Kinetic Study of the Activation Process of a Latent Mushroom (*Agaricus bisporus*) Tyrosinase by Serine Proteases

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Latent mushroom tyrosinase can be considered as a zymogen when activated by proteases because the activation process fulfilled all of the kinetic dependencies predicted by a theoretical zymogen activation model previously reported. The activation was studied under two assay conditions: high and low ratio of latent tyrosinase/serine protease (trypsin and subtilisin Carlsberg) concentrations, in the presence and in the absence of a serine protease inhibitor (aprotinin). The size of the latent enzyme was 67 kDa, determined by denaturing SDS−PAGE electrophoresis and Western blot assays. After proteolytic activation, the size was 43 kDa, with an intermediate band of 58 kDa. The values of the catalytic (k^S_{cat}) and Michaelis (K^S_m) constants for the active forms of tyrosinase resulting from the activation by subtilisin, trypsin, or sodium dodecyl sulfate on the substrate *tert*-butylcatechol were slightly different, which could support the idea of "one activator−one different active tyrosinase". Vacuum infiltration experiments tried to reproduce in vivo the role of mushroom serine proteases in the activation of latent tyrosinase. The use of serine protease inhibitors is proposed as a new alternative tool to prevent melanin formation.

Keywords: *Activation; Agaricus bisporus; kinetics; latent; serine proteases; tyrosinase*

INTRODUCTION

Tyrosinase or polyphenol oxidase (EC 1.14.18.1; PPO) is largely responsible for enzymatic browning in fruits and vegetables, which is mostly a commercially undesirable reaction in foods (Prota, 1988; Whitaker, 1995). Tyrosinase catalyzes the hydroxylation of monophenols to *o*-diphenols (monophenolase activity) and the oxidation of *o*-diphenols to *o*-quinones (diphenolase activity). The *o*-quinones produced are very reactive and can tan proteins by covalent bonds or autoxidize, producing brown pigments. The enzyme is ubiquitous and found in a variety of vertebrate, invertebrate, plant, and fungal organisms. It can be found in either a latent form or an active form. It is also possible that both forms occur at the same time (Whitaker, 1995).

Many enzymes occur as inactive precursors, named proenzymes or zymogens, to avoid undesirable intracellular reactions. Zymogen activation is involved in many physiological processes such as complement activation (Havsteen and Varón, 1990), proinsulin−insulin conversion (Mihalyi, 1972), blood clotting (Wohl et al., 1980), digestion of proteins in the intestine (Cohen, 1976), and pro-tyrosinase activation (Peñafiel et al., 1982; Galindo et al., 1983), among others. The kinetic analysis of several zymogen activation models has been previously reported in several theoretical studies (Varón et al., 1987, 1988, 1990, 1993; Vázquez et al., 1993).

Latent PPO from different sources can be activated by a variety of treatments including acid shock (Kenten, 1957), fatty acids (Sugumaran and Nellaiappan, 1991), detergents (Moore and Flurkey, 1990; Nellaiappan and Sugumaran, 1996), alcohols (Asada et al., 1993), and proteases (Galindo et al., 1983; King and Flurkey, 1987; Robinson and Dry, 1992; Chosa et al., 1997). This activation can also result from the attack of pathogens with the subsequent browning of the mushroom (Soler-Rivas et al., 1997).

During postharvest storage and senescence of mushrooms (*Agaricus bisporus*) several biochemical changes take place. There is a decrease of protein content upon storage, with a concomitant increase in the protease activity and browning of the mushroom (Rai and Saxena, 1988; Burton, 1988, Burton et al., 1993, 1994, 1997). A serine protease has been previously purified from senescent sporophores of *A. bisporus* (Burton et al., 1993). This protease (3% of soluble protein in postharvest mushrooms) was classified as a subtilisin-like serine protease according to its molecular weight (27 kDa), wide optimum pH (6.5−11), isoelectric point (8.9), and inhibitor sensitivity (PMSF).

Proteases from pathogens could also be involved in the activation of latent tyrosinase (*Verticillium fungicola* uses subtilisin-like serine proteases to attack the cell wall of the mushroom cells; Kalberer, 1984; Gea et al., 1995; Calonje et al., 1997; Leger et al., 1997).

Approximately 98−99% of tyrosinase in mushrooms occurs in a latent form (Yamaguchi et al., 1970; Van Leeuwen and Wichers, 1999). Therefore, the prevention of its activation is a possible approach to avoid further enzymatic browning in mushrooms. To properly approach the inhibition of activation of the latent tyrosinase, it is important to understand the activation process itself.

