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Evidence of *p*-quinoid enamine formation during the oxidative desamination of *p*-hydroxy-D-phenylglycine catalyzed by D-amino acid oxidase

Estrella Núñez-Delicado [a], Francisco García-Carmona [b], Álvaro Sánchez-Ferrer [b,*]

[a] *Department of Food Technology, Catholic University San Antonio of Murcia, Campus de Los Jerónimos s/n, 30107 Guadalupe, Murcia, Spain*
[b] *Department of Biochemistry and Molecular Biology-A, Faculty of Biology, University of Murcia, Campus de Espinardo, E-30071 Murcia, Spain*

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Abstract

An enzymatic process for obtaining the corresponding α-keto acid of *p*-hydroxy-D-phenylglycine, a unnatural aromatic amino acid used in the synthesis of β-lactam antibiotics, was developed. For this, *p*-hydroxy-D-phenylglycine was oxidized by D-amino acid oxidase in the presence of catalase at pH 8.0. The gradual development of a 370 nm maximum was initially observed, which evolved to generate another maximum at 335 nm. The latter represented a product that was purified by anionic exchange chromatography and identified by NMR as the α-keto acid corresponding to *p*-hydroxy-D-phenylglycine. The intermediate with a maximum at 370 nm was taken as direct evidence that the enamine is formed as result of the chemical stabilization of the first imine form released by the enzyme. This imine–enamine equilibrium was demonstrated spectrophotometrically due to the particular structure of *p*-hydroxy-D-phenylglycine, which allows chemical stabilization via the formation of a *p*-quinone enamine. When the reaction was carried out with D-amino acid oxidase and catalase co-immobilized on Eupergit C, the enamine intermediate was separated from the enzyme, and its chemical decomposition constant was obtained ($k = 0.16\ \text{min}^{-1}$). However, this chemical evolution was not enough to explain the levels of enamine accumulated, when the enzyme concentration was increased. This observation indicates for the first time that the enzyme also contributes to the evolution of enamine into α-keto acid. A reaction scheme is proposed to explain the above-mentioned results.

Keywords: D-Amino acid oxidase; α-Keto acid; Imino acid; Enamine; Enzyme immobilization

1. Introduction

D-Amino acid oxidase, DAAO [D-amino acid: O_2 oxidoreductase (deaminating) (E.C.1.4.3.3)] is a flavoprotein that uses flavin-adenine dinucleotide (FAD) as cofactor to catalyze the oxidation of D-amino acids into imino acids and hydrogen peroxide (Fig. 1). The imino acid thus formed evolves rapidly to yield the corresponding α-keto acid and ammonium ion. In the presence of hydrogen peroxide, the α-keto acid product decarboxylates spontaneously.

This enzyme is of major biotechnological interest because it is used in many processes including the production of 7-aminocephalosporanic acid from cephalosporin C [1,2], of biosensors [3,4], of L-amino acids by the resolution of racemic amino acid mixtures [5] and of α-keto acids [6,7]. In addition, it is also used in the qualitative and quantitative analysis of D-amino acids [8].

The formation of α-imino acids as transient intermediates in the oxidative deamination of α-amino acids was postulated by Knoop in 1910 [9]. However, such intermediates have never been isolated from enzymatic reaction mixtures due to their extreme instability in aqueous solutions. However, their formation by amino acid oxidases was supported

* Corresponding author. Fax: +34 968 364147.
E-mail address: alvaro@um.es (Á. Sánchez-Ferrer).

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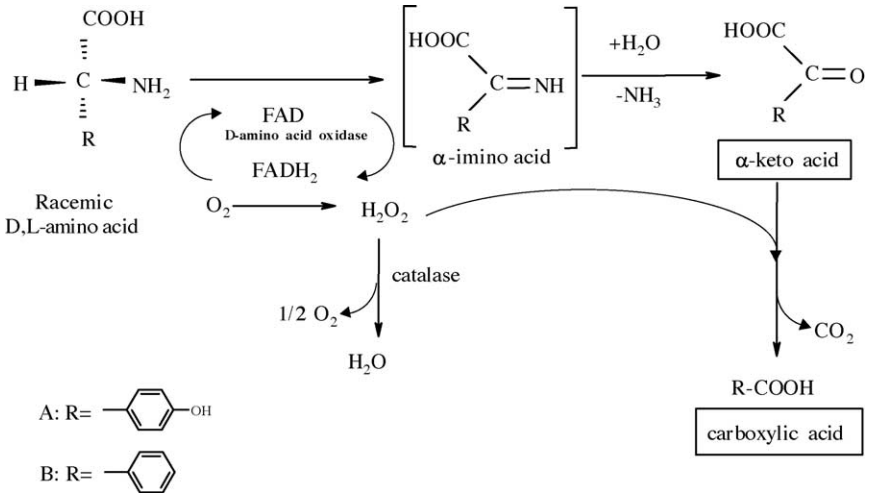

