Oxidation of Salsolinol by Banana Pulp Polyphenol Oxidase and Its Kinetic Synergism with Dopamine

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Salsolinol, a tetrahydroisoquinoline present in banana and biosynthesized from dopamine, was oxidized by banana pulp polyphenol oxidase to its corresponding salsolinol-*o*-quinone. This oxidation was pH-dependent and showed a maximum at acidic pH values. At physiological pH of 5.0, the values obtained for the kinetic parameter (V_m and K_m) were 62.5 μM/min and 1.7 mM, respectively. When dopamine was added to the reaction medium to imitate physiological conditions, salsolinol was co-oxidized by dopamine-quinone. When this phenomenon was studied oxygraphically, an unexpected activation of dopamine oxidation was found in the presence of salsolinol. This activation was related with the enzyme's kinetic mechanism and was named "kinetic synergism", because a bad substrate activated a good one. A possible physiological role is discussed.
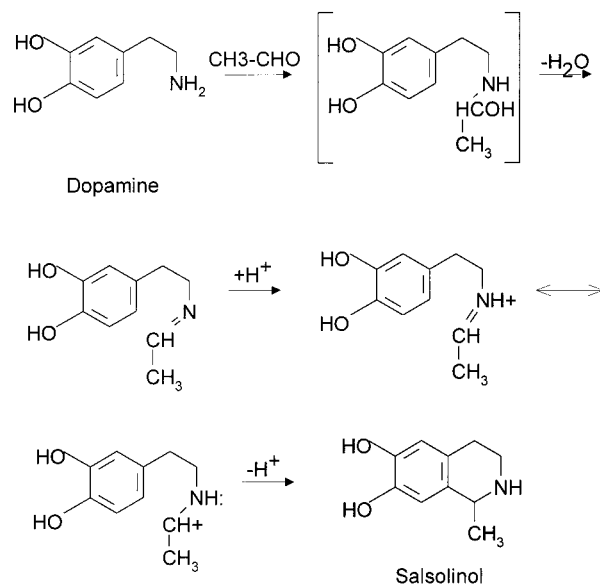
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INTRODUCTION

The metabolism of tetrahydroisoquinolines (TIQ) is of interest because these heterocyclic substrates are formed in mammals during certain diseases (e.g., alcoholism or Parkinson's) (Sandler et al., 1973; Dostert et al., 1996), although they are also found in the urine of healthy volunteers after the intake of dried banana (Deng et al., 1997). Banana has large quantities of dopamine and norepinephrine, and during the later stages of the ripening process the concentration of salsolinol (SAL) (6,7-dihydroxy-1,2,3,4-isoquinoline) increases (Riggin et al., 1976). This complex *o*-diphenol is biosynthesized by nonenzymatic Pictet–Spengler condensation (Scheme 1), in which a phenylethylamine, such as dopamine, complexes with acetaldehyde to form an imine, which subsequently cyclizes to the TIQ (Whaley and Govindachari, 1951). The acetaldehyde is enzymatically generated from ethanol during the postclimacteric phase in banana (Riggin et al., 1976).

Salsolinol is known to be involved in the black appearance of overripe bananas (Riggin et al., 1976). The route involved probably entails the oxidation of SAL to a quinone, which couples with other brown pigments to give melanins (Riggin et al., 1976). However, no detailed study of the oxidation of SAL by endogenous banana enzymes has been carried out. Banana pulp has a high level of polyphenol oxidase (PPO), the diphenolase activity of which is able to catalyze the oxidation of *o*-diphenols to their corresponding *o*-quinones. Recently, a new mild purification method for untanned banana pulp PPO has been developed by our group using a combination of two phase separations in Triton X-114 and in PEG 8000/phosphate (Sojo et al., 1998a,b). The enzyme has diphenolase activity toward several *o*-diphenols, including SAL. The purpose of this work was to kinetically characterize the oxidation of SAL by banana pulp PPO and to study the effect of other

Scheme 1. Biochemical Pathway of SAL Biosynthesis via a Pictet−Spengler Condensation (Whaley and Govindachari, 1951)

o-diphenols, such as dopamine, on this oxidation. From this study a novel kinetic synergism was demonstrated between dopamine and SAL, with possible physiological implications.

