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Stereospecific inactivation of tyrosinase by L- and D-ascorbic acid

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ABSTRACT

A kinetic study of the inactivation of tyrosinase by L- and D-ascorbic acid isomers has been carried out. In aerobic conditions, a suicide inactivation mechanism operates, which was attributed to the enzymatic form *oxy*tyrosinase. This suicide inactivation is stereospecific as regards the affinity of the enzyme for the substrate but not as regards the speed of the process, which is the same for both isomers, reflecting the influence of the chemical shift of the carbon C-2 (δ_2) and C-3 (δ_3) as seen by ^{13}C-NMR. The inactivation of *deoxy*tyrosinase and *met*tyrosinase observed in anaerobic conditions, is irreversible and faster than the suicide inactivation process, underlining the fact that the presence of oxygen protects the enzyme against inactivation.

1. Introduction

Enzymatic browning in fruits and vegetables is one of the most serious alteration processes for the food industry, where, it has been estimated, about 50% of economic losses are due to this phenomenon [1]. The control and prevention of this undesirable process is the key for minimising these losses and for prolonging the shelf life of these fresh products. The most important reactions intervening in browning are enzymatic processes catalysed mainly by peroxidases and, especially, polyphenol oxidases [2].

Polyphenol oxidase or tyrosinase (EC 1.14.18.1) is present in the tissues of plants and is widely distributed through the animal, vegetal and fungal kingdoms. tyrosinase has two coppers in its active site, which may be in three coordination states or forms: (*i*) Cu^{2+}–Cu^{2+}, *met*-tyrosinase, (*ii*) Cu^{2+}–O_2^{2-}–Cu^{2+} with a peroxide group in its active site, *oxy*-tyrosinase or (*iii*) Cu^{1+}–Cu^{1+}, *deoxy*-tyrosinase [3]. Tyrosinase catalyses two types of reaction in which molecular oxygen intervenes: (a) the hydroxylation of the monophenols to *o*-diphenols (monophenolase activity) and (b) the oxidation of *o*-diphenols to *o*-quinones (diphenolase activity) which, in turn, are polymerized to brown, red or black pigments [4,5]. Moreover, this enzyme is of central importance in vertebrate pigmentation. It is directly responsible for the conversion of the amino acid, L-tyrosine, to one of several types of melanin pigment [6].

Due to poor specificity of tyrosinase for phenolic substrates and for oxidisable compounds in general, several catalytic activities have been described for this enzyme, including catalase [7,8] or ascorbate oxidase [9–11].

Several strategies are being used to minimize or reduce tyrosinase-catalyzed browning in fruits and vegetables. For example, several inhibitors of this enzyme and/or antioxidants have been added [12]. Many inhibitors of enzymatic browning have been described, including 4-hexylresorcinol [13] and ascorbic acid itself (Scheme 1) [14]. However, the reaction mechanisms of these compounds still have to be elucidated. There has also been much discussion concerning the role of ascorbic acid as inhibitor/inactivator. For example, it has been suggested that it acts as an antioxidant, reducing the *o*-quinones generated by tyrosinase back to the initial *o*-diphenols, or that it interacts directly with the active site of the enzyme, thereby inactivating it [14,15]. Studies carried out in our laboratory several years ago pointed to the role of ascorbic acid as alternative substrate of tyrosinase, thus influencing melanogenesis [9,10].

In the present work, we attempt to ascertain whether ascorbic acid is a substrate that can inactivate tyrosinase. We propose mechanisms to explain the inactivation of the different enzymatic species of tyrosinase in both aerobic and anaerobic conditions. The kinetic analysis of the mechanism in aerobic conditions provides explicit equations for oxygen consumption with time, from which an experimental design can be described which permits the kinetic characterisation of the enzyme's action on the possible inactivating substrate. The mechanism is based on that previously proposed to

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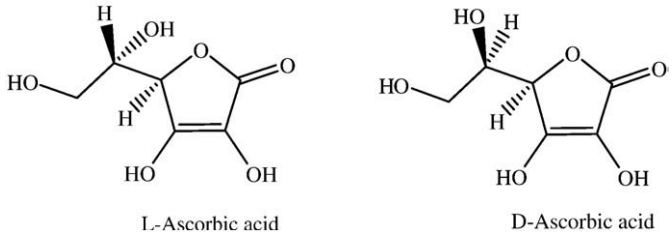


Scheme 1. Chemical structures of L- and D-ascorbic acid.

explain the suicide inactivation of tyrosinase as it acts on phenolic substrates [16]. Applying the same methodology, the stereospecificity of the enzyme in this process is studied, using the isomers L and D-ascorbic acid. The kinetic analysis of the mechanisms in anaerobic conditions provides explicit equations for the residual activity *vs.* time, from which the experimental design permits the kinetic characterisation of these inactivation processes.

1.1. Abbreviations

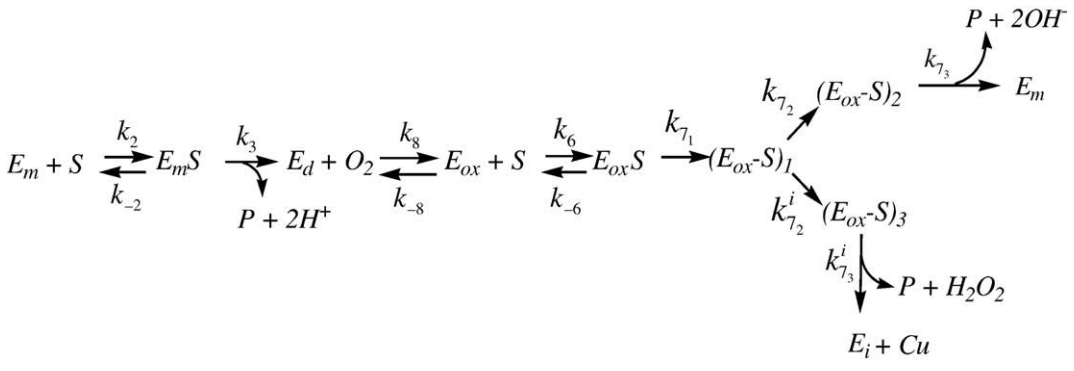
For clarity and for the sake of brevity, the following abbreviations will be used in the text.