Fig. 1. General scheme for the oxidative deamination of D-amino acid by D-amino acid oxidase. In the case of *p*-hydroxy-D-phenylglycine (OHDPG), R is the A side chain, and of D-phenylglycine (DPG), R is the B side chain (see text for details).

by some indirect evidences [10–15]. For example, Taborsky in 1955 [12] and Pitt in 1958 [13] obtained spectrophotometric evidence for the formation of an intermediate absorbing at 300 nm in the oxidation of L-tyrosine by snake venom L-amino acid oxidase. However, the data presented did not permit any unequivocal conclusion to be reached concerning the exact nature of this intermediate. Yagi et al. in 1970 [10] reported evidence for the formation of a product, less basic than either the starting amino acid or ammonia, in the oxidation of D-alanine by DAAO. These data were interpreted as an imino acid being released from the enzyme as the primary oxidation product, giving rise to an equilibrium imino–enamine. Later, in 1971, Hafner and Wellner [14] showed that imino acids could be formed by allowing the amino acid oxidation reaction to proceed in the presence of $NaBH_4$, during which time the imino acid is reduced to the corresponding racemic amino acid. Thus, when $NaBH_4$ was added to a mixture of DAAO and D-alanine, or LAAO and L-leucine, significant amounts of L-alanine and D-leucine, respectively, were formed [14]. Finally, in 1972, Porter and Bright [15], in studying the oxidation of D-phenylalanine by DAAO, observed a short lag period (<5 s) in the accumulation curve of the keto product, which was not present in the oxygen consumption curve. These results indicated the presence of a reaction intermediate, which could have been the imino–enamine form. Until now, these have been the evidences supporting the formation of an imino–enamine intermediate in the oxidation of amino acids by amino acid oxidases.

Unexpectedly, a clear spectrophotometric evidence concerning the presence and evolution of the imino–enamine acid intermediate during the oxidation of D-amino acid by DAAO was obtained during our study of the oxidation of *p*-hydroxy-D-phenylglycine (OHDPG) by DAAO of *Trigonopsis variabilis*. This observation is due to the special chemical structure of *p*-hydroxy-D-phenylglycine, which permits the formation of a relatively stable resonant enamine compared to those previously studied.

2. Experimental

2.1. Materials

p-Hydroxy-D-phenylglycine, *p*-hydroxybenzoic acid, Amberlite IRA 400 and Eupergit C were obtained from Sigma (Madrid, Spain). Catalase from bovine liver (301,500 U/ml) was purchased from Fluka (Madrid, Spain).

DAAO was obtained from *T. variabilis* CBS 4091. *T. variabilis* was cultivated in rich medium (20 g/L of malt extract, 20 g/L of glucose, 10 g/L of bacto peptone and 5 g/L of yeast extract, pH 6.0), until the optical density at 546 nm of the suspension was 65–70. DAAO was induced by the presence of DL-methionine [16]. The enzyme was purified by ammonium sulfate fractionation between 30 and 55% as described by Sánchez-Ferrer et al. [17]. Catalase from *Micrococcus lysodeikticus* (Sigma) was mixed with DAAO in 200-fold excess under the immobilization conditions described elsewhere [18].

2.2. Measurement of D-amino acid oxidase activity

The oxidation of OHDPG by DAAO was followed spectrophotometrically in an HP8452A diode array spectrophotometer (Hewlett-Packard) from 200 to 450 nm at pH 8.0 and 37 °C. The standard reaction medium contained 50 mM potassium phosphate, 62.5 μM OHDPG and 0.22 U in a final volume of 1 ml.