MATERIALS AND METHODS

Materials. Bananas (*Musa acuminata* subgr. Cavendish var. Spanish Pequeña Enana) were obtained from a commercial source in Murcia (COPLACA, S.A.). After holding for 24 h in ethylene gas in the dark, the samples were stored at 17 °C for 72 h until they were at stage 4 with the peel "more yellow than green" (Giami and Alu, 1994) and with a soluble solids content of 10%.

Biochemicals were purchased from Sigma (Madrid, Spain) and used without further purification. Triton X-114 (TX-114) was obtained from Fluka (Madrid, Spain) and condensed three

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times as described by Bordier (1981). Phenolic substrates (dopamine and salsolinol) were purchased from Sigma (Madrid, Spain). All other chemicals used were of analytical grade.

Purification of PPO. Banana pulp PPO was obtained following the method of Sojo et al. (1998a). In brief, a 30 g pulp sample was homogenized with 45 mL of 4% (w/v) TX-114 in 100 mM sodium phosphate buffer (pH 7.3) for 1 min. The homogenate was centrifuged at 15000g for 15 min at 4 °C. The supernatant was subjected to temperature-induced phase partitioning by increasing the TX-114 concentration by an additional 6% (w/v) at 4 °C and then warming to 37 °C for 15 min. After 10 min, the solution became spontaneously turbid due to the formation, aggregation, and precipitation of large mixed micelles of detergent that contained hydrophobic protein and phenolic compounds. This solution was centrifuged at 10000g for 15 min at 25 °C. The detergent-rich phase was discarded, and the clean supernatant containing the banana PPO was subjected to a different aqueous two-phase system based on PEG 8000 (5% w/w)/potassium phosphate buffer (pH 7.0). After 15 min of stirring at room temperature, the solution was centrifuged at 10000g for 10 min at 25 °C. The upper PEG-rich phase was discarded and the phosphate-rich phase containing the banana PPO brought to 15% saturation with solid $(NH_4)_2SO_4$ under continuous stirring at 4 °C. After 1 h, the solution was centrifuged at 60000g for 30 min at 4 °C, and the pellet was discarded. $(NH_4)_2SO_4$ was added to the clean supernatant to give 30% saturation and stirred for 1 h at 4 °C. The precipitate obtained between 15 and 35% was collected by centrifugation at the same rotor speed and dissolved in a minimal volume of 100 mM sodium phosphate buffer (pH 7.3). The enzyme was stored at −20 °C. This simple and fast protocol gave a 5-fold purification and 10-fold activation with SDS of the enzyme extract, which preserved its monophenolase activity (Sojo et al., 1998b). The purified enzyme used in this paper has a specific activity of 51.92 enzyme units (EU)/mg on dopamine as substrate.

Enzyme Activity. The oxidation of the two diphenolic substrates used was measured spectrophotometrically, in a Uvikon 940 (Kontron Instruments) or HP8452A (Hewlett-Packard) diode array spectrophotometer, at their respective absorption maxima, which are 475 nm for dopaminochrome (ϵ = 3300 M^{-1} cm^{-1}) and 400 nm for salsolinol-o-quinone.

The salsolinol-o-quinone absorption coefficient (ϵ) was calculated at different pH values (3.5−8.5) with increasing concentrations of SAL, an excess of metaperiodate acting as chemical oxidant, and different 10 mM buffers depending on the pH values. The standard reaction medium at 25 °C contained 150 μg/mL PPO (0.05 EU), 2 mM SDS to activate latent enzyme (Sojo et al., 1998a), and 4 mM SAL in 10 mM acetate buffer (pH 5.0) in 1 mL. The reference cuvette contained all of the components of the reaction medium except the enzyme. One EU is the amount of enzyme that produces 1 μmol of salsolinol-o-quinone per minute. The phenolic solutions were freshly prepared.

