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# *CERCOSPORA BETICOLA* TOXINS. DETERMINATION OF $O_2^-$ · SCAVENGING ACTIVITY OF BETICOLIN-1*

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**Key Word Index**—*Cercospora beticola*; fungus; beticolin; superoxide anion ($O_2^-$ ·); guaiacol; tiron; luminol; vitamin E.

**Abstract**—The $O_2^-$ · scavenging properties of beticolin-1, a fungal toxin, have been studied using peroxidase-mediated luminol chemiluminescence. A comparison between beticolin-1, vitamin E and tiron is reported: beticolin-1 displays an anti-radical effect without inhibiting peroxidase activity in a larger range of concentrations ($1 \times 10^{-8}$–$1 \times 10^{-5}$ M) than vitamin E ($3 \times 10^{-7}$–$1 \times 10^{-5}$ M) or tiron ($5 \times 10^{-7}$–$4 \times 10^{-5}$ M). Maximal scavenging efficacy was higher for beticolin-1 and vitamin E than for tiron (88, 80 and 52%, respectively).

## INTRODUCTION

Beticolins are yellow toxins produced by a phytopathogenic fungus, *Cercospora beticola*. They are constituted of partially hydrogenated anthraquinone and xanthone moieties (Fig. 1) [2–6]. Many chemical compounds containing phenolic groups are known for their antiradical activities [7–9]. This feature led us to search for a potential $O_2^-$ · scavenging activity of beticolins. The peroxidase-mediated luminol chemiluminescence was used, since superoxide anions are generated through this reaction. This method is widely documented for determination of active oxygen species and the following scheme for the reaction has been proposed [10, 11]:

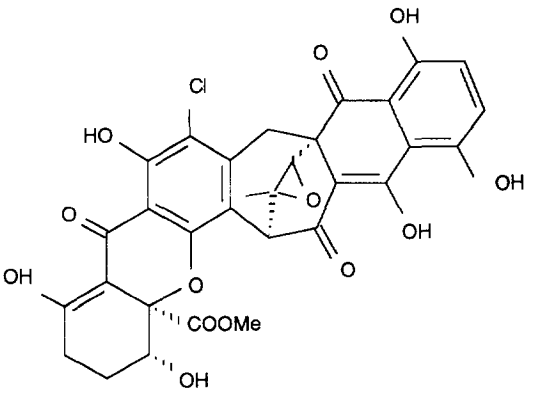


Fig. 1. Structure of beticolin-1.

2 Luminol (LH) + $H_2O_2$
+ Peroxidase → 2 $L^-$ · + 2 $H_2O$

$L^-$ · + $O_2$ → $O_2^-$ ·

$L^-$ · + $O_2^-$ · → $LOO^{2-}$ endoperoxide

$LOO^{2-}$ → 3-Aminophthalate dianion* + $N_2$

3-Aminophthalate dianion* → 3-aminophthalate dianion + $h\nu$

However, in such an experiment, a decrease in luminescence could also result either from an inhibition of peroxidase activity or from metabolism of $H_2O_2$ (catalase-like activity). In order to rule out these two possibilities and to point out the scavenging activity of a given compound, a simultaneous assay based on peroxidase-dependent guaiacol oxidation by $H_2O_2$ was performed [12]. In these conditions, guaiacol is oxidized in tetraguaiacol (determined spectrophotometrically) without involvement of $O_2^-$ ·. Thus, an $O_2^-$ · scavenger would be characterized by its inhibitory action of the luminol oxidation without any effect on the guaiacol one.

Using these dual experimental approaches, beticolin-1, one of the major compounds present in the fungal *C. beticola* extracts, appeared as an effective anti-radical molecule. Moreover, a comparison with two other antioxidants, vitamin E (presenting a large spectrum of anti-radical activity [13, 14]) and tiron (an $O_2^-$ · -specific scavenger [15, 16]) is reported.

*Part XIII in the series '*Cercospora beticola* Toxins'. For Part XII see ref. [1].

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## RESULTS AND DISCUSSION

The effects of beticolin-1, vitamin E and tiron on the luminol oxidation (open symbols) and on the guaiacol oxidation (closed symbols) are shown in Figs. 2, 3 and 4, respectively. Results are expressed as percentage of respective controls.