Reaction products were analyzed by HPLC (Kontron-420) in an Inertsil-ODS-2 column (GL Science Inc., 5 μm particles, 4.6 mm × 150 mm) at 286 nm (OHDPG and keto acid) and 370 nm (enamine form), using a Kontron 430 UV

detector. Samples were injected using a Rheodyne 7125 loop injector (20 μl). Products were eluted isocratically with 15 mM potassium phosphate buffer, pH 6.5, at a flow rate of 1 mL/min.

2.3. Isolation and characterization of the α-keto acid

The α-keto acid generated from OHDPG by DAAO and catalase co-immobilized on Eupergit C, was isolated from the reaction mixture by chromatography on Amberlite IRA 400 (Sigma), an anionic exchange column, equilibrated in 50 mM potassium phosphate buffer, pH 8.0. Under these conditions amino acids are eluted, whereas α-keto acid remains bound to the matrix, but can be eluted with a solution of 40% acetonitrile and 1 M hydrochloric acid [19].

The absorption coefficients (at 310 and 362 nm) of the α-keto acid thus isolated were calculated at pH 8.0 using increasing concentrations of the α-keto acid ($\varepsilon_{310\,nm} = 10{,}680\ M^{-1}\ cm^{-1}$ and $\varepsilon_{362\,nm} = 3870\ M^{-1}\ cm^{-1}$).

NMR spectra were recorded with a Bruker 300 MHz spectrometer and chemical shifts are reported in ppm. For NMR analysis, the *p*-OH-benzoylformate resulting from OHDPG was dissolved in D_2O and the spectrum obtained confirmed the nature of the compound: ^{1}H NMR (D_2O): $\delta = 7.81$ (d, 2H, $^3J = 8.8$ Hz), 6.86 (d, 2H, $^3J = 8.8$ Hz); ^{13}C NMR (D_2O): $\delta = 189.81$ (CO), 168.86 (COOH), 162.66 (C-4), 133.01 (C-2/C-6), 123.80 (C-1) and 115.78 (C-3/C-5).

2.4. Measurement of accumulation rate of enamine and α-keto acid in the reaction media

The initial accumulation rate (v_0) of enamine form in the reaction medium was measured at 362 nm, which corresponds to the isosbestic point between the enamine and α-keto acid forms.

The accumulation rate of α-keto acid in the reaction medium was measured not at its λ_{max} (335 nm) but at 310 nm, where the contribution of enamine form is minimal. The accumulation rate was calculated in the linear portion after the lag period.

2.5. Measurement of maximum level and decomposition rate of enamine accumulated in the reaction media

The maximum level of the enamine form accumulated in the reaction media and the apparent decomposition rate of this form were measured at 370 nm (λ_{max} of enamine form). This decomposition constant was determined by measuring the maximal decrease in absorbance after the maximum level of enamine form was reached.

3. Results and discussion

p-Hydroxy-D-phenylglycine (OHDPG) is a non-proteinogenic aromatic amino acid with a hydroxyl group

