Activation of a Latent Mushroom (*Agaricus bisporus*) Tyrosinase Isoform by Sodium Dodecyl Sulfate (SDS). Kinetic Properties of the SDS-Activated Isoform

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This study reports the activation of a latent mushroom tyrosinase isoform by sodium dodecyl sulfate (SDS). The activation process of latent mushroom tyrosinase by SDS is characterized by the presence of a lag period (τ) prior to the attainment of a steady-state rate (V_{ss}). This could be related to a slow conformational change of the latent enzyme to render the active isoform. The molecular size of the latent isoform was 67 kDa as determined by SDS−PAGE and western-blotting assays. This size did not change after activation by SDS. The molecular size of the protease-activated isoform was 43 kDa. τ and V_{ss} displayed a sigmoidal relationship to the concentration of SDS, but τ was not dependent on *o*-diphenol or enzyme concentration. Increasing SDS concentrations decreased τ, but then lower V_{ss} values were detected because of a possible excess of unfolding and subsequent denaturation of the protein. The same reaction mechanism operated in both SDS-activated and protease-activated tyrosinase isoforms despite their different kinetic features. A possible mechanism for the activation of this latent tyrosinase by SDS is proposed.

INTRODUCTION

Enzymatic browning in fruits and vegetables is predominantly catalyzed by a copper-containing enzyme, tyrosinase (EC 1.14.18.1; tyrosine, L-Dopa:oxygen oxidoreductase; catecholase, diphenol oxidase, polyphenol oxidase). Tyrosinase catalyzed in the presence of molecular oxygen the hydroxylation of monophenols (monophenolase activity) and the oxidation of *o*-diphenols (diphenolase activity) to *o*-quinones. The latter chemically evolve to give rise to melanins, which are brown, black, or red heterogeneous polymers responsible for the loss of quality in many crops (Prota, 1988; Martínez and Whitaker, 1995; Sánchez-Ferrer et al., 1995).

This enzyme is present throughout the phylogenetic tree (mammals, fungi, plants, arthropods...). It can be found in either a latent or an active form as well as in both forms at the same time in many sources (Whitaker, 1995). Mushroom (*Agaricus bisporus*) tyrosinase is present as a latent form in ~98−99% of total tyrosinase activity (Yamaguchi et al., 1970; van Leeuwen and Wichers, 1999).

Latent tyrosinases can be activated by different treatments such as acid shock (Kenten, 1957), fatty acids (Sugumaran and Nellaiappan, 1991), detergents (Moore and Flurkey, 1990; Marques et al., 1995; Nellaiappan and Sugumaran, 1996), alcohols (Asada et al., 1993) and proteases (King and Flurkey, 1987; Robinson and Dry, 1992; Chosa et al., 1997). This activation can also result from the attack of pathogens (Soler-Rivas et al., 1997).

The activation of latent tyrosinases from several sources by sodium dodecyl sulfate (SDS) has been well reported (Moore and Flurkey, 1990; Nellapaian and Sugumaran, 1996; Chazarra et al., 1996; Jiménez and García-Carmona, 1996; Escribano et al., 1997). It is assumed that SDS binds to the latent enzyme below its critical micellar concentration and induces conformational changes that cause the activation of the latent enzyme (Moore and Flurkey, 1990; Nellapaian and Sugumaran, 1996). Moreover, it has been previously reported that the behavior of tyrosinase at different pH values could depend on the agent used to induce the activation (SDS or trypsin) (Jiménez and García-Carmona, 1996).

The aim of the study presented here is to characterize the activation of a latent mushroom tyrosinase by SDS. Kinetic characteristics of the SDS-activated enzyme are compared to those of a tyrosinase isoform that was activated by endogenous proteases. The previously reported kinetic reaction mechanism for tyrosinase (Rodríguez-López et al., 1992; Ros et al., 1994a; Espín et al., 1995a,b, 1996, 1997a−f, 1998a−d) is compared to both tyrosinase isoforms. A possible mechanism for the activation by SDS is proposed.

MATERIALS AND METHODS

Reagents. 4-*tert*-Butylcatechol (TBC), L-DOPA, dopamine, tyramine, and SDS were purchased from Sigma (Holland). Ampholites for preparative isoelectric focusing were obtained from Bio-Rad (Grand Junction, CO). All other reagents were of analytical grade and also supplied by Sigma. Milli-Q system (Millipore Corp., Bedford, MA) ultrapure water was used throughout this research.

Preparation of a Latent Mushroom Tyrosinase Isoform. Boxes of compost spawned with U1 *Agaricus bisporus* and covered with casing soil from the Mushroom Experimental Station (Horst, The Netherlands) were transported, after bud initiation, to a climate room at ATO-DLO (18 °C, 80% relative

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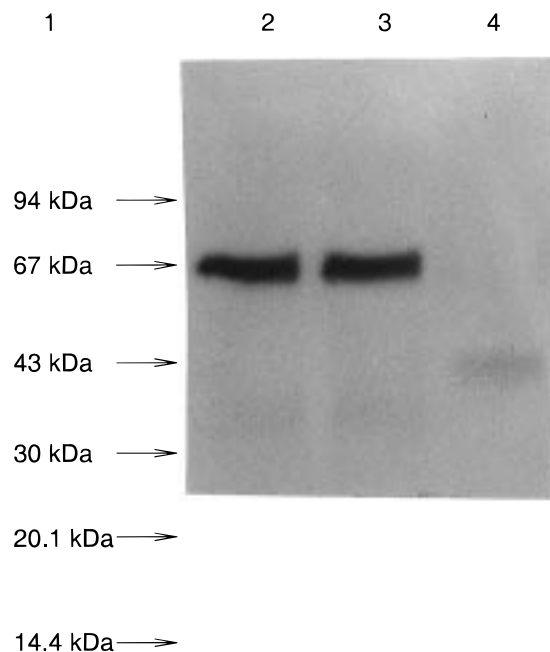
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1 2 3 4