PPO activity was also determined by monitoring O$_2$ consumption with a Clark-type electrode when dopamine and SAL were present in the same reaction medium. Samples of 2 mL were prepared containing 2 mM SDS and different concentrations of dopamine and salsolinol in 10 mM acetate buffer (pH 5.0) at 25 °C. These were transferred to the stirred thermostated oxygraph chamber (Hansatech Ltd., Norfolk, U.K.). The reaction was started by injection of the enzyme to reach a final concentration of 6 μg/mL. Quantitative measurements were made by using an oxygen calibration method (Rodriguez-López et al., 1992), and the activity was expressed as O$_2$ consumed in micromolar per minute.

The protein content was determined according to the Bradford (1976) dye binding method using bovine serum albumin (BSA) as a standard.

RESULTS AND DISCUSSION

Salsolinol, in contrast to other o-diphenols, is quite stable to autoxidation for periods of up to 10 h at pH
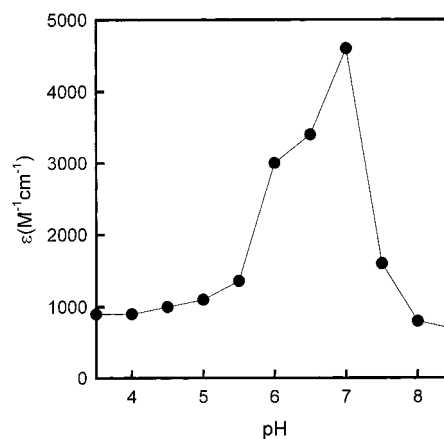

Figure 1. Effect of pH on SAL-o-quinone molar extinction coefficient. The values were obtained at 25 °C by changing SAL concentration (0−75 μM) in different 10 mM buffers (acetate, pH 3.5−5.5; phosphate, pH 6.0−7.5; and glycine−NaOH, pH 8.0−8.5) under a 5-fold excess (375 μM) of chemical oxidant, sodium metaperiodate (λ = 390 nm).

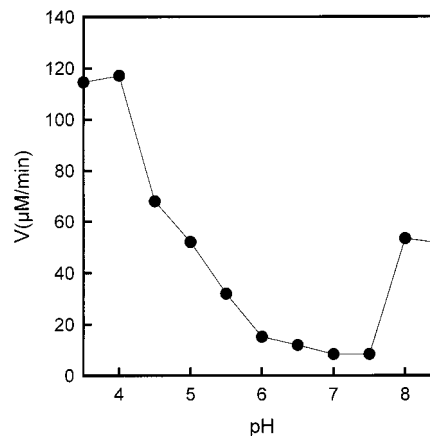**Figure 2.** Effect of pH on the oxidation of SAL by banana pulp PPO. The reaction medium at 25 °C contained 150 μg/mL PPO, 2 mM SDS, and 4 mM SAL in 10 mM acetate buffer (pH 3.5−5.5), 10 mM phosphate buffer (pH 6.0−7.5), or 10 mM glycine−NaOH buffer (pH 8.0−8.5), respectively.

values ranging from 7.0 to 4.0 (Fa and Dryhurst, 1991a). In the presence of a suitable catalyst it is oxidized to a wide range of products depending on pH, as has been shown in the exhaustive electrochemical study of Fa and Dryhurst (1991a−c). However, a detailed spectrophotometric study of its chemically generated quinone with pH has not been carried out. The results showed changes in the quinone extinction coefficient in the pH range 3.5−8.5 (Figure 1), the value being greater at neutral pH than at acidic pH.