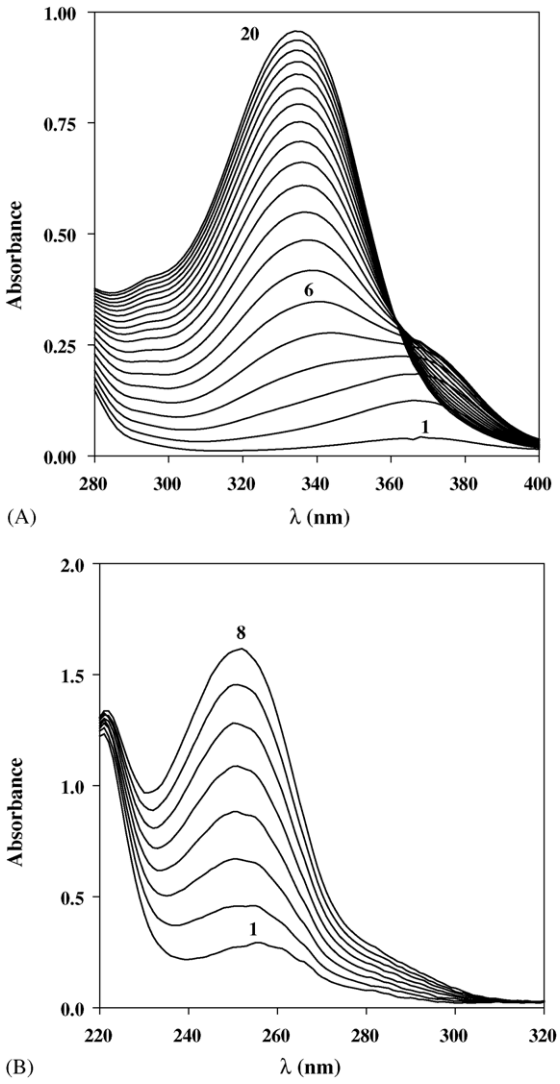

Fig. 2. (A) Oxidation of OHDPG by DAAO in the presence of catalase. The reaction medium contained 50 mM potassium phosphate buffer, pH 8.0, 62.5 μM OHDPG, 300 U/mL catalase and 0.22 U/mL DAAO. Scan rate 0.5 min. (B) Oxidation of DPG by DAAO in the presence of catalase. The reaction medium contained 50 mM potassium phosphate buffer, pH 8.0, 625 μM DPG, 300 U/mL catalase and 0.22 U/mL DAAO. Scan were recorded every 1 min.

in the *para* position of the benzene ring (Fig. 1, side chain A). When OHPG was incubated at pH 8.0 with *T. variabilis* DAAO in the presence of catalase to avoid keto decarboxylation, a complex spectral change was observed (Fig. 2A). A maximum at 370 nm was gradually developed (scans 1–3), which evolved to generate another maximum at 335 nm (scans 4–20), generating an isosbestic point at 362 nm. These spectral changes (Fig. 2A) pointed to the presence of an intermediate with a maximum at 370 nm. In order to clarify the nature of this intermediate, the oxidation of D-phenylglycine (DPG) (Fig. 1, side chain B) in the same conditions was studied. The spectral changes in this case showed the development of one sole maximum at 252 nm (Fig. 2B), which corresponds to the maximum of

the spectrum of the commercially available benzoyl formic acid, as is to be expected [20].

To identify the reaction product with a maximum at 335 nm formed during the oxidation of OHDPG by the DAAO–catalase system (Fig. 2A), an enzymatic bioreactor system was developed using co-immobilized DAAO and catalase on Eupergit C. The course of the reaction was followed by HPLC, until the substrate was fully converted. The solution obtained after enzyme support separation was purified by anionic exchange chromatography on Amberlite IRA-400 and dried under vacuum. The solid was analyzed by NMR and identified as the α-keto acid corresponding to the OHDPG (see Section 2 for details).

Given that the final product of the reaction was identified as α-keto acid, the reaction intermediate with a maximum at 370 nm could not have been the imino form, as has been previously described for other D-amino acids [21], since this imino form would not have presented a different absorption spectrum from the substrate. The only possibility to explain such a maximum is that the imino form had stabilized through imino–enamine equilibrium [15]. In the case of the imine from OHDPG, the stabilization of its particular structure would involve the formation of a *p*-quinone enamine (Fig. 3) with the typical absorption band of the *p*-quinonic structures at 370 nm. The surprising chemical stability of this enamine, its different spectrum, and the different retention time from that of both D-amino and α-keto acid are all due to the presence of a hydroxyl group in the *para* position of the ring. This permits the formation of an enamine with a *p*-quinoid structure, which is not possible during DPG oxidation, because of its structure.

To further characterize this stable enamine, OHDPG was oxidized by DAAO in the presence of catalase at low enzyme concentration. In these conditions, the enamine form was accumulated (Fig. 4), confirming that the oxidation of OHDPG by DAAO in the presence of catalase involves a first step during which stable enamine form is generated, which is subsequently liberated from the active center of the enzyme, evolving finally to α-keto acid and ammonia.