Up to *ca* $1 \times 10^{-8}$ M, beticolin-1 had no effect either on the luminol response or on the guaiacol one. At higher concentrations ($1 \times 10^{-8}$–$1 \times 10^{-5}$ M), beticolin-1 did not affect significantly the guaiacol oxidation whereas the luminol response was inhibited from 100 to 10% in a dose-dependent manner (Fig. 2). Between $1 \times 10^{-9}$ and $3 \times 10^{-6}$ M vitamin E concentrations, no change in guaiacol oxidation was detected. Between $3 \times 10^{-7}$ to $3 \times 10^{-6}$ M, vitamin E decreased luminol oxidation up to 20% of the control level (Fig. 3). Curves obtained with tiron (Fig. 4) presented similar shapes as the ones plotted in Fig. 3. However, the inhibition of guaiacol oxidation was noticeable from $1 \times 10^{-5}$ M tiron concentration whereas luminol oxidation started to decrease from $5 \times 10^{-7}$ M tiron concentration.

All these data show that beticolin-1, vitamin E and tiron exhibit an $O_2^- \cdot$ scavenging activity. In order to assess more accurately the potential scavenging activity of these three compounds, we plotted the curves of scavenging efficacy (Fig. 5) found by subtracting the values obtained by chemiluminescence from those obtained with guaiacol for each effector concentration. Two parameters could be analysed: the maximal efficacy and the concentration ranges where scavenging activity is observed without inhibiting peroxidase activity. First, the maximal efficacy for beticolin-1 and vitamin E were similar (88 and 80%, respectively) and better than that of tiron (52%). Moreover, these maxima were obtained for $1 \times 10^{-5}$ M for beticolin-1 and vitamin E, and for $4 \times 10^{-5}$ M for tiron. Second, the concentration ranges where scavenging activities are observed without inhibiting peroxidase activity are $1 \times 10^{-8}$–$1 \times 10^{-5}$ M, $3 \times 10^{-7}$–$1 \times 10^{-5}$ M and $5 \times 10^{-7}$–$4 \times 10^{-5}$ M for beticolin-1, vitamin E and tiron, respectively (Fig. 5).

This comparison shows that antioxidant activity of beticolin-1 occurs at lower concentration than for