Taking this into account, the effect of pH on the oxidation of SAL by banana pulp PPO was carried out. Figure 2 shows that enzymatic activity was greater at acid pH than at neutral, with a maximum at pH 4.0. However, at this pH the SAL-o-quinone was quite unstable (Figure 3, curve a) compared to that generated at pH 5.0 (Figure 3, curve b), which had a clear maximum at 400 nm (Figure 3, inset). This last datum, together with the fact that banana pulp has a physiological pH of 4.8 (Cano et al., 1990), makes pH 5.0 ideal for calculating the kinetic parameters V_m and K_m. As shown in Figure 4, the values obtained were 62.5 μM/min and 1.7 mM, respectively, using a Lineweaver−Burk plot (Figure 4, inset). These values cannot be
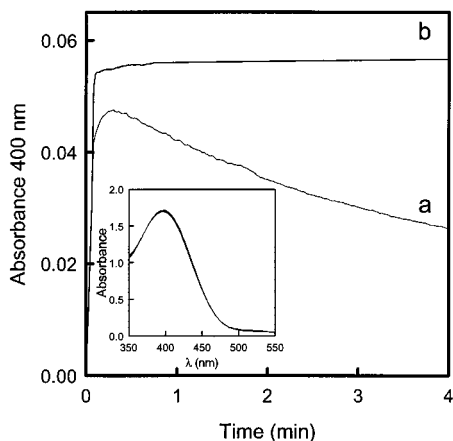
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Figure 3. Effect of pH on the stability SAL-*o*-quinone. The reaction medium at 25 °C contained 50 μM SAL and 375 μM IO_4^- in 10 mM acetate buffer (pH 4.0, curve a; and pH 5.0, curve b). (Inset) Stability of SAL-*o*-quinone at pH 5.0. The reaction medium at 25 °C contained 10 mM acetate buffer (pH 5.0), 1.5 mM SAL, and 1.5 mM IO_4^-.
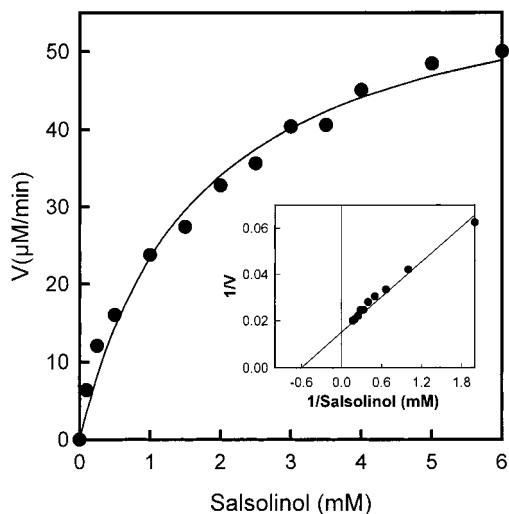

Figure 4. Effect of the SAL concentration on PPO activity at pH 5.0 The reaction medium at 25 °C contained 10 mM acetate buffer (pH 5.0), 2 mM SDS, 150 μg/mL PPO, and increasing concentrations of SAL (0–6 mM). (Inset) Lineweaver–Burk plot for calculating V_{max} and K_m.

compared because no data on SAL oxidation by any PPO have been reported in the literature.

When dopamine, SAL, and PPO were present in the reaction medium at pH 5.0 in order to imitate physiological conditions, the SAL-*o*-quinone maximum at 400 nm was the most surprising feature of the spectrum (Figure 5), with no contribution from dopaminechrome (475 nm) being observed compared with the same experiment in the absence of SAL (Figure 5, inset). These scans showed that dopaminequinone chemically oxidized SAL to SAL-*o*-quinone. To confirm this, the pH was increased to pH 7.0, at which value the intramolecular cyclization of dopaminoquinone to dopaminechrome is favored (García-Cánovas et al., 1982) compared with the external addition. At this pH, SAL-*o*-quinone and dopaminechrome coexist (Figure 6), with the latter making a substantial contribution to the spectrum compared with the contribution it makes during the oxidation of dopamine at pH 7.0 in the absence of SAL (Figure 6, inset). Both Figures 5 and 6 are compatible with Scheme 2, in which, at neutral pH
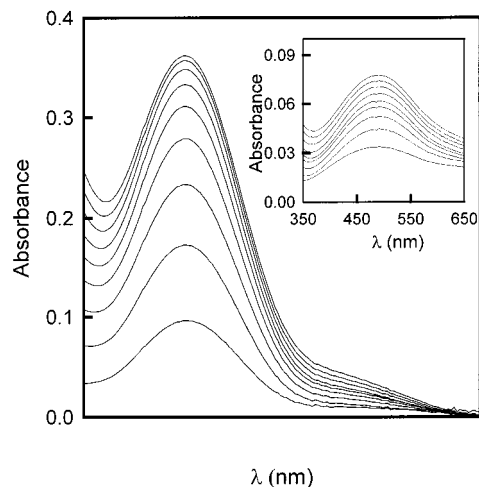