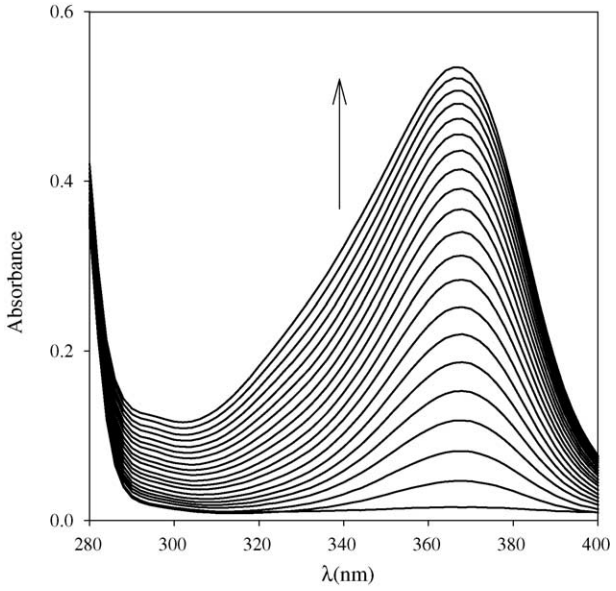

Fig. 4. Oxidation of OHDPG at low DAAO concentration in the presence of catalase. The reaction medium contained 50 mM potassium phosphate buffer, pH 8.0, 62.5 μM OHDPG, 300 U/mL catalase and 0.022 U/mL DAAO. Scan were recorded every 0.5 min.

To check whether the evolution of the enamine form to generate α-keto acid was strictly chemical or whether an enzymatic component was involved, the kinetics of the appearance of both species and the disappearance of enamine was studied in the presence of increasing concentrations of DAAO. The initial rate of enamine generation, obtained from the initial part of the accumulation curve, was followed at the isosbestic point generated at 362 nm ($\varepsilon_{362\,nm} = 3870\ M^{-1}\ cm^{-1}$) (Fig. 5, curve a). The α-keto acid generation rate in the steady state (in the linear portion, after the lag period) was followed not at its absorption maximum (335 nm) but at 310 nm ($\varepsilon_{310\,nm} = 10{,}680\ M^{-1}\ cm^{-1}$), where the contribution of enamine form is minimal (Fig. 5, curve b). The disappearance rate of enamine was calculated as the maximal slope obtained after the maximum level of concentration was reached at its absorption maximum (370 nm) (Fig. 5, curve c).

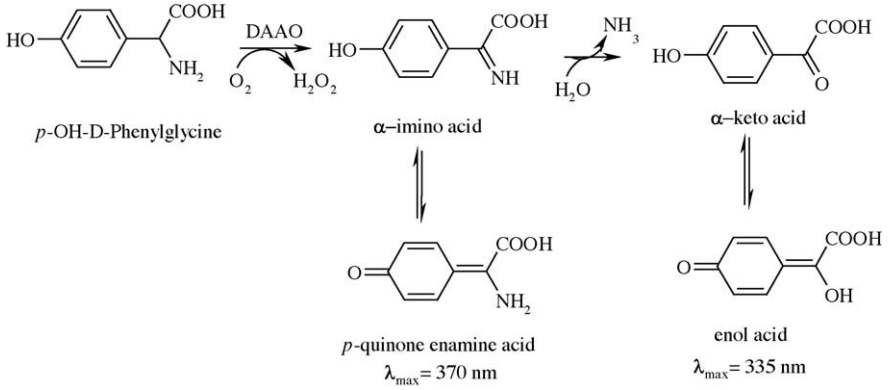

Fig. 3. Scheme for the generation of α-keto acid from OHDPG via *p*-quinone enamine, in the presence of DAAO–catalase.

