was 0.3156 mM  $\Delta A_{380}/\text{min}$  mM. The value of the catalytic constant,  $k_{+2}$ , is calculated as the ratio of the maximum rate and enzyme concentration, amounting 0.0011  $\Delta A_{380}/\text{min}$  nM. The efficiency of the enzyme activity was calculated on the basis of the values of  $k_{+2}$  and  $K_m$ , amounting 0.01356 min/mM nM.

The subject of our further study will be the identification of the products of enzymatic modification as well as testing of their pharmacological activities.

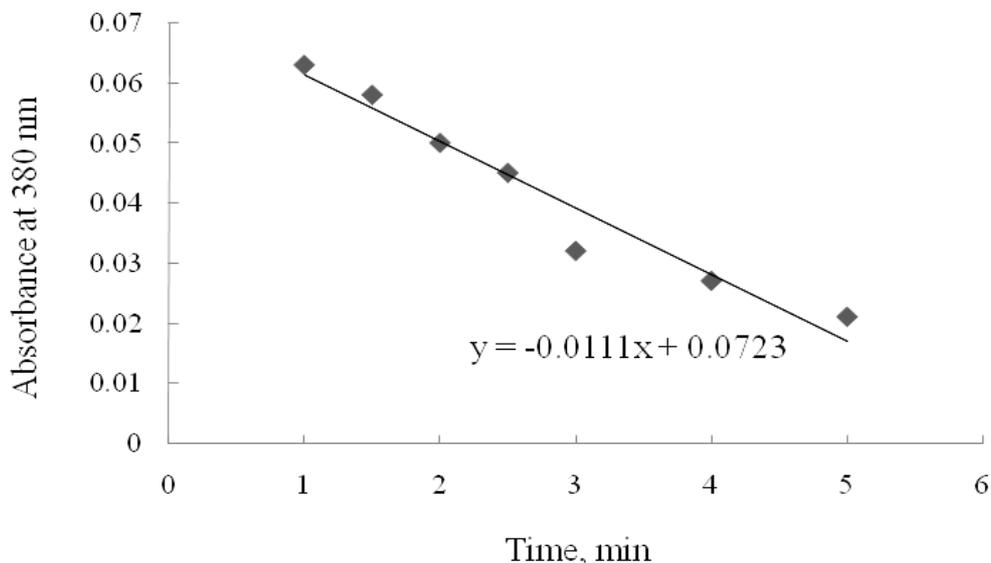


Figure 2. Dependence of absorbance in the function of reaction time at quercetin concentration of 0.06 mM

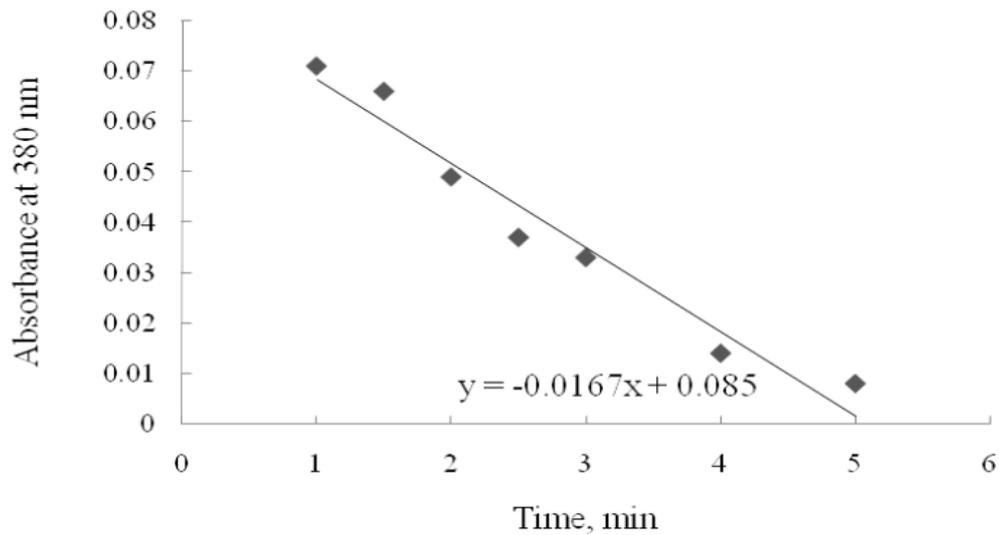


Figure 3. Dependence of absorbance in the function of reaction time at quercetin concentration of 0.16 mM