94 kDa →

67 kDa →

43 kDa →

30 kDa →

20.1 kDa →

14.4 kDa →

Figure 1. Identification of mushroom tyrosinase by Western blotting on SDS–PAGE: (lane 1) markers; (lane 2) latent mushroom tyrosinase (10 μg/mL); (lane 3) mushroom tyrosinase (10 μg/mL) activated by SDS (0.35 mM); (lane 4) protease-activated mushroom tyrosinase (1 μg/mL). Markers (Pharmacia), which were stained with Coomassie blue, were phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa). See Materials and Methods for details.

humidity). Mushrooms were harvested at stage 5 (Hammond and Nichols, 1976) and frozen in liquid nitrogen immediately after picking. The freeze-dried mushrooms were ground under liquid nitrogen to a fine powder in a mortar and pestle. The powder was rehydrated with 10 mM PB (sodium phosphate buffer) containing 10 mM ascorbic acid and mixed thoroughly on a vortex shaker. This homogenate was then extracted for 25 min on ice and centrifuged at 12000*g* for 10 min.

The supernatant was immediately applied to an anion exchange column (DEAE-Sepharose Fast Flow, length = 75 cm, diameter = 5 cm, Pharmacia, Uppsala, Sweden). The column was previously equilibrated with 20 mM BIS-TRIS buffer, pH 6, and eluted with a stepwise gradient of increasing sodium chloride (NaCl) concentrations (3 mL/min). The different fractions were assayed with TBC in the absence and in the presence of SDS to discriminate between active and latent tyrosinase isoforms. A major latent isoform with an isoelectric point of 5.6 (determined by analytical isoelectric focusing, results not shown) was eluted at 50 mM NaCl concentration. The proportion of latent tyrosinase (100%) was determined by comparing the activity in the presence and in the absence of SDS. This latent tyrosinase isoform showed a band of 67 kDa determined by SDS–PAGE and Western-blotting analysis (Figure 1).

Preparation of a Protease-Activated Tyrosinase Isoform. The homogenate was obtained and centrifuged as described above. After centrifugation, the supernatant was kept at room temperature for 2 h and further dialyzed against ultrapure water for 1 day at 4 °C by using a dialysis tube of 12 kDa cutoff, which allowed removal of the components with low molecular weight. However, proteases which are abundant in the *Agaricus* genus (Burton et al., 1997) were not removed. During the dialysis process, in these conditions the proportion of latent tyrosinase decreased until it completely disappeared. The extract with fully active tyrosinase was applied to a Rotofor preparative isoelectric focusing system (Bio-Rad). The cell was loaded with 3.4 mL of Bio-Lyte ampholites, pH range 4–6, and 0.55 mL of Tween 20 in 45 mL of ultrapure water.

Focusing conditions were controlled by limiting the power to 12 W (maximum = 2000 V and 20 mA). The sample was focused for 4 h. The fraction with tyrosinase activity was dialyzed against ultrapure water to remove ampholites according to the manufacturer's instructions. Several isoforms were detected, of which the most abundant (∼80% of activity) showed an isoelectric point of 4.5. Addition of SDS to the fraction did not increase the activity but even decreased it. This fully active tyrosinase isoform showed a band of 43 kDa determined by SDS–PAGE and Western-bloting analysis (Figure 1). This isoform will be hereafter termed "protease-activated isoform".

Electrophoresis. SDS–PAGE electrophoresis experiments were performed under denaturing conditions in 10% polyacrylamide gels with a minigel Bio-Rad system. Samples were diluted with 50 mM Tris-chlorhydric buffer (Tris-HCl buffer, TCB), pH 7, containing 0.5 mM β-mercaptoethanol, 2% SDS, 1% bromophenol blue, and 10% glycerol. Electrophoresis was conducted at a constant voltage of 200 V in a buffer (pH 6.8) containing 3 g/L Tris base, 14.4 g/L glycine, and 1 g/L SDS. For molecular mass determination, the calibration kit of SDS–PAGE standards (low range of M_r) from Bio-Rad was used. After electrophoresis, the gels were equilibrated for 30 min at 4 °C in transfer buffer (TB) containing 3 g/L Tris base, 14.4 g/L glycine, and 20% methanol (v/v). Then the gels were blotted onto an Immobilon-P transfer membrane (PVDF) for 1 h at constant voltage of 100 V in a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad). The ECL protocol (Amersham International, Buckinghamshire, U.K.) was followed to develop tyrosinase bands on the gel. This method is based on light emission for detection of immobilized specific antigens. After electroblotting, the membrane was rinsed in Tris-HCl buffer saline (TBS) and incubated in the block solution (low-fat dried milk) for 1 h. After incubation, the membrane was washed in Tris-HCl buffer saline with 0.05% Tween 20 (TBST) for 25 min and incubated with the first antibody (polyclonal antibody anti-tyrosinase developed in mouse; AbPPO) diluted in TBST solution for 2 h. After incubation with AbPPO, the membrane was washed in TBST for 25 min and then incubated with the secondary antibody (goat anti-mouse IgG conjugated peroxidase antibody; HRP-Ab) diluted in TBST solution for 1 h. After incubation with HRP-Ab, the membrane was washed in TBST for 35 min. To detect the bands, the reagents of the kit were mixed (1:1) according to the manufacturer's instructions. After 1 min of incubation, the membrane was immediately exposed to a photographic film for 1 min.