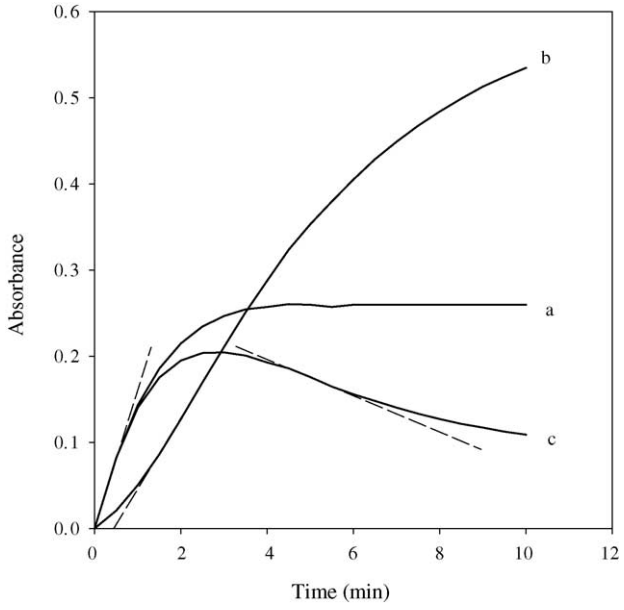

Fig. 5. Accumulation curves derived from the oxidation of OHDPG by DAAO in the presence of catalase at selected wavelengths. (a) Accumulation curve of enamine at 362 nm (isosbestic point). (b) Accumulation curve of α-keto acid at 310 nm. (c) Accumulation and disappearance curve of enamine at its absorption maximum (370 nm). The reaction medium contained 50 mM potassium phosphate buffer, pH 8.0, 62.5 μM OHDPG, 300 U/mL catalase and 0.22 U/mL DAAO.

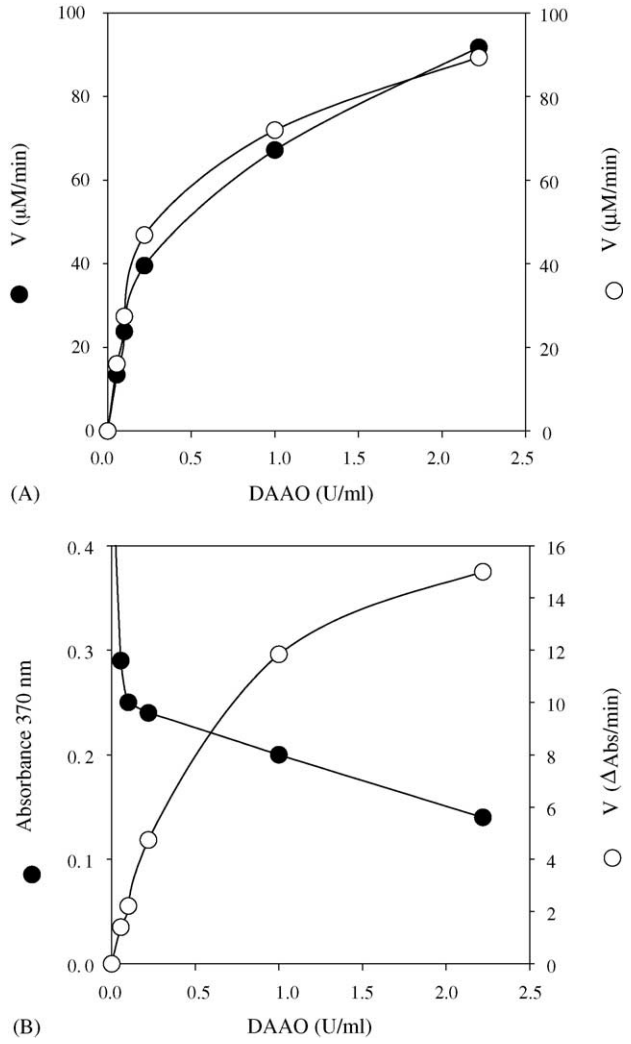

Fig. 6. Effect of enzyme concentration on the oxidation of OHDPG by DAAO in the presence of catalase. (A) Effect on the formation rate of enamine measured at 362 nm (isosbestic point) (●) and of keto acid measured at 310 nm, where the contribution of enamine is minimal (○). (B) Effect on the enamine accumulation (●) and enamine decomposition rate (○), both measured at 370 nm. The reaction medium contained 50 mM potassium phosphate buffer, pH 8.0, 62.5 μM OHDPG, 300 U/mL catalase and 0–2.2 U/mL DAAO.

As the enzyme concentration was increased up to 2.2 U/ml, a non-linear generation of both enamine and α-keto acid was observed (Fig. 6A). However, the maximum enamine level decreased (Fig. 6B, ●), whereas its rate of decomposition increased as enzyme concentration was raised to 2.2 U/ml (Fig. 6B, ○). These results are not in accordance with a simple reaction scheme, in which the enamine intermediate would evolve chemically to subsequently generate imino and then α-keto acid. If this had been the case, the concentration of the enamine intermediate would have increased with the enzyme concentration. However, the results presented in Fig. 6B show that the enamine level decreased with increasing DAAO concentration, clearly indicating, for the first time in DAAO kinetics, the contribution of the enzyme to the decomposition of the enamine form. In addition, an increase in substrate concentration produced an increase in the enamine level (data not shown), indicating that the substrate competes with the enamine form for DAAO's active centre.