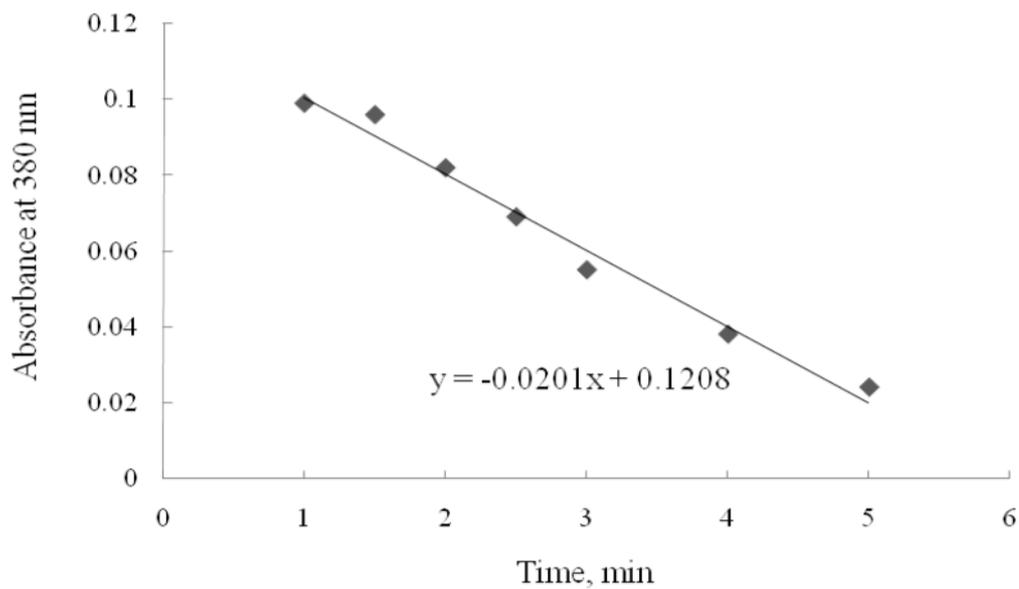


Figure 4. Dependence of absorbance in the function of reaction time at quercetin concentration of 0.24 mM

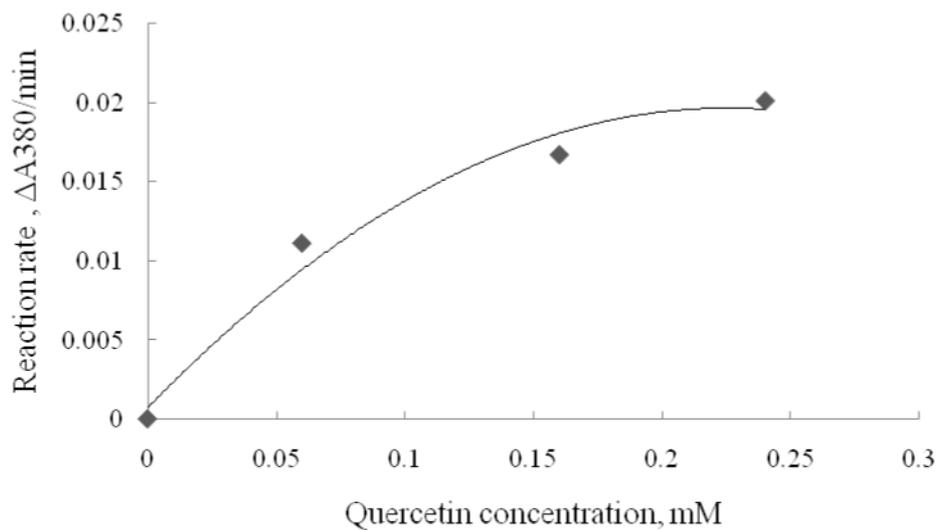


Figure 5. Dependence of the rate of quercetin nitration in the function of substrate concentration