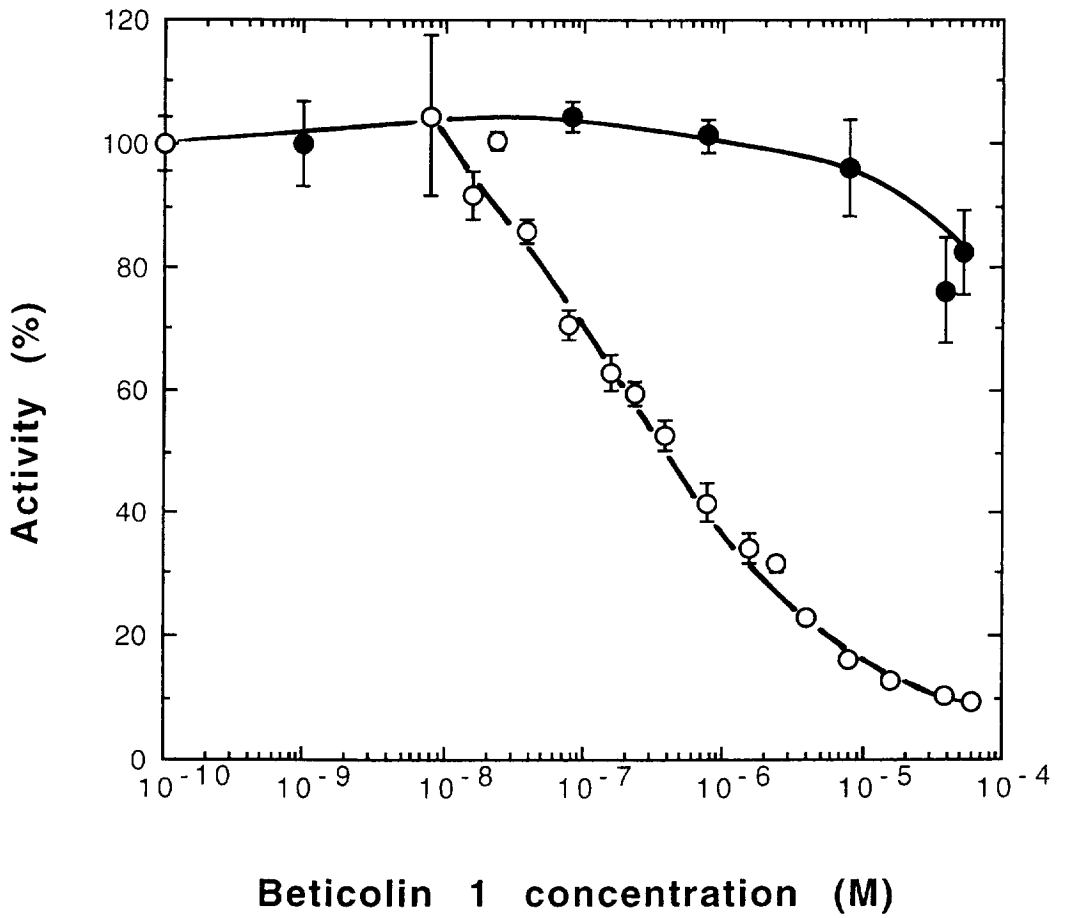


Fig. 2. Effect of beticolin-1 concentration on the peroxidase-mediated luminol chemiluminescence (○) and peroxidase activity (●) expressed as a percentage of the control containing the same concentration of DMSO (each point is the mean of three replicates).

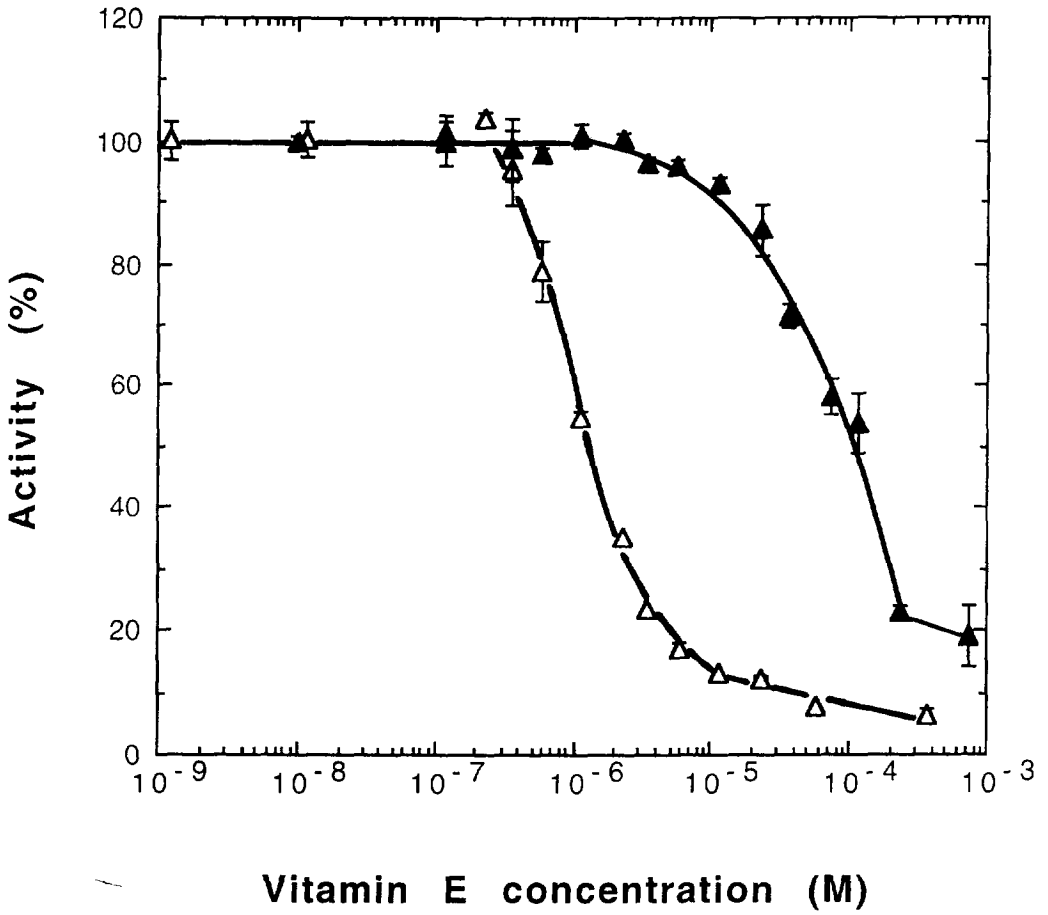


Fig. 3. Effect of vitamin E concentration on the peroxidase-mediated luminol chemiluminescence (△) and peroxidase activity (▲) expressed as a percentage of the control containing the same concentration of DMSO (each point is the mean of three replicates).