To complete the study, the chemical decomposition constant of the imino form was measured. To characterize this constant, OHDPG was oxidized using co-immobilized DAAO–catalase on Eupergit C and the course of the reaction was followed by HPLC at 286 and 370 nm for substrate, keto and enamine, respectively. Fig. 7A shows the conversion of OHDPG into its corresponding α-keto acid at 286 nm. The peak area of α-keto acid (which has a retention time higher than the substrate) increased with the reaction time, whereas the concentration of the substrate decreased, being practically negligible after 30 min. On the other hand, the transient appearance of the enamine form at 370 nm (Fig. 7B) showed a maximum level 5.5 min after the reaction was started, after which it decreased, accompanied by a concomitant increase in the α-keto acid product (with a higher retention time in the chromatogram, Fig. 7B).

Once the kinetics of the reaction with immobilized enzyme had been characterized, the enzyme was removed from the reaction medium at 5.5 min, and the chemical evolution of the enamine form thereafter was followed spectrophotometrically at 370 nm. A decrease in absorbance was observed (data not shown), indicating the chemical decomposition following first-order kinetics, of the enamine intermediate. These results were subjected to Guggenhein analysis [22], in which it is not necessary to know the experimental values of the

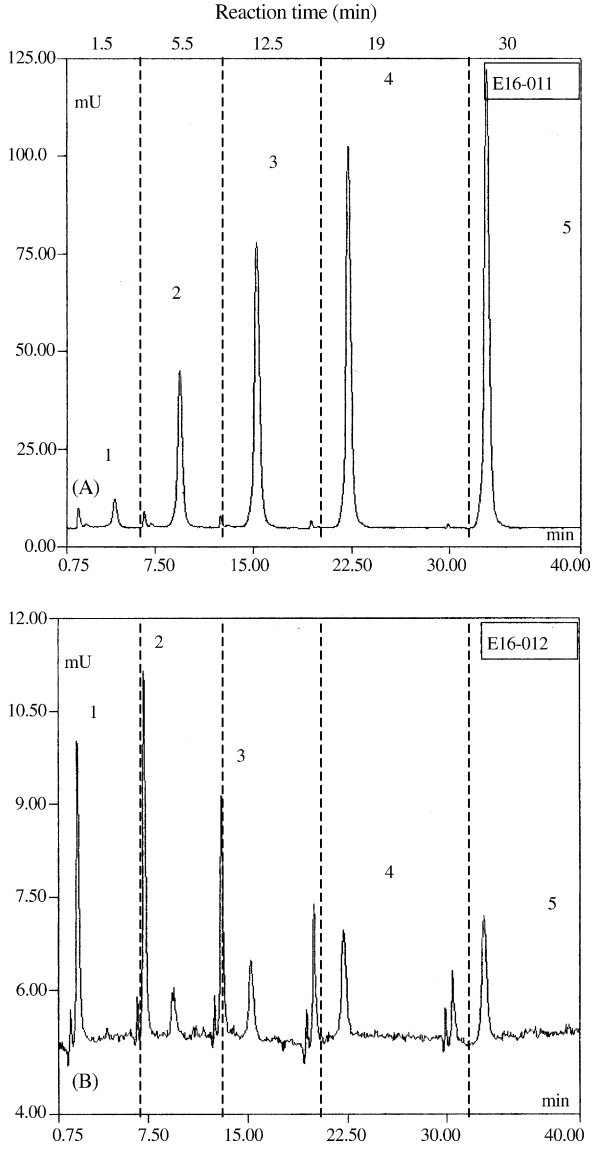

Fig. 7. Time course of the oxidation of OHDPG by co-immobilized DAAO and catalase followed by HPLC. OHDPG and its keto acid were detected at 280 nm (A) and enamine form was detected at 370 nm (B). (1) Sample injected at 1.5 min after the start of the reaction. (2) Sample injected at 5.5 min after the start of the reaction. (3) Sample injected at 12 min after the start of the reaction. (4) Sample injected at 19 min after the start of the reaction. (5) Sample injected at 30 min after the start of the reaction. The reaction medium contained 50 mM potassium phosphate buffer, pH 8.0, 0.5 mM OHDPG, 300 U/mL catalase and 0.073 U/mL DAAO.

enamine (P) in order to calculate its chemical decomposition constant (k). In this analysis, two values of enamine, P_i and P'_i, were measured at t_i and t'_i, where $t'_i = t + T'$ and T' = constant.