Enzymatic Assays. Tyrosinase activity on TBC was monitored spectrophotometrically at 400 nm by measuring the accumulation of 4-(*tert*-butyl)benzo-1,2-quinone (TBQ). This *o*-quinone was highly stable at every pH assayed and for a longer period than those used in the activity measurements (Waite, 1976; Ros et al., 1994b; Escribano et al., 1997). One unit of active form of tyrosinase was defined as the amount of the enzyme that produces 1 μmol of TBQ per minute. The final volume of assay was 1 mL.

Tyrosinase activity on L-DOPA (484 nm), dopamine (476 nm), and tyramine (476 nm) was determined by using 3-methyl-2-benzothiazolinone hydrazone (MBTH), which is a potent nucleophile that traps the enzymatically generated *o*-quinones to render MBTH-quinone adducts with high molar extinction coefficients (Espín et al., 1995a, 1996, 1997a, 1998a).

The spectrophotometric assays were recorded in an ultraviolet–visible Perkin-Elmer Lambda-2 spectrophotometer (Überlingen, Germany), on-line interfaced to a Pentium-100 microcomputer (Ede, The Netherlands). Temperature was controlled at 25 °C with a circulating bath with heater/cooler and checked using a precision of ±0.1 °C.

Kinetic Data Analysis. The values of K_m and V_m were calculated from triplicate measurements of the steady-state rate, V_{ss}, for each initial substrate concentration ([S]$_0$). The substrate concentration to determine the kinetic constants K_m and V_{max} ranged from $K_m/5$ to $5K_m$ at every pH. The reciprocals of the variances of V_{ss} were used as weighting factors to the nonlinear regression fitting of V_{ss} versus [S]$_0$ to the Michaelis equation (Endrenyi, 1981). Curve-fitting was carried out by

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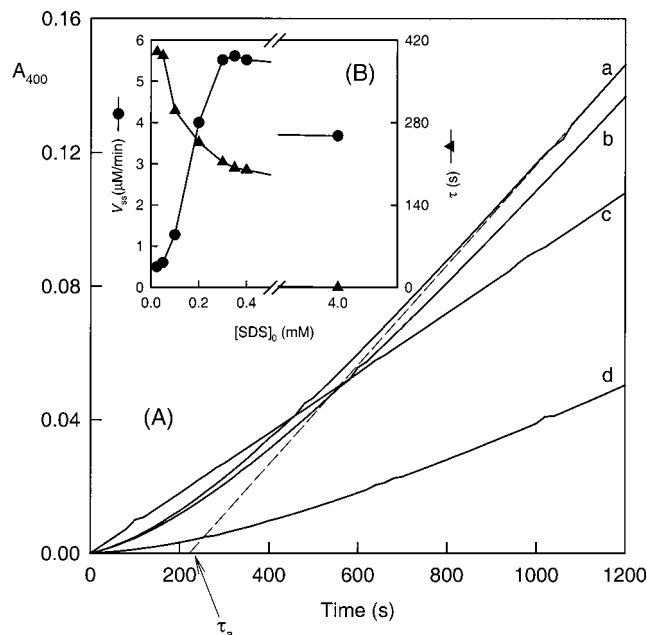
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Figure 2. (A) Spectrophotometric recordings for the activation of a latent mushroom tyrosinase isoform by SDS. Conditions were as follows: PB, 50 mM, pH 6; TBC, 0.8 mM; 0.05 μg/mL latent tyrosinase; and SDS (a) 0.35 mM, (b) 0.3 mM, (c) 4 mM, and (d) 0.1 mM. (B) Dependence of V_{ss} (●) and τ (▲) on [SDS]$_0$. Conditions were as in (A).

using a Gauss–Newton algorithm (Marquardt, 1963) implemented in the Sigma Plot 2.01 program for Windows.

Experimental data from thermal inactivation experiments were fitted by nonlinear regression to a decreasing uniexponential equation:

$$S = S_0(e^{-\lambda t}) \qquad (1)$$

The apparent constant that describes the velocity of thermal inactivation (λ) was calculated as well as the time required to inactivate half of the enzyme ($t_{1/2}$).

Other Methods. Protein content was determined by using the method of Bradford (1976) using bovine serum albumin as standard.

RESULTS AND DISCUSSION

Activation Assays. The activation of latent tyrosinases by SDS has been widely and well reported (Moore and Flurkey, 1990; Nellapaian and Sugumaran, 1996; Chazarra et al., 1996; Jiménez and García-Carmona, 1996; Escribano et al., 1997). However, this activation has not been systematically studied for latent mushroom tyrosinase.

Increasing SDS concentrations in the activation process of the latent isoform yielded increasing steady-state rates (V_{ss}) after a lag period (τ) (Figure 2A). To our knowledge, this behavior in the activation by SDS has not been previously reported for any latent enzyme. If the activation process is monitored spectrophotometrically over a too short period, then the V_{ss} values are underestimated because the steady state is not properly reached. In this way, in Figure 2A, in the first 200 s, the maximum velocity is observed for the curve with 4 mM SDS (Figure 2A, curve c). However, it is clear that the highest steady-state rate was reached with 0.35 mM SDS (Figure 2A, curve a). The lag period decreased with increasing SDS concentrations with a concomitant increase in the final steady-state rate. At a certain SDS concentration, the lag period was abolished but V_{ss}
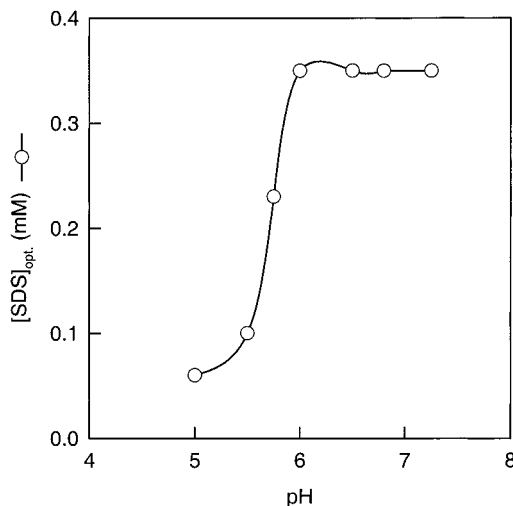