Figure 5. Effect of dopamine on SAL oxidation by PPO at pH 5.0. The reaction medium at 25 °C contained 10 mM acetate buffer (pH 5.0), 50 μg/mL PPO, 2 mM SDS, 1.5 mM SAL, and 0.1 mM dopamine. (Inset) Oxidation of dopamine by PPO at pH 5.0 in the absence of SAL. The reaction medium at 25 °C contained 10 mM acetate buffer (pH 5.0), 0.1 mM dopamine, 2 mM SDS, and 5 μg/mL PPO. The time between scans was 20 s.
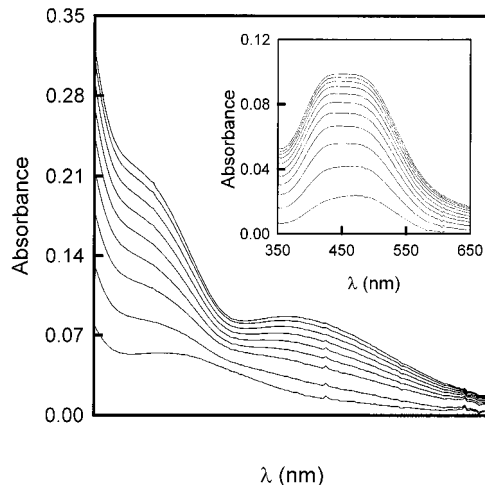

Figure 6. Effect of dopamine on SAL oxidation by PPO at pH 7.0. The reaction medium at 25 °C contained 50 μg/mL PPO, 2 mM SDS, 1.5 mM SAL, and 0.1 mM dopamine in 10 mM phosphate buffer (pH 7.0). (Inset) Oxidation of dopamine by PPO at pH 7.0 in the absence of SAL. The reaction medium at 25 °C contained 0.1 mM dopamine, 2 mM SDS, and 5 μg/mL PPO in 10 mM phosphate buffer (pH 7.0). The time between scans was 20 s.

values, there is competition between the cyclization of dopaminequinone-H^+ to dopaminechrome and the chemical oxidation of SAL to SAL-*o*-quinone mediated by dopaminequinone-H^+, both maxima developing at 400 and 475 nm (Figure 6). In contrast, at acidic pH values, the cyclization reaction is very slow and the cooxidation of SAL by dopaminequinone-H^+ prevails, giving a clear maximum at 400 nm (Figure 5). The coexistence of these two absorbing species, dopaminechrome and SAL-*o*-quinone, with different molar ratios depending on experimental conditions, hindered the use of spectrophotometric methods to follow this oxidation of SAL in the presence of dopamine. Thus, an alternative oxygraphic method was used.

Figure 7 shows how in the presence of SAL the oxidation of dopamine was greater as the concentration increased. Such activation found was in the range of

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Table 1. Oxygen Consumption during the Oxidation of Dopamine by Banana PPO in the Presence of SAL[a]

dopamine (mM)	O₂ consumption (μM/min) at 0 mM SAL	O₂ consumption (μM/min) at 1 mM SAL			O₂ consumption (μM/min) at 3 mM SAL		
		real	theoretical addition	ΔO₂ (activation fold)	real	theoretical addition	ΔO₂ (activation fold)
0	0	0.8			1.3		
0.05	13.1	16.7	13.9	1.2	20.5	14.4	1.4
0.1	27.4	38.0	28.2	1.4	41.7	28.7	1.5
0.15	36.2	49.6	37.0	1.3	54.7	37.5	1.5
0.25	60.9	71.7	61.7	1.2	75.0	62.2	1.2

