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Inhibitory activity of flavonols towards the xanthine oxidase enzyme

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Summary

The spectrophotometric methods of analysis adopted for the study of the inhibition of the enzyme, xanthine oxidase, are very different as regards the time required for the assay. With a view to studying the inhibitory effect of flavonoids, we examined the stability and the inhibitory activity of quercetin and myricetin in relation to environment and time. The results show that, in a buffer solution of pH 7.6 at 20°C, these flavonols undergo transformation in accordance with the environment and that their transformation products have a lower inhibitory capacity towards xanthine oxidase. The method that assesses the initial rate of formation of uric acid is therefore that which affords the most reliable results regarding the activity of flavonols; accordingly, this was the method used to determine the kinetic parameters of the enzymatic inhibition exerted by quercetin and myricetin.

Introduction

Flavonoids are naturally occurring substances possessing various pharmacological properties and are able, amongst other things, to inhibit the enzyme xanthine oxidase to a greater or lesser degree (Hayashi et al., 1988). This enzyme, which in vivo exists predominantly as an NAD^+-dependent oxidase, can be transformed during tissue ischemia to an oxygen-dependent oxidase, and the activity of this form of the enzyme at the moment of reperfusion produces oxygen-free radicals which cause severe tissue damage (Jones and McCord, 1987).

The spectrophotometric methods of analysis used for the study of the in vitro activity of xanthine oxidase are based on the amount of uric acid formed starting from xanthine or hypoxanthine at the wavelength of 295 nm. However, there are two different approaches: the first involves determining the rate of formation of uric acid during the initial phase of reaction (Bindoli et al., 1985); the second entails measuring the amount of uric acid formed after 45 min (15 min incubation of the reaction mixture without substrate followed by 30 min enzymatic reaction) (Noro et al., 1983). Both approaches have been

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used in the study of xanthine oxidase inhibition by flavonols with different results (Bindoli et al., 1985; Iio et al., 1985). Furthermore, it is known that the enzyme-inhibiting capacity of flavonoids may vary in accordance with the solvent used (Shimizu et al., 1984). Since we wished to obtain kinetic parameters that would serve for subsequent quantitative structure-activity relationships (QSAR), we studied: (a) the stability of the inhibitor in relation to environment and time and (b) the inhibitory action of the flavonols in question and of their transformation products.

Having estabilished these points, we then determined the type and kinetics of the inhibition exerted by quercetin and myricetin dissolved in methylsulfoxide (DMSO) and the inhibitory capacity of myricetin solubilized in DMSO, then placed in phosphate buffer.

Materials and Methods

Materials

Xanthine, xanthine oxidase (EC 1.2.3.2) (from buttermilk, 1.36 U/mg protein), quercetin and myricetin were purchased from Fluka. Quercetin and myricetin stock solutions were always prepared using DMSO (0.8×10^{-3} M). At the concentrations used (maximum 1% v/v) DMSO had no effect on xanthine oxidase activity. All the enzymatic assays were carried out in phosphate buffer, pH 7.6 (Normex Carlo Erba) at 20°C. All the spectrophotometric measurements were performed using a Perkin-Elmer UV/Vis λ 15 spectrophotometer with a thermostatted cuvette holder (Haake F3-C).

Transformation of quercetin and myricetin in phosphate buffer saturated with nitrogen, air or oxygen

Quercetin (17.64 μM final concentration) and myricetin (16.81 μM final concentration) were dissolved in DMSO and added to a phosphate buffer (pH 7.6) at 20°C saturated with nitrogen, air or oxygen, the cuvettes being hermetically sealed. Saturation was achieved by bubbling nitrogen, air or oxygen inside the cuvette containing buffer for at least 15 min. The transformation reaction of the flavonols was followed at their peak absorption maximum (378 nm for quercetin, 380 nm for myricetin).

Transformation of quercetin and myricetin in the presence of xanthine-xanthine oxidase

The transformation of quercetin (17.64 μM final) and myricetin (16.81 μM final) was observed through the variation of their absorption peaks (378 and 380 nm, respectively) in air-saturated phosphate buffer in the presence of xanthine (42.82 μM) and xanthine oxidase (0.038 U/ml).

Reaction of quercetin and myricetin with KO_2

A small grain of KO_2 was added to the reaction mixture (35.2 μM quercetin, 33.6 μM myricetin in phosphate buffer, pH 7.6) and the UV spectra were recorded after 5 min.