Figure 3. Dependence of optimum SDS concentration ([SDS]$_{opt}$) on pH. Conditions were as follows: AB, 50 mM, pH 5 and 5.5; PB, 50 mM, pH 5.75–6.8; TBC, 4 mM; and 0.07 μg/mL latent mushroom tyrosinase.

decreased (Figure 2B). The typical sigmoid curve in the activation by SDS (Figure 2B) has also been reported in the activation of other latent tyrosinases (Moore and Flurkey, 1990; Nellaiappan and Sugumaran, 1996).

The optimum of 0.35 mM for the SDS concentration was found for pH ≥6 (Figure 3).

The SDS-activated enzyme showed a pH optimum (6.0) different from that of the protease-activated isoform (6.8) (Figure 4). This could be related to the displacement of the sensitive pK_a values of the enzyme caused by the interaction with SDS molecules (Jiménez and García-Carmona, 1996) (Figure 3). The optimum pH for the protease-activated isoform corresponded to what was previously reported for commercial tyrosinases (Ros et al., 1994a; Espín et al., 1997b) (Figure 4). The lag period changed with a sigmoid profile versus pH, showing the above-mentioned effect of SDS and pH. The latent isoform without SDS did not show any activity at the optimum pH for the SDS-activated isoform. This latent enzyme showed a small peak of activity at pH 4–4.5 because of acid shock (Figure 4A) (Kenten, 1957). The difference found in the optimum pH could also be related to the different conformation of the active site of the enzyme. The tyrosinase isoforms that we are comparing differ in the way they have been activated to render the final active isoform. Latent tyrosinase represents ~98–99% of the total tyrosinase activity in mushrooms (Yamaguchi et al., 1970; van Leeuwen and Wichers, 1999). SDS, below its micellar concentration, (Moore and Flurkey, 1990; Nellaiappan and Sugumaran, 1996), induces a conformational change of the protein. This could give rise to an active isoform with catalytic properties different from those of the protease-activated isoform. The activation of latent tyrosinase by proteases implies a proteolytic cleavage in the protein with release of one or more peptides (Robinson and Dry, 1992; Chosa et al., 1997). Therefore, the presence of a different optimum pH could support the overall idea of one activator–one different active isoform.

The V_{ss} values in the activation process by SDS depended on the initial latent enzyme concentration (Figure 5). However, the period of time required to completely activate the latent enzyme (lag period) was the same for every latent enzyme concentration (Figure
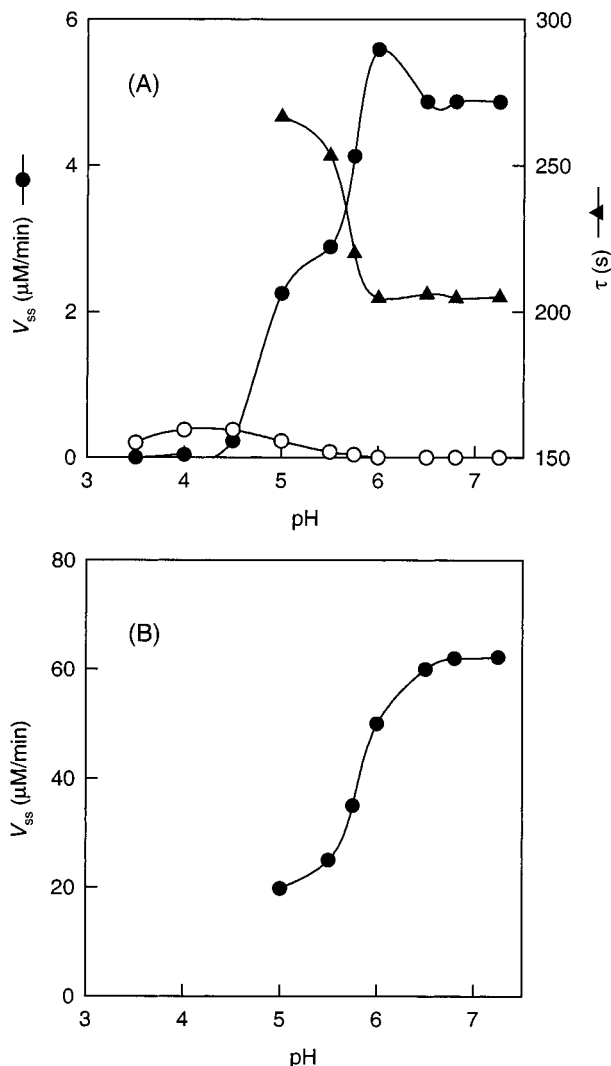
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Figure 4. (A) Dependence of V_{ss} (●, ○) and τ (▲) on pH in the activation of a latent mushroom tyrosinase by SDS. Conditions were as follows: AB, 50 mM pH 4−5.5; PB, 50 mM, pH 5.75−7.25; TBC, 0.8 mM, [SDS]$_{opt}$ at every pH (● = plus SDS; ○ = without SDS); and 0.05 μg/mL latent tyrosinase. (B) Dependence of V_{ss} (●) on pH in the activity of protease-activated tyrosinase. Conditions were the same as in (A) but without SDS, 1 mM TBC, and 0.6 μg/mL protease-activated tyrosinase.