[a] The real value corresponds to the oxygen measured in the different reaction conditions. Theoretical addition was calculated by the sum of the oxygen consumption obtained in the different reaction conditions with dopamine and SAL alone. The ΔO₂ was calculated as the ratio between the real value and the theoretical value. The reaction medium at 25 °C contained 2 mM SDS, 6 μg/mL banana PPO, an SAL concentration ranging from 0 to 3 mM, and a dopamine concentration ranging from 0 to 0.25 mM in 10 mM sodium acetate buffer (pH 5.0).
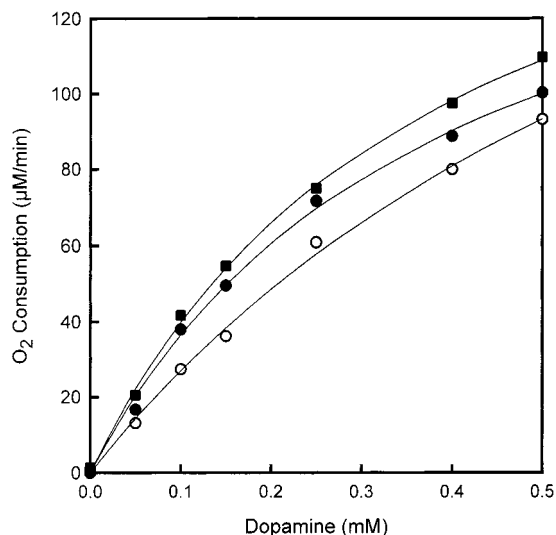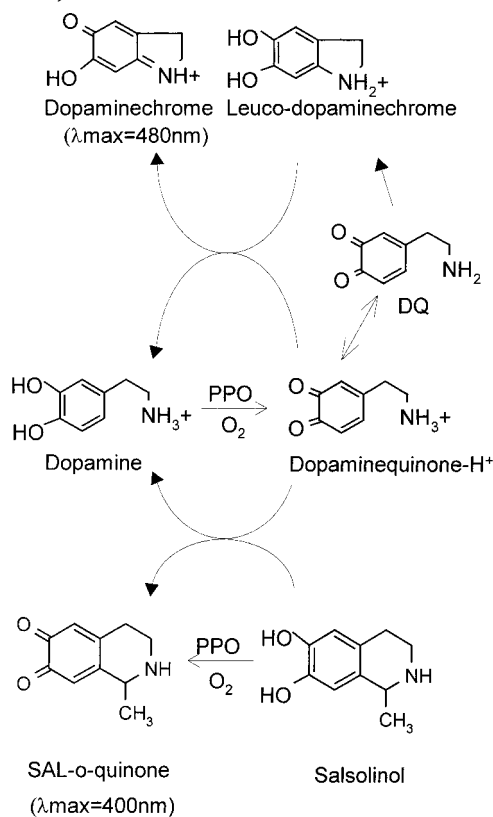

Figure 7. Activation of dopamine oxidation in the presence of SAL by PPO at pH 5.0. The reaction medium at 25 °C contained 10 mM acetate buffer (pH 5.0), 12 μg of PPO, 2 mM SDS, and increasing dopamine concentrations from 0 to 0.5 mM at different levels of SAL: none (○), 1 mM (●), and 3 mM (■).

1.4−1.5-fold (Table 1) when the ratio between real oxygen consumption in the presence of SAL was compared with the corresponding sum of the oxygen consumed during individual oxidations of SAL and dopamine (for example, 16.7 μM O₂/min in a reaction medium with 0.05 mM dopamine and 1 mM SAL, divided by the sum of 13.1 μM O₂/min for dopamine plus 0.8 for SAL). This unexpected increase in the activity of banana pulp PPO was not due to the chemical steps involved because only O₂ consumption was measured, but it does seem to be related with the enzyme.