vitamin E and tiron. Moreover, the concentration range usable for scavenging activity is wider and the maximal scavenging activity higher for beticolin-1 than that for vitamin E or tiron. It was surprising to see that tiron in the millimolar range displays a low scavenging activity, concentrations which have been used to assess $O_2^-$ · production in biological systems [17, 18]. Peroxidase-mediated luminol chemiluminescence is a rapid and convenient method to analyse $O_2^-$ · production but, in order to search for the scavenging activity of a given compound, its effect on peroxidase activity has to be controlled.

## EXPERIMENTAL

*Reagents and chemicals.* Peroxidase (EC 1.11.1.7) from horseradish (HRP), type VI A, 1100 diammonium salt substrate units $mg^{-1}$ solid, luminol (5-amino-2,3-dihydro-1,4-phthalazinedione), guaiacol (2-methoxy-phenol) and vitamin E (dl-α-tocopherol) were obtained from Sigma. $H_2O_2$ (9% soln) was supplied by Gifrer Barbezat (France). Beticolin-1 was purified from a mycelial extract of a *C. beticola* strain (CM) according to ref. [19].

*Chemiluminescence (CL).* This was measured with a Beckman LS 6000 TA scintillation counter in a single photon mode. HRP, $H_2O_2$ and tiron were dissolved in $H_2O$ (0.45 $\mu$g $ml^{-1}$, 20 and 10 mM, respectively) and kept at 4°. Luminol (3 mM), vitamin E (0.23 mM) and beticolin-1 (0.16 mM) were dissolved in DMSO/$H_2O$. The final DMSO concn did not exceed 1%. Appropriate controls were run.

In the dark, in a scintillation vial containing 10 $\mu$l luminol, 500 $\mu$l MES buffer (175 mM mannitol, 0.5 mM $CaCl_2$, 0.5 mM $K_2SO_4$, 10 mM MES, pH 6.5), 50 $\mu$l $H_2O_2$ and 50 $\mu$l of the chemical assayed (beticolin-1, vitamin E or tiron), 50 $\mu$l of HRP were added. The final vol. was adjusted to 1 ml with $H_2O$. The pH of the final reaction mixt. was 6.5. CL measurements were recorded 40 sec after addition of HRP.

*Guaiacol peroxidase assays.* Measurements of *A* were performed with a Beckman DU 7400 spectrophotometer. Guaiacol (100 mM) was dissolved in DMSO/$H_2O$ in such conditions that the final concn of