5). This lag period was also independent of the nature of the *o*-diphenolic substrate as well as of its concentration (results not shown).

The preincubation of the latent isoform with 0.35 mM SDS (optimum concentration) induced the shortening of τ prior to the attainment of the same V_{ss} (Figure 6). The lag period was completely abolished after 15 min of preincubation. However, progress curves for the activation process showed that the complete activation was reached after ~3.5 min. A possible explanation is that the presence of traces of substrate could facilitate the interaction between the latent enzyme and SDS in a synergistic effect in a process that finally becomes independent of nature and substrate concentration.

Both final active isoforms differ in their thermal stabilities. Experiments of thermal inactivation were carried out at 50 °C. At several times, aliquots of both isoforms were removed to measure residual activity at their optimal assay conditions. Figure 7 shows that the thermal lability of the SDS-activated isoform was higher than that for the protease-activated isoform. Nonlinear
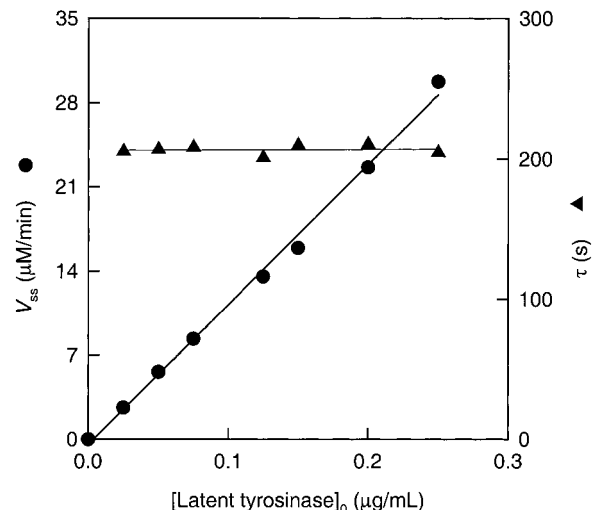
Figure 5. Dependence of V_{ss} (●) and τ (▲) on latent tyrosinase concentration in the activation of latent tyrosinase by SDS. Conditions were as follows: PB, 50 mM, pH 6; TBC, 0.8 mM; SDS, 0.35 mM; and 0.025−0.25 μg/mL latent tyrosinase.
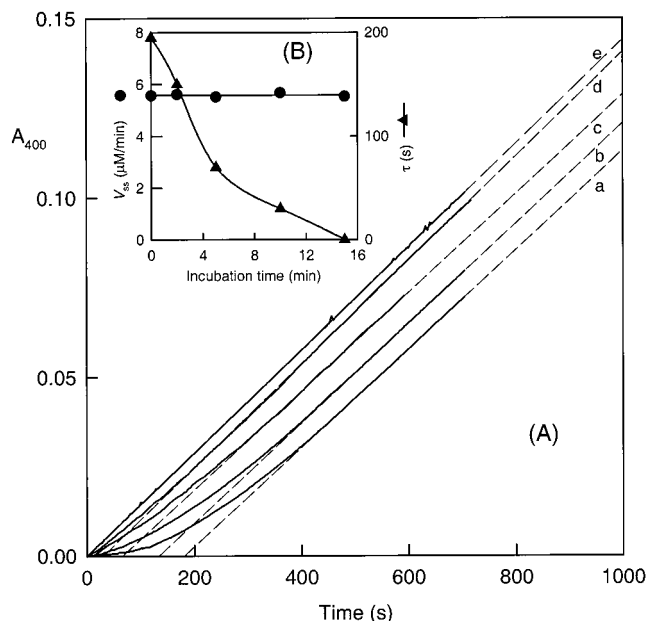

Figure 6. Preincubation of latent tyrosinase with SDS. (A) Spectrophotometric recordings for the activation of a latent mushroom tyrosinase by SDS with different preincubation times: (a) 0 min; (b) 2 min; (c) 5 min; (d) 10 min; (e) 15 min. (−) Experimental results; (- - -) linear regression fits to the final portions of the curves to estimate V_{ss} and τ. Conditions were as follows: PB, 50 mM, pH 6; SDS, 0.35 mM; TBC, 0.8 mM; and 0.05 μg/mL latent tyrosinase. (B) Dependence of V_{ss} (●) and τ (▲) on preincubation time of latent enzyme and SDS. Conditions were as in (A).

regression fits to eq 1 (see Materials and Methods) yielded the value of the apparent inactivation constant (λ) for the thermal inactivation of both isoforms. The $t_{1/2}$ values were 3.65 min for the SDS-activated enzyme (λ = 0.19 min^{-1}) and 11.55 min for the protease-activated enzyme (λ = 0.06 min^{-1}).