Species and concentrations

E_m	*met*-tyrosinase.
$[E_m]_0$	initial concentration of *met*-tyrosinase
E_d	*deoxy*-tyrosinase.
$[E_d]$	instantaneous concentration of *deoxy*-tyrosinase.
$[E_d]_0$	initial concentration of *deoxy*-tyrosinase.
E_{ox}	*oxy*-tyrosinase.
E_mS	*met*-tyrosinase/substrate complex (diaxial).
$E_{ox}S$	*oxy*-tyrosinase/substrate complex.
$(E_{ox}-S)_1$	*oxy*-tyrosinase/substrate complex axially bound to a Cu atom.
$(E_{ox}-S)_2$	*oxy*-tyrosinase/substrate complex axially bound to two Cu atoms.
$(E_{ox}-S)_3$	*oxy*-tyrosinase/substrate complex axially bound to one Cu atom and the deprotonated hydroxyl group of C-3.
E_i	inactive enzyme originated by suicide inactivation.
E_{di}	inactive enzyme resulting from denaturalisation of E_d in anaerobic conditions.
E_{dSi}	inactive enzyme obtained by irreversible inhibition of E_d and E_m by L- and D-ascorbic acid.
$[E]_0$	initial concentration of tyrosinase.
E_a	active enzyme.
$[E_a]$	instantaneous concentration of E_a.
$L\text{-}AH_2$	L-ascorbic acid.
$D\text{-}AH_2$	D-ascorbic acid.
S	L-ascorbic acid ($L\text{-}AH_2$) or D-ascorbic acid ($D\text{-}AH_2$) acting as suicide substrate.
$[S]_0$	initial concentration of substrate.
P	Product of the enzymatic reaction, dehydroascorbic acid (*L-A*, *D-A*).
$[P]_\infty$	Product concentration at $t \to \infty$.
PPO	tyrosinase or polyphenol oxidase.
$[O_2]$	instantaneous concentration of oxygen.
$[O_2]_0$	initial concentration of oxygen.
$[\dot{O}_2]$	instantaneous rate of the variation of $[O_2]$.
$[O_2]_f$	Oxygen remaining at $t \to \infty$.
$[O_2]_\infty$	Oxygen consumed at $t \to \infty$. $[O_2]_\infty = [O_2]_0 - [O_2]_f$
$[E_S]$	total concentration of set of enzyme species involved in a restricted steady–state, Schemes 2 and 3, i.e.: $[E_S]=[E_m]+[E_mS]+[E_d]+[E_{ox}]+[E_{ox}S]+[(E_{ox}-S)_1]+[(E_{ox}-S)_2]$.
$[\dot{E}_s]$	instantaneous rate of the variation of $[Es]$.
$f_{(E_{ox}-S)_1}$	concentration factor corresponding to enzyme species $(E_{ox}-S)_1$ with regard to E_s : $f_{(E_{ox}-S)_1} = \frac{[(E_{ox}-S)_1]}{[E_s]}$.

Kinetic parameters

λ	apparent inactivation constant.
λ^d	apparent inactivation constant of E_d.
λ^m	apparent inactivation constant of E_m.
λ^{ox}	apparent inactivation constant of E_{ox}.
k_d^d	inactivation constant of E_d in anaerobic conditions.
k_i^d	inactivation constant of E_dS.
K_9	dissociation constant of E_dS complex.
$V_0^{O_2}$	initial rate of the catalytic pathway.
k_x	rate constants of the catalytic pathway (Scheme 2).
k_{7_1}	substrate binding constant to copper atom through C-2 hydroxyl (axial).
k_{7_2}	substrate binding constant to copper atom through C-3 hydroxyl (axial).
k_x^i	rate constants of the inactivation pathway (Scheme 2).
$k_{7_2}^i$	rate constant of transfer of a H^+ to the protonated peroxide.
k_{7_3}	rate constant of substrate oxidation through complex $(E_{ox}-S)_2$.
$k_{7_3}^i$	inactivation constant.
r	partition ratio $r=k_{7_2}/k_{7_2}^i=k_{cat}/\lambda_{max}^{ox}$.
K^{O_2}	dissociation constant of E_{ox}.
$K_m^{O_2}$	Michaelis constant of oxygen.
$K_{m_1}^S$	Michaelis constant of *met*–tyrosinase for *S*.
$K_{m_2}^S$	Michaelis constant of *oxy*–tyrosinase for *S*.
K_m^S	Michaelis constant of tyrosinase for *S*.
k_{cat}	catalytic constant of the catalytic pathway ($k_{cat}=\lambda_{max}^{ox}r$).
λ_{max}^{ox}	maximum value of λ^{ox} for saturating *S*.
$V_{max}^{O_2}$	maximum value of $V_0^{O_2}$ for saturating *S*.



Scheme 2. Kinetic mechanism proposed to explain the suicide inactivation of tyrosinase acting on ascorbic acid.