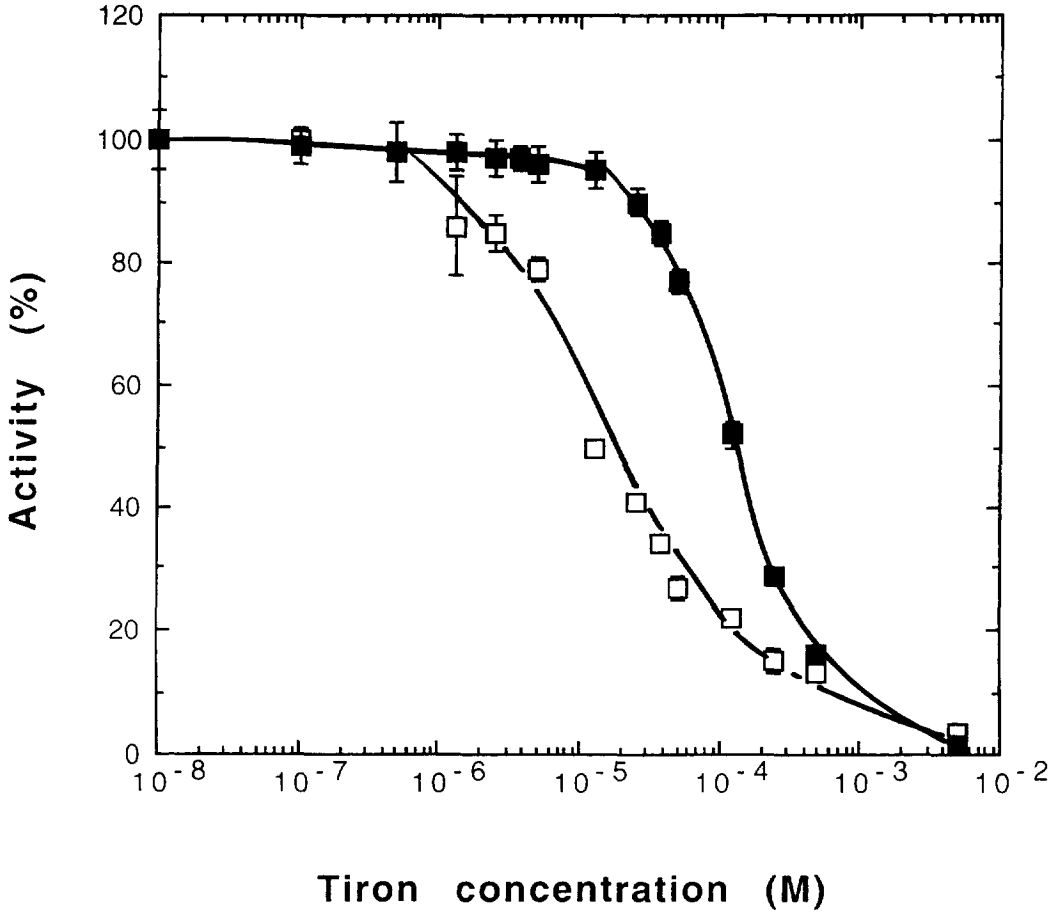


Fig. 4. Effect of tiron concentration on the peroxidase-mediated luminol chemiluminescence (□) and peroxidase activity (■) expressed as a percentage of the control containing the same concentration of DMSO (each point is the mean of three replicates).

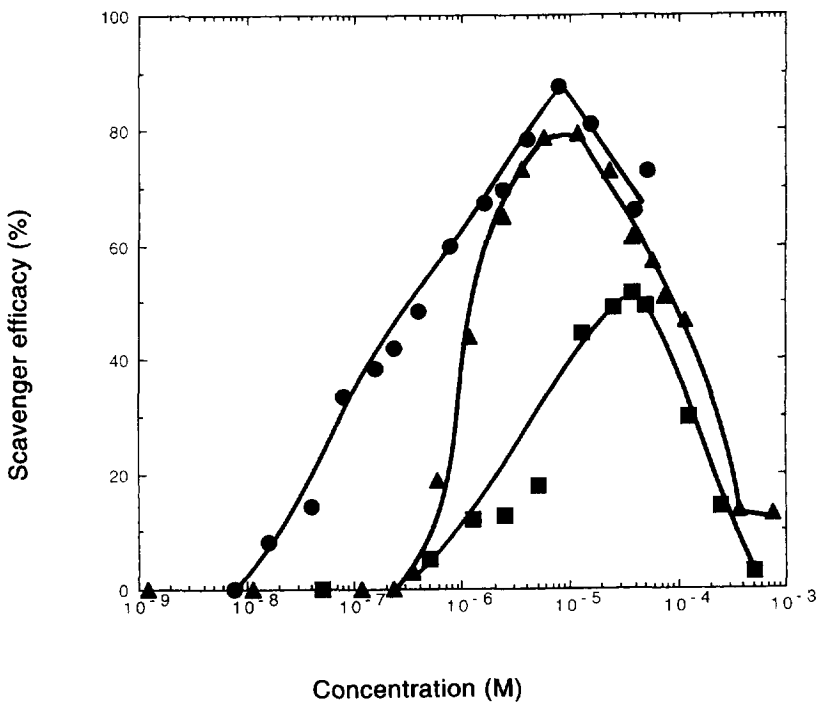


Fig. 5. Scavenging efficacy of beticolin-1 (●), vitamin E (▲) and tiron (■). Each plot was obtained by subtracting the value obtained by chemiluminescence assay from that obtained by guaiacol oxidation for each concentration assayed (open and closed symbols of Figs 2–4).

DMSO did not exceed 1%. In the dark, in the cuvette containing 50 $\mu$l guaiacol, 500 $\mu$l MES buffer, 50 $\mu$l $H_2O_2$ and 50 $\mu$l chemical assayed (beticolin-1, vitamin E or tiron), 50 $\mu$l HRP were added. The final vol. was adjusted to 1 ml with $H_2O$. Oxidation of guaiacol was determined by measuring $A$ at 470 nm, 10 min after HRP addition. Controls with and without DMSO have been performed.

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