Kinetic Reaction Mechanism. To check the kinetic reaction mechanism previously proposed for tyrosinase (Rodríguez-López et al., 1992; Ros et al., 1994a; Espín et al., 1995a,b, 1996, 1997a−f, 1998a−d), V_{max} and K_m values were determined at different pH values for both tyrosinase isoforms on different substrates. Despite the different kinetic features of the SDS-activated isoform
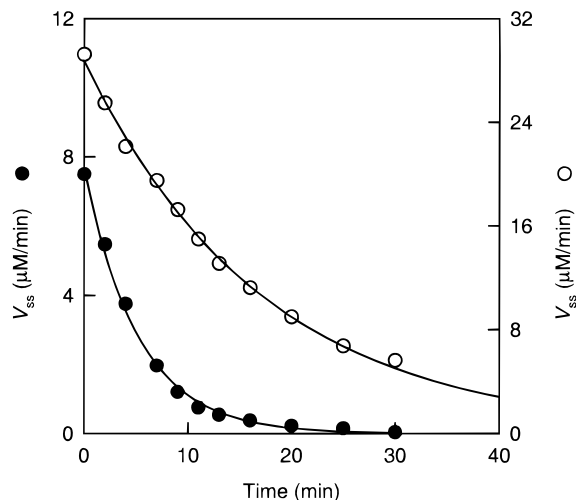
Figure 7. Thermal inactivation experiments for SDS-activated (●) and protease-activated (○) tyrosinase isoforms at 50 °C. Conditions for SDS-activated enzyme were as follows: PB, 50 mM, pH 6; SDS, 0.35 mM; TBC, 2 mM; and 0.07 μg/mL latent tyrosinase. $\lambda = 0.19$ min^{-1}; $t_{1/2} = 3.65$ min. Conditions for protease-activated enzyme were as follows: PB, 50 mM, pH 6.8; TBC, 2 mM; 0.3 μg/mL protease-activated tyrosinase. $\lambda = 0.06$ min^{-1}; $t_{1/2} = 11.55$ min.

with respect to protease-activated enzyme, the same reaction mechanism operated because the values of V_{max} remained constant with pH and the values of K_m increased when pH decreased (Figure 8). This phenomenon is explained by the reaction mechanism of tyrosinase. At low pH, tyrosinase is protonated and the affinity on the substrates is low, resulting in high K_m values (Figure 8). The effect of pH was related to two significant pK_a values in the case of the SDS-activated enzyme (5.8 and 6.3) and only one (5.8) in the case of the protease-activated enzyme (Figure 2). These pK_a values corresponded to the free enzyme forms and not to the enzyme–substrate complexes (Figure 8) (Ros et al., 1994a; Espín et al., 1995b, 1997b–f).

The monophenolase activity of the SDS-activated tyrosinase isoform was assayed with tyramine. Both the steady-state rate of the monophenolase activity (V_{ss}^M) and its lag period (τ^M) increased with monophenol concentration. V_{ss}^M linearly increased and τ^M was shortened with enzyme concentration; V_{ss}^M increased with the same sigmoid pattern as that for the diphenolase activity (activity on TBC, L-DOPA or dopamine). τ^M also increased with a sigmoid pattern on pH (results not shown). Therefore, the kinetics of the SDS-activated isoform corresponded to all previously reported characteristics (Rodríguez-López et al., 1992; Ros et al., 1994a; Espín et al., 1995a,b, 1997a–f, 1998a–d) for the monophenolase activity of tyrosinase. For all of these experiments, the latent enzyme was incubated with optimum SDS to eliminate the lag period. Thus, in the monophenolase activity only the characteristic lag period of this activity was observed. Some differences were observed when the monophenolase activity of both isoforms was assayed. For instance, different kinetic values were obtained for the monophenolase activity on tyramine: $V_{max} = 5$ μM/min and $K_m = 7$ mM when 0.5 μg/mL protease-activated isoform was assayed and $V_{max} = 0.8$ μM/min and $K_m = 5$ mM when 0.5 μg/mL SDS-activated isoform was assayed.

It is of note that this general reaction mechanism for tyrosinase has been corroborated for tyrosinases from many sources (mushroom, frog epidermis, grape, apple,
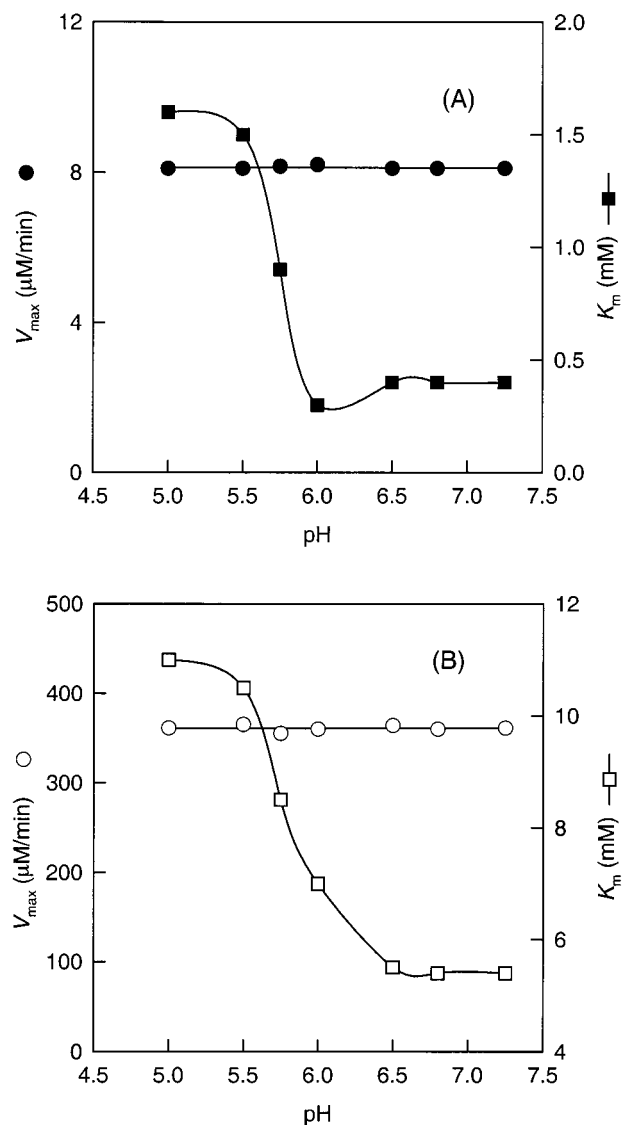