If we accept that the chemical decomposition has a first order kinetic, the following equations can be written:

$$P_\infty - P_i = P_\infty e^{-kt_i} \quad (1)$$

$$P_\infty - P'_i = P_\infty e^{-k(t_i+T')} \quad (2)$$

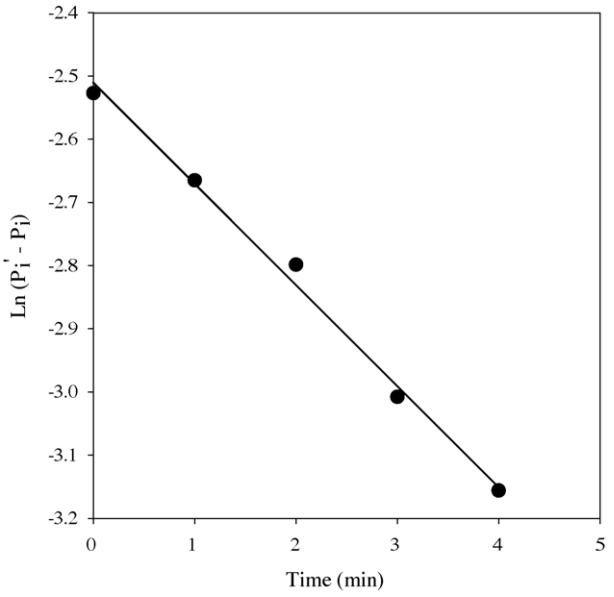

Fig. 8. Guggenheim's representation for the chemical decomposition of α-imino acid (see text for details).

Subtracting Eq. (2) from Eq. (1) would give:

$$P'_i - P_i = P_\infty(1 - e^{-kT'})e^{-kt_i} \quad (3)$$

$$\ln(P'_i - P_i) + kt_i = \text{constant} \quad (4)$$

$$\ln(P'_i - P_i) = \text{constant} - kt_i \quad (5)$$

When $\ln(P'_i - P_i)$ is plotted versus t_i, a straight line is obtained (Fig. 8), whose slope represents $-k$. The value of k, the chemical decomposition constant of the enamine form corresponding to OHDPG thus obtained, was 0.16 min^{-1}.

In order to explain the above-mentioned results, the kinetic scheme shown in Fig. 9 is proposed. In this scheme, the only possibility is that the enzyme (DAAO) alters the dynamic between enamine, imino and α-keto acid forms. This can only be possible if the enzyme uses the enamine as substrate analogue (unsaturated α-amino acid), binding it to the active centre and thus accelerating the transformation towards the imino form. This process produces an increase in the level of E – imino which makes it possible to see the E – imino + water minority pathway (Fig. 9, box), in which a water molecule close to the active centre is able to convert the imino into α-keto before the latter is released from the enzyme.

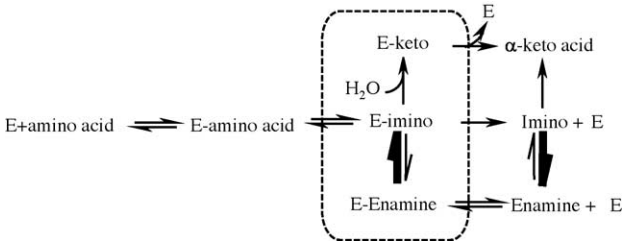

Fig. 9. Proposed reaction mechanism for the oxidation of OHDPG by DAAO. E means DAAO.

The proposed model explains how an intermediate (imino ↔ enamine), which normally evolves chemically to the product (α-keto acid), is affected by the presence of the enzyme due to the analogy between enamine and substrate. Moreover, the fact that an increase in substrate concentration led to an increase in enamine level can be explained because of the competition between the substrate and enamine form for the active centre of the enzyme.

In conclusion, the results shown in this paper and the kinetic scheme proposed open up the possibility of designing new DAAO substrates with stable imino–enamine forms to further characterize its complex kinetic mechanism.

Acknowledgements

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