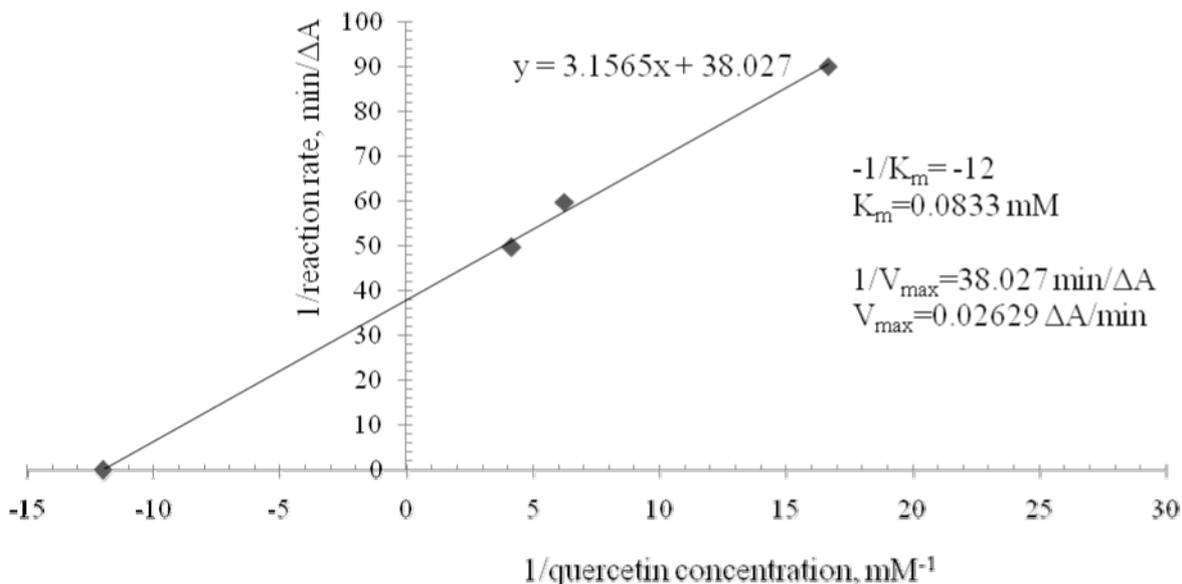


Figure 6. Lineweaver–Burk plot of quercetin nitration by horseradish peroxidase

### Conclusions

The kinetics of nitration of quercetin by horseradish peroxidase was studied. Based on the obtained results, the following conclusions can be drawn:

- under the conditions described in this paper the enzymatic nitration of quercetin was carried out; reducing the concentration of quercetin in the mixture indicates the formation of products;
- quercetin nitration was followed by recording of spectral changes over the time at 380 nm;
- the reaction rate increases with increasing of the quercetin concentration and follows Michaelis-

Menten type kinetics;

- kinetic constants  $K_m$  and  $V_{max}$  amounted to 0.0833 mM and 0.02629 ΔA/mM, respectively;
- efficiency of the enzyme activity amounted to 0.01356 min/mM nM;
- to the best of our knowledge, this is the first report on the nitration of quercetin by horseradish peroxidase.

### Acknowledgements

The financial support of this work by Ministry of Science and Technological Development of Serbia (Project OI 172044) is gratefully acknowledged.