Figure 8. (A) Dependence of V_{max} (●) and K_m (■) on pH for the SDS-activated tyrosinase isoform. Conditions were as follows: AB, 50 mM, pH 5, 5.5; PB, 50 mM, pH 5.75–7.25; TBC concentration, from 5/K_m to 5K_m at every pH, [SDS]$_{opt}$ at every pH; and 0.05 μg/mL latent tyrosinase. (B) Dependence of V_{max} (○) and K_m (□) on pH for the protease-activated tyrosinase isoforms. Conditions were the same as in (A) but without SDS and with 0.6 μg/mL active tyrosinase.

strawberry, avocado, pear, artichoke...) (Rodríguez-López et al., 1992; Ros et al., 1994a; Espín et al., 1995a,b, 1997a–f, 1998a–d). Every tyrosinase has its own kinetic features regarding optimum pH, different catalytic constant on substrates, etc., but all fully accomplished this mechanism. Therefore, it is logical that these active isoforms activated by different activators (SDS and endogenous mushroom proteases) can differ in their catalytic properties but fit the same general kinetic reaction mechanism.

Activation Mechanism. A tentative model to explain the activation of latent mushroom tyrosinase is shown in Figure 9. Taking into account that SDS binds to the enzyme to induce a conformational change which activates it (Nellapaian and Sugumaran, 1996), we could assume that this unfolding of the protein, to expose the active site, is rather slow. The molecular size of the latent isoform did not change after activation by SDS, which corresponds to an activation through a
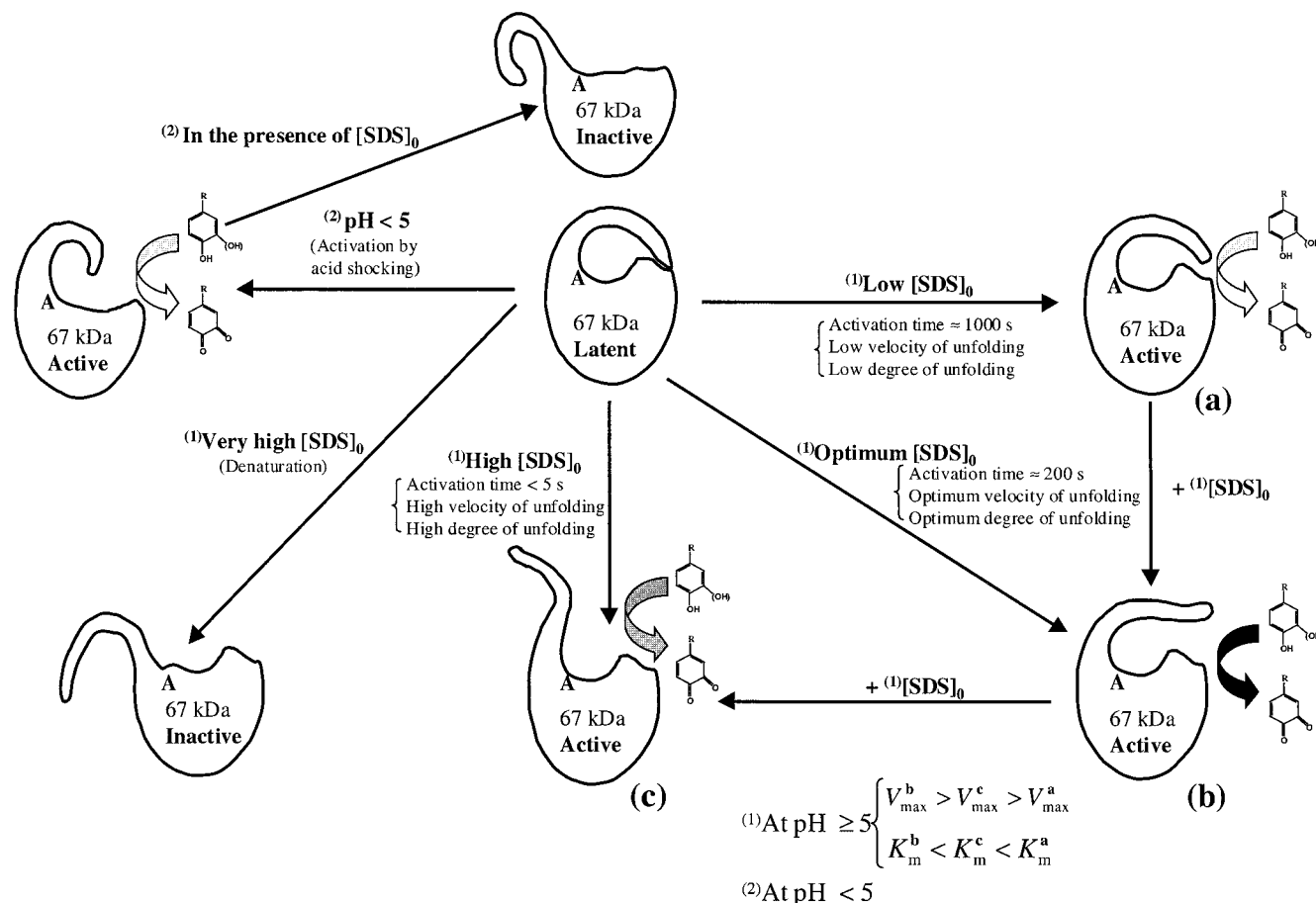
Figure 9. Tentative model to describe the activation of latent mushroom tyrosinase by SDS through a conformational change of the enzyme: (A) active site; $+ [SDS]_0$, increasing initial SDS concentration. The more densely shaded the thick arrow is, the higher the catalytic power of tyrosinase on its substrate (monophenol or o-diphenol) is. (1) Activation process at pH $\geq$5. (2) Activation process at pH <5.