This effect can be explained only by taking into account the kinetic mechanism described by Cabanes et al. (1987) [for a review see Sánchez-Ferrer et al. (1995)]. In this (Scheme 3), only the oxy- and met-forms of the enzyme are capable of binding the diphenol (Makino and Mason, 1973), whereas the deoxy-form binds molecular oxygen. This gives rise to two quinones and the consumption of one oxygen molecule per turnover. The increase in oxygen consumption according to this scheme can be explained only by a different combination of the two quinone-forming steps compared with what occurs with dopamine alone (steps 1 and 3) or with SAL alone (steps 2 and 4) as substrate. This new combination (step 1 plus step 4 or step 2 plus step 3) means that SAL opens up a faster way for the turnover of dopamine, which leads to higher oxygen
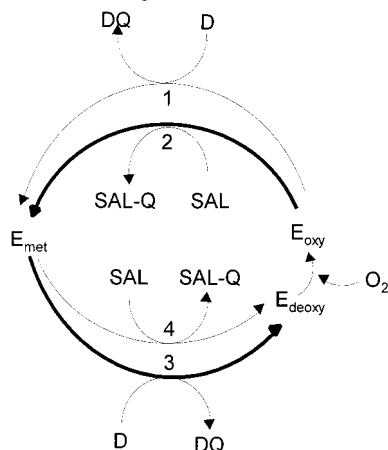
Scheme 2. Reaction Sequence of the Oxidation of SAL in the Presence of Dopamine and PPO (See Text for Details)

consumption. This phenomenon might be termed "kinetic synergism", in which a bad substrate speeds up the oxidation of a good substrate, in contrast to the competitive inhibition usually described (Laidler and Bunting, 1973). Even so, the level of activation is moderate, as can be seen from the data presented in Table 1. This degree of activation was up to 1.6 times greater than than with the best substrate (dopamine) compared with the rate observed in the presence of both good and bad (salsolinol) substrates. In addition, it should be noted that this effect also supposes a change from a competitive inhibition (Laidler and Bunting, 1973) to an activation. This work opens up the possibility of finding new combinations of substrates to augment this process.

This research shows for the first time that banana pulp PPO can oxidize SAL to its corresponding SAL-*o*-quinone and that this oxidation was enhanced by the presence of small amounts of dopamine. In this case, dopamine was first oxidized to dopaminequinone-H⁺,

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Scheme 3. Model for Explaining the Kinetic Synergism Found in the Oxidation of Dopamine in the Presence of SAL by PPO (See Text for Details)[a]

[a] D, dopamine; DQ, dopaminequinone, SAL, salsolinol; SAL-Q, salsolinol-*o*-quinone; E_{met}, enzyme in met-form; E_{deoxy}, enzyme in deoxy-form; E_{oxy}, enzyme in oxy-form.

which chemically co-oxidized SAL to SAL-*o*-quinone. In addition, when oxygen consumption was measured, a new unexpected phenomenon was found, in which SAL activated 1.4-fold the oxidation of dopamine to dopaminequinone by acting directly on the PPO catalytic cycle. This effect might explain the browning of banana during the postclimacteric stages, in which the level of SAL increases and the levels of PPO and dopamine decrease, indicating that, in pulp, not only does co-oxidation occur but a kinetic synergism takes place. A combination of both effects might act as a regulatory mechanism during postclimacteric stages.

ABBREVIATIONS USED

TIQ, tetrahydroisoquinolines; SAL, salsolinol (6,7-dihydroxy-1,2,3,4-isoquinoline); PPO, polyphenol oxidase; TX-114, Triton X-114; PPO, polyphenol oxidase; EU, enzyme unit; SDS, sodium dodecyl sulfate; PEG 8000, polyethylene glycol, MW 8000 Da.

LITERATURE CITED

Bordier, C. Phase separation of integral membrane proteins in Triton X-114 solution. *J. Biol. Chem.* **1981**, *256*, 1604–1607.

Bradford, M. M. A rapid and sensitive method for the quantification of microgrm quantities of proteins utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254.

Cabanes, J.; García-Cánovas, F.; Lozano, J. A.; García-Carmona, F. A kinetic study of the melanization patway between L-tyrosine and dopachrome. *Biochim. Biophys. Acta* **1987**, *923*, 187–195.

Cano, P.; Marín, M. A.; Fúster, C. Effects of some thermal treatments on polyphenol oxidase and peroxidase activities of banana (*Musa cavendishii*, var *enana*). *J. Sci. Food Agric.* **1990**, *51*, 223–231.