## References

1. Dueñas M, González-Manzano S, González-Paramás A, Santos-Buelga C. Antioxidant evaluation of *O*-methylated metabolites of catechin, epicatechin and quercetin. *Journal of Pharmaceutical and Biomedical Analysis*. 2010; 51: 443-9. [[CrossRef](#)]
2. Dehghan G, Dolatabadi JEN, Jouyban A, Zeynali KA, Ahmadi SM, Kashanian S. Spectroscopic studies on the interaction of quercetin-terbium (III) complex with calf thymus DNA. *DNA and Cell Biology*. 2011; 30: 195-201. [[CrossRef](#)]
3. Pourcel L, Routaboul J, Cheynier V, Lepiniec L, Debeaujon I. Flavonoid oxidation in plants: from biochemical properties to physiological functions. *Trends in Plant Science*. 2007; 12: 29-36. [[CrossRef](#)]
4. Hirota S, Nishioka T, Shimoda T, Miura K, Ansai T, Takahama U. Quercetin glucosides are hydrolyzed to quercetin in human oral cavity to participate in peroxidase-dependent scavenging of hydrogen peroxide. *Food Science and Technology Research*. 2001; 7: 239-45. [[CrossRef](#)]
5. Butterwerck V, Juergeniemi G, Nahrstedt A, Winterhoff H. Flavonoids from *Hypericum perforatum* show antidepressant activity in the forced swimming test. *Planta Medica*. 2000; 66: 3-6. [[CrossRef](#)]
6. Havsteen BH. The biochemistry and medical significance of the flavonoids. *Pharmacology & Therapeutics*. 2002; 96: 67-202. [[CrossRef](#)]
7. Cos P, Ying L, Calomme M, Hu JP, Cimanga K, Van Poel B, et al. Structure-activity relationship and classification of flavonoids as inhibitors of xanthine oxidase and superoxide scavengers. *Journal of Natural Products*. 1998; 61: 71-6. [[CrossRef](#)]
8. Da Silva EL, Tsushida T, Terao J. Inhibition of mammalian 15-lipoxygenase-dependent lipid peroxidation in low-density lipoprotein by quercetin and quercetin monoglucosides. *Archives of Biochemistry and Biophysics*. 1998; 349: 313-20. [[CrossRef](#)]
9. Budde CL, Beyer A, Munir IZ, Dordick JS, Khmelnsky YL. Enzymatic nitration of phenols. *Journal of Molecular Catalysis B: Enzymatic*. 2001; 15: 55-64. [[CrossRef](#)]
10. Fenoll LG, Garcia-Ruiz PA, Varon R, Garcia-Canovas F. Kinetic study of the oxidation of quercetin by mushroom tyrosinase. *Journal of Agricultural and Food Chemistry*. 2003; 51: 7781-7. [[CrossRef](#)]
11. Hirota S, Takahama U, Ly TN, Yamauchi R. Quercetin-dependent inhibition of nitration induced by peroxidase/H<sub>2</sub>O<sub>2</sub>/nitrite systems in human saliva and characterization of an oxidation product of quercetin formed during the inhibition. *Journal of Agricultural and Food Chemistry*. 2005; 53: 3265-72. [[CrossRef](#)]

## KINETIKA NITROVANJA KVERCETINA PEROKSIDAZOM IZ RENA

*Andrija Šmelcerović, Nataša Sokolov, Živomir Petronijević*

U ovom radu je ispitivana kinetika nitrovanja kvercetina peroksidazom iz rena. Tok reakcije nitrovanja kvercetina praćen je spektrofotometrijski na 380 nm. Brzina reakcije se povećava sa povećanjem koncentracije kvercetina i sledi Mihaelis-Mentenovu kinetiku. Određeni su kinetički parametri ispitivane enzimske reakcije. *Acta Medica Medianae* 2013;52(2):5-9.

**Ključne reči:** kvercetin, nitrovanje, peroksidaza iz rena