Variation in the inhibitory capacity of quercetin and myricetin in relation to the environment and incubation time

Nitrogen-saturated buffer A solution in phosphate buffer (pH 7.6, 20°C) containing quercetin or myricetin solved in DMSO and enzyme was kept under nitrogen (the buffer had previously been saturated with nitrogen for 20 min). At fixed intervals, an aliquot was taken and placed in the cuvette of a spectrophotometer where it was diluted 30 fold with aereated buffer (final concentration of quercetin or myricetin in the reaction mixture: 1.37 and 1.48 μM, respectively). The reaction was initiated by the addition of the substrate (xanthine: 42.82 μM) and followed at 295 nm (uric acid formation). The percent inhibition was then calculated by comparing the initial reaction rate of the solutions containing inhibitor with that of the control reaction without inhibitor.

Air-saturated buffer. A solution of quercetin or myricetin in DMSO and enzyme was placed in air-saturated phosphate buffer (pH 7.6, 20°C). At fixed intervals, an aliquot was taken and placed in the cuvette of the spectrophotometer (final concentration in the reaction mixture: 1.37 and 1.48 μM, respectively). The inhibitory capacity was then determined as above.

Incubation of myricetin alone (air-saturated buffer) Myricetin (1.43 μM final) in DMSO was

added to 3 ml of buffer and left to stand for 90 min. Xanthine and xanthine oxidase were then added and the procedure described above was repeated.

Xanthine oxidase activity assay

Xanthine oxidase activity was assayed spectrophotometrically at 20°C by determining the increase in uric acid concentration at 295 nm (Bindoli et al., 1985). Kinetic studies were performed with three concentrations (0.24, 0.49, 0.99 μM in the case of quercetin, 0.26, 0.52, 1.05 μM in the case of myricetin) of inhibitors, solved in DMSO. For these studies, the concentration of the substrate, xanthine, was varied (21.55, 10.77, 5.38, 4.04, 2.69 μM) while the inhibitor was held constant. The reaction was initiated by the addition of the enzyme and data collection began no more than 20 s after the addition of quercetin and myricetin. The nature of the inhibition produced by each concentration of inhibitor was determined by analysis of the plot of reciprocal