Deng, Y.; Maruyama, W.; Kawai, M.; Dostert, P.; Yamamura, H.; Takahashi, T.; Naoi, M. Assay for the (R)- and (S)-enantiomers of salsolinols in biological samples and foods with ion-pair high-performance liquid chromatography using α-cyclodextrin as a chiral mobile phase additive. *J. Chromatogr. B* **1997**, *689*, 313–320.

Dostert, P.; La Croix, R.; Maruyama, W.; Naoi, M.; Strolin-Benedetti, M. Effects of dopamina-derived isoquinolines on hydroxyl radical formation by dopamine autoxidation. *Biogenic Amines* **1996**, *12*, 149–460.

Fa, Z.; Dryhurst, G. Interactions of salsolinol with oxidative enzymes. *Biochem. Pharmacol.* **1991a**, *42*, 2209–2219.

Fa, Z.; Dryhurst, G. Electrochemical and enzyme-mediated oxidations of tetrahydropapaveroline. *J. Org. Chem.* **1991b**, *56*, 7113–7121.

Fa, Z.; Dryhurst, G. Oxidation chemistry of the endogenous central nervous system alkaloid salsolinol. I. Electrochemical studies. *Bioorg. Chem.* **1991c**, *19*, 384–397.

Galeazzi, M. A.; Sgarbieri, V. C.; Constantinides, S. M. Isolation, purification and physicochemical characterization of polyphenol oxidases (PPO) from a Dwarf variety of banana (*Musa cavendishii*, L.). *J. Food Sci.* **1981**, *46*, 150–155.

García-Cánovas, F.; García-Carmona, F.; Vera, J.; Iborra, J. L.; Lozano, J. A. The role of pH in the melanine biosynthesis pathway. *J. Biol. Chem.* **1982**, *257*, 8738–8744.

Giami, S.; Alu, D. Changes in composition and certain functional properties of ripening plantain (*Musa spp.*, AAB group) pulp. *Food Chem.* **1994**, *50*, 137–140.

Laidler, K.; Bunting, P. S. *The Chemical Kinetic of Enzyme Action*; Clarendon Press: Oxford, U.K., 1973.

Makino, N.; Mason, H. S. Reactivity of oxytyrosinase toward substrates. *J. Biol. Chem.* **1973**, *248*, 5731–5735.

Riggin, R. M.; McCarthy, M. J.; Kissinger, P. T. Identification of salsolinol as a major dopamine metabolite in the banana. *J. Agric. Food Chem.* **1976**, *24*, 189–191.

Rodríguez-López, J. N.; Ros-Martínez, J. R.; Varón, R.; García-Cánovas, F. Calibration of a Clark-type oxygen electrode by tyrosinase-catalized oxidation of 4-*tert*-butylcatechol. *Anal. Biochem.* **1992**, *202*, 356–360.

Sánchez-Ferrer, A.; Rodríguez-López, J. N.; García-Cánovas, F.; García-Carmona, F. Tyrosinase: a comprehensive review of its mechanism. *Biochim. Biophys. Acta* **1995**, *1247*, 1–11.

Sandler, M.; Bonham Carter, S.; Hunter, H. R.; Stern, G. M. Tetrahydroisoquinoline alkaloids: in vivo metabolites of L-Dopa in man. *Nature* **1973**, *241*, 439–443.

Sojo, M. M.; Nuñez-Delicado, E.; Sánchez-Ferrer, A.; García-Carmona, F. Partial purification of a banana polyphenol oxidase using Triton X-114 and PEG 8000 for removal of polyphenols. *J. Agric. Food Chem.* **1998a**, *46*, 4924–4930.

Sojo, M. M.; Nuñez-Delicado, E.; Sánchez-Ferrer, A.; García-Carmona, F. Monophenolase activity of latent banana pulp polyphenol oxidase. *J. Agric. Food Chem.* **1998b**, *46*, 4931–4936.

Whaley, W. M.; Govindachari, T. R. The Pictet–Spengler synthesis of tetrahydroisoquinolines and related compounds. *Org. React.* **1951**, *6*, 151–190.