conformational change (Figure 1). Depending on SDS concentration both the final unfolding and the velocity of the activation process changed, and a different enzyme resulted with an active site more or less available to the substrate. This unfolding can be accelerated by increasing SDS concentrations, but in this case, if the unfolding is too fast, a modified final active isoform might result with slightly different catalytic properties (Figure 9). V_{max} and K_m values for TBC with the SDS-activated isoform with 4 mM SDS were 5.6 μM/min and 1.7 mM, respectively, and 1.8 μM/min and 3 mM with 0.1 mM SDS, respectively. In the same assay conditions, these values for the SDS-activated isoform with 0.35 mM SDS (optimum concentration) were 8.2 μM/min and 0.3 mM, respectively. The isoform activated with 4 mM SDS showed less affinity on this substrate as well as less efficiency in the catalysis of its oxidation. The unfolding of the enzyme in the presence of high SDS concentrations (4 mM) cannot be followed by standard spectrophotometry because of the long dead time to start the reaction (this dead time usually involves ~10−15 s to open the lid of the spectrophotometer, to insert the cuvette, to close the lid, and to start the recording of the data points). With this standard equipment, in these assay conditions, $\tau = 0$. However, we think that the determination of the transient phase before the enzyme is activated under these assay conditions could also be determined by using stopped-flow techniques.

At pH <5 a slight activation was observed due to acid shock (Figure 4A). However, in the presence of SDS, at

pH <5, negligible activity was observed. This could be due to a previously reported antagonistic effect between protons and SDS (Jiménez and García-Carmona, 1996). Maybe, proton activation involves (in the case of latent mushroom tyrosinase at pH <5) a fast and drastic conformational change. In the presence of SDS the unfolding is very high, which renders an inactive enzyme (Figure 9).

The protease-activated isoform showed a smaller molecular weight (43 kDa) than the latent isoform (67 kDa) (Figure 1). This is due to the proteolytic attack of the endogenous proteases of mushroom (Burton et al., 1997) to the latent tyrosinase. The kinetic and structural mechanisms for the activation of latent tyrosinase by proteases are currently under study.

The existence of this 67 kDa latent mushroom tyrosinase has not been previously reported. This finding agrees with the previously reported putative tyrosinase cDNA clone that encoded a protein of ~64 kDa (Wichers et al., 1995; van Gelder et al., 1997).

It is noteworthy that the SDS-activated isoform showed higher V_{max} values in the oxidation of dopamine and similar values in the oxidation of both TBC and L-DOPA. However, the K_m values were much lower for TBC than for L-DOPA and dopamine (results not shown). On the other hand, the protease-activated isoform showed V_{max} values much higher for TBC than for dopamine and L-DOPA. However, the K_m values were also much higher for TBC than for L-DOPA and dopamine (results not shown). This suggests a possible

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different active site with different preference for substrates, according to the protein's or the substrate's charge. This also supports the idea of one activator−one different active enzyme.

CONCLUSIONS

We can conclude the following from this study:

(1) The activation of a latent mushroom tyrosinase by SDS is provoked by a slow conformational change of the protein showing a characteristic lag period that was not previously reported (Figures 1, 2, 6, and 9). This activation process showed several dependencies on SDS concentration (Figures 2 and 3), pH (Figures 3 and 4), latent enzyme concentration (Figure 5), and substrate concentration.

(2) The same reaction mechanism operated in both SDS-activated and protease-activated tyrosinase isoforms (Figure 8), despite their different kinetic features (Figures 4−7).

(3) According to the results presented here (Figures 1−7), different final active tyrosinase isoforms can be obtained depending on the activator used ("one different activator−one different final active form").

(4) The understanding of the activation process of latent mushroom tyrosinase (≈98−99% in mushroom) is critical in the design of alternative tools to prevent enzymatic browning.

ABBREVIATIONS USED

AB, sodium acetate buffer; AbPPO, primary antibody (polyclonal antibody anti-tyrosinase developed in mouse); BIS-TRIS, (bis[2-hydroxyethyl]iminotris[hydroxymethyl]-methane); DMF, *N,N*-dimethylformamide; DEAE, diethylaminoethyl, L-DOPA, L-3,4-dihydroxyphenylalanine; dopamine, 3,4-dihydroxyphenethylamine; HRP-Ab, secondary antibody (goat anti-mouse IgG conjugated peroxidase antibody); K_m, Michaelis constant of active tyrosinase toward *o*-diphenol; M_r, molecular mass; MBTH, 3-methyl-2-benzothiazolinone hydrazone; PB, sodium phosphate buffer; PVDF, Immobilon-P transfer membrane, pore size 0.45 μm (Millipore); SDS, sodium dodecyl sulfate; [SDS]$_{opt}$, optimum SDS concentration; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel; τ, lag period; TB, transfer buffer, pH 8.3; TBC, 4-*tert*-butylcatechol; TBQ; 4-(*tert*-butyl)benzo-1,2-quinone; TBS, TCB saline, pH 7.5; TCB or Tris-HCl buffer, tris[hydroxymethyl]aminomethane chlorhydric buffer; Tris, tris[hydroxymethyl]aminomethane; TBST, TCB saline, pH 7.5, with 0.05% Tween 20; V_{ss}, steady-state rate; tyramine, 4-hydroxyphenethylamine; V_{max}, maximum steady-state rate.

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