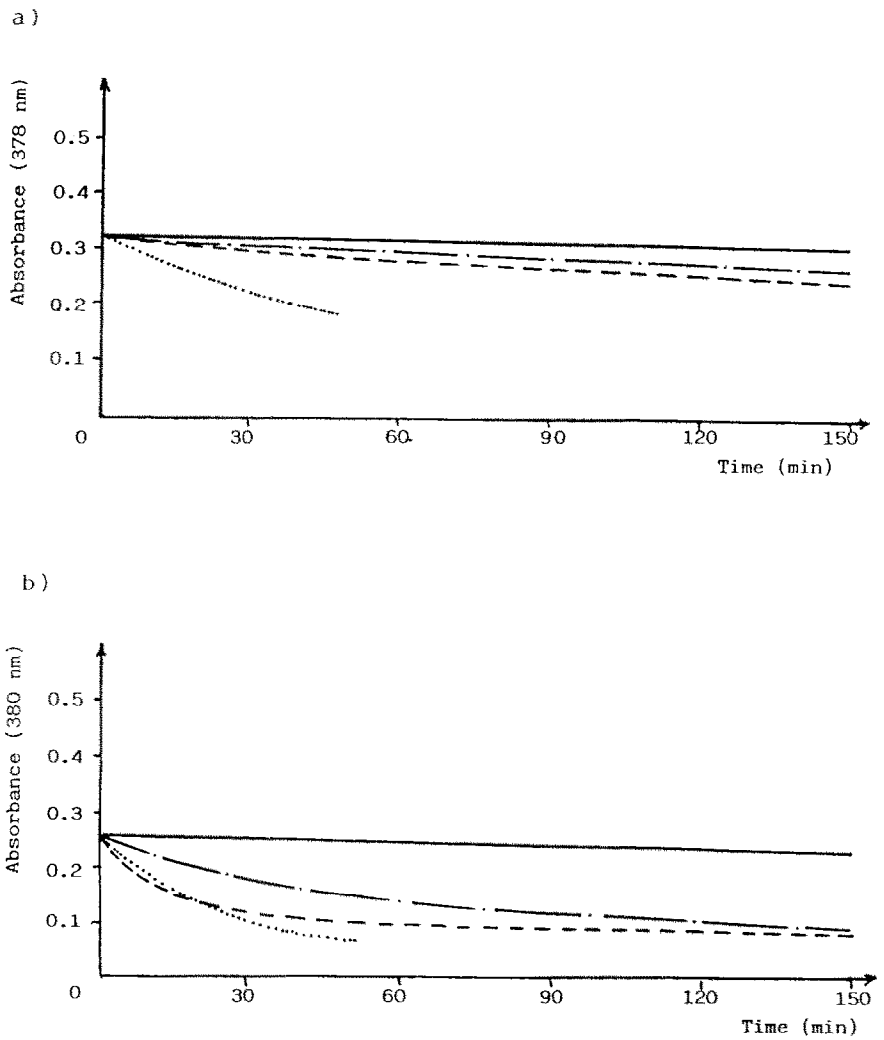

Fig. 1. Behaviour of quercetin and myricetin in the presence of nitrogen-, air- and oxygen-saturated phosphate buffer (pH 7.6, 20°C) or in the presence of xanthine oxidase activity. (a) Quercetin transformation (λ 378 nm) vs time; (b) myricetin transformation (λ 380 nm) vs time.

initial velocities vs reciprocal substrate concentrations with and without inhibitor present. These plots were generated by a least-squares fit to the data (Tallarida and Murray, 1987) and the effect of the inhibitors on K_m and V_{max} was verified. The absorbance values were converted to μmol by using $\epsilon = 9600\ M^{-1}\ cm^{-1}$ for uric acid formation.

K_{EI} and K_{EIS} (the dissociation constants of the enzyme-substrate complex and those of the enzyme-substrate-inhibitor complex) were determined from the slopes and intercepts in double-reciprocal plots.

Inhibition kinetics exerted by myricetin maintained in air-saturated phosphate buffer pH 7.6

5 ml of a solution of myricetin in DMSO were made up to 100 ml with phosphate buffer (pH 7.6) at room temperature and left to stand for 4 h. Various tests were then carried out with the inhibitor at a fixed concentration (5.48 and 21.89 μM expressed as myricetin), but varying the concentration of the substrate as described above.

Results and Discussion

In the case of myricetin, an earlier observation (Hodnick et al., 1986), namely, that this flavonol undergoes a transformation in a solution of phosphate buffer (pH 7.6, 20°C) saturated with air, was confirmed. The same phenomenon, albeit at a lower reaction rate, was observed in the case of quercetin (Fig. 1). Qualitatively, the reaction rate for both flavonols was faster in oxygen-saturated and slower in air-saturated buffer.

Similarly, quercetin (Takahama, 1983a) and myricetin were affected by KO_2, a substance which produces O_2^- in water (Takahama, 1983a), and by xanthine oxidase activity (Fig. 1). It is known that this enzyme is able to produce superoxide and hydroxyl radicals (Kuppusamy and Zweier, 1989). In the presence of air, oxygen, KO_2 or xanthine oxidase activity the respective absorption peaks of the flavonols at 378 and 380 nm disappeared and a new absorption peak appeared at about 330 nm with an isosbestic point at 350 nm. It has been suggested that this spectral behaviour may be due to the saturation of the double bond with the contemporaneous formation of a 2,3-diol (Hosel and Barz 1972; Takahama, 1983b, 1985).

The transformation products of the flavonols, on the other hand, had a diminished inhibitory capacity. This was borne out by the observation that when quercetin and myricetin were incubated in a nitrogen-saturated buffer, in which the flavonols are stable, their inhibitory capacity re-

TABLE 1

Time-dependent inhibition of xanthine oxidase enzyme during preincubation with quercetin (1.37 μM) or myricetin (1.48 μM) [in the presence (a) and absence (b) of enzyme] in nitrogen- or air-saturated buffer (pH 7.6)

	Incubation time (min)	% inhibition			
		Quercetin		Myricetin	
		N_2-saturated buffer	Air-saturated buffer	N_2-saturated buffer	Air-saturated buffer
(a)	0	63	61	79	70
	20	64	59	77	63
	40	64	58	77	51
	60	64	58	75	39
	80	61	56	74	31
	100	61	53	73	25
	120	60	52	74	22
(b)	0				72
	90				23

Control reaction (no inhibitor): 0.050 ΔA/min

mained uninpaired, whereas when the buffer was saturated with air this capacity diminished with time. In the case of quercetin, this phenomenon was barely perceptible; in that of myricetin, however, it was more marked (Table 1).

The degradation of myricetin in the reaction mixture did not appear to affect the intrinsic catalytic capacity of the enzyme; irrespective of whether the enzyme was present in the incubation solution, its remaining activity is virtually the same (Table 1).

Therefore, following the method whereby the initial rate at which uric acid forms is measured, we constructed Lineweaver-Burk plots for quercetin and myricetin (Figs 2 and 3) solved in DMSO and also for myricetin solubilized in DMSO and then placed in air-saturated buffer for 4 h before the assays (Fig. 3).

Table 2 sets out the K_m and V_{max} readings and also the K_{EI} and K_{EIS}, obtained by replotting the slopes and intercepts of the lines of the double-reciprocal plots as a function of the inhibitor concentration. When myricetin is kept in buffer at pH 7.6 for a certain period of time (4 h), the resultant inhibition constants K_{EI} and K_{EIS} are very different, of the order of 4 and 23 μM respectively, i.e., 2 orders of magnitude greater than those of the unchanged flavonol.

To conclude, the method whereby the rate at which uric acid forms in the first few minutes of reaction time is measured turns out to be the most reliable, for the concentration of the less active transformation products is as low as is possible and the inhibitory activity can be wholly attributed to the original flavonol. At the same time, these findings highlight the importance of

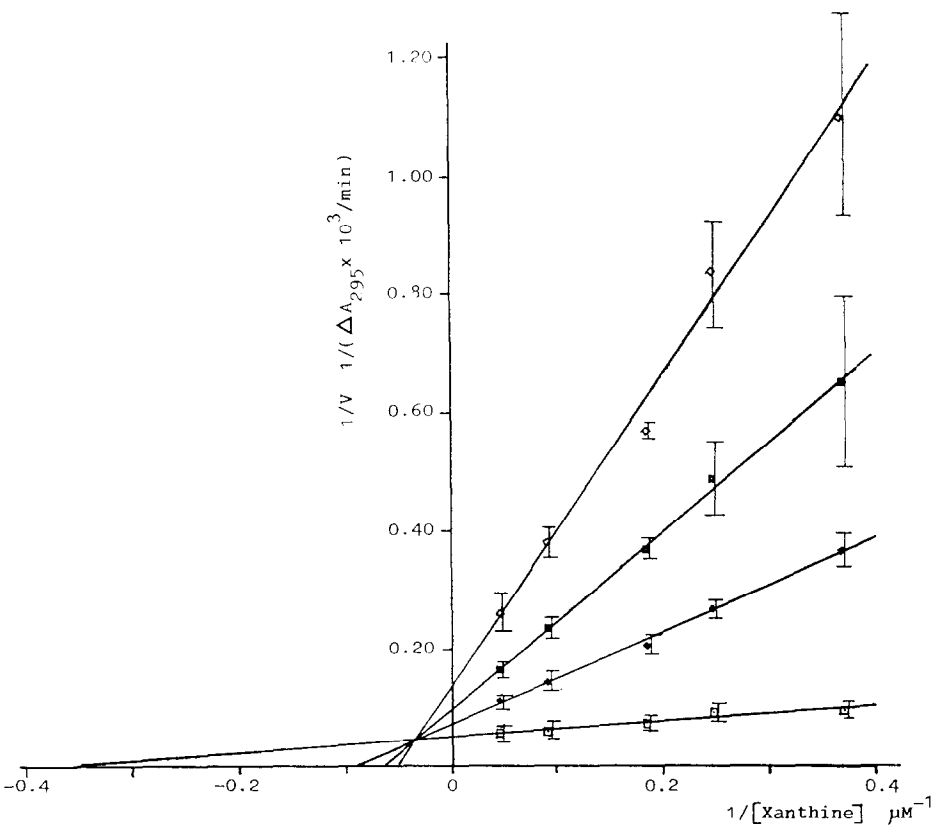

Fig. 2. Double-reciprocal plot of initial enzyme velocity vs concentration of substrate in the presence or absence of quercetin. (□) No inhibitor; (◆) 0.24 μM quercetin; (■) 0.49 μM quercetin; (◇) 0.99 μM quercetin. The bars represent 2 × S.D., $n = 3$.

TABLE 2

Kinetic data for quercetin and myricetin inhibition of xanthine oxidase

Additions	K_m ($\times 10^6$) (M)	V_m (μmol/min)	
None	2.18	2.07	
0.24 μM quercetin	11.31	1.48	
0.49 μM quercetin	15.82	1.08	
0.99 μM quercetin	20.22	0.79	
0.26 μM myricetin	17.74	2.04	
0.52 μM myricetin	20.19	1.10	
1.05 μM myricetin	23.65	0.71	
Flavonol	Mode of inhibition	K_{EI} (μM)	K_{EIS} (μM)
Quercetin	mixed type	0.074	0.612
Myricetin	mixed type	0.033	0.387

choosing an appropriate solvent with no effects on xanthine oxidase activity and in which the flavonols are stable, such as DMSO (Hodnick et al., 1986).

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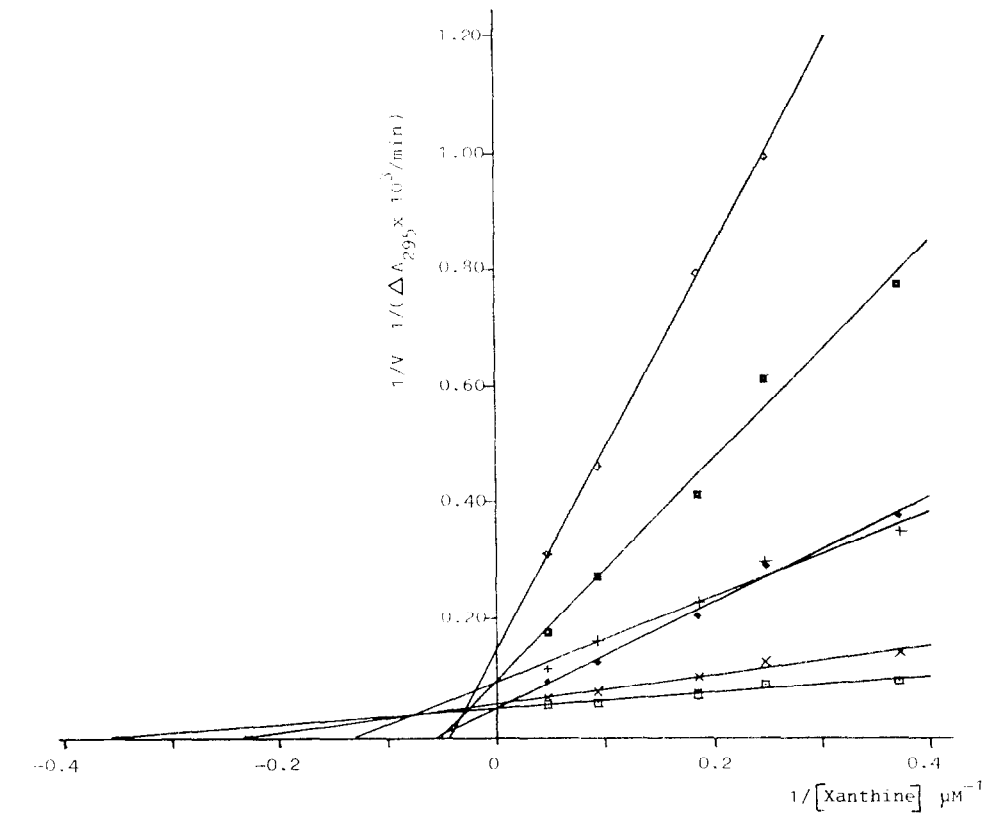

Fig. 3. Double-reciprocal plot of initial enzyme velocity vs concentration of substrate in the presence or absence of myricetin. (□) No inhibitor; (◆) 0.26 μM; (■) 0.52 μM; (◇) 1.05 μM; (×) 5.48 μM myricetin after 4 h incubation in phosphate buffer pH 7.6*; (+) 21.89 μM myricetin after 4 h incubation in phosphate buffer pH 7.6*. S.D. values are of the same order of magnitude as those of quercetin; $n = 3$; * $n = 2$.

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