The aim of the work presented here is to kinetically characterize the activation process of latent mushroom tyrosinase by serine proteases (subtilisin Carlsberg and trypsin). The activation of the latent enzyme is fitted to a kinetic model in the presence and in the absence of

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a serine protease inhibitor (aprotinin). Moreover, the dependencies of some parameters of the activation process on $[Z]_0$, $[E]_0$, $[S]_0$, and temperature are studied with two sets of assay conditions in the activation process: low and high ratio of zymogen (latent tyrosinase) ($[Z]_0$) and serine protease ($[E]_0$) concentrations ($[E]_0 \gg [Z]_0$ and $[Z]_0 \gg [E]_0$, respectively), which might reflect microenvironmental conditions in the mushroom. Vacuum infiltration experiments are carried out to try to correlate in vitro activation experiments with possible in vivo situations to find possible physiological implications of such a system. The use of the serine protease inhibitors (illustrated with aprotinin) is proposed as an alternative tool to prevent enzymatic browning in mushrooms.

MATERIALS AND METHODS

Reagents. 4-*tert*-Butylcatechol (TBC), bovine pancreas trypsin (type XIII, treated with TPCK to remove contamination by chymotrypsin), subtilisin Carlsberg from *Bacillus licheniformis* (type VIII), and aprotinin from bovine lung were purchased from Sigma (St. Louis, MO). 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. Stock solutions of trypsin (1 mM) were prepared in 1 mM HCl and subtilisin solutions (1 mM) in water (Flannery et al., 1989).

Preparation of a Latent Mushroom Tyrosinase. Boxes of compost spawned with U1 *A. 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 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 with a mortar and pestle. The powder was rehydrated with 10 mM sodium phosphate buffer (PB) 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 equilibrated with 20 mM Bis-Tris buffer (pH 6). A stepwise gradient of increasing sodium chloride (NaCl) concentrations was applied (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 activities 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 blot analysis. 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 which encoded a protein of ~64 kDa (Wichers et al., 1995; van Gelder et al., 1997). After proteolytic activation, a final band of 43 kDa appeared (Figure 1) with an intermediate one of 58 kDa. Latent enzyme concentration was calculated by taking the value of $M_r = 67$ kDa.

Enzymatic Assays. The rate of active tyrosinase formation in the activation processes was estimated by coupling this process to the oxidation of TBC, which was used for the stability of the 4-(*tert*-butyl)benzo-1,2-quinone (TBQ) obtained as product of the tyrosinase activity (Waite, 1976; Ros et al., 1994a). To measure total tyrosinase activity, 0.35 mM SDS was included in the assay medium. Tyrosinase activity was determined by measuring TBQ accumulation at 400 nm. TBQ was very stable at pH 7 and at every temperature assayed (4−35 °C). One unit of active form of tyrosinase was defined as the amount of the enzyme that produces 1 μmol of TBQ per minute. The assay mixture contained Tris-HCl buffer (pH 7.0) to find a balanced medium for the optimum activity of the serine proteases (Bond, 1989; Espín and Tudela, 1994) and mushroom tyrosinase (Rodríguez-López et al., 1992; Ros et al., 1994b; Espín et al., 1997a,b, 1998a,d). The presence of chloride ions in the medium because of the Tris-HCl buffer did not affect the tyrosinase activity.

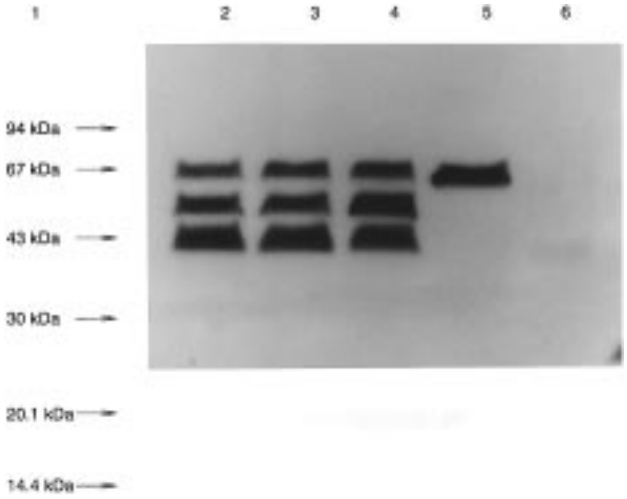


Figure 1. Identification of mushroom tyrosinase by Western blotting on denaturing SDS−PAGE: lane 1, markers; lanes 2−4, mushroom tyrosinase (10 μg/mL) activated by trypsin 10 nM (45, 15, and 5 min, respectively); lane 5, latent mushroom tyrosinase (10 μg/mL); lane 6, commercial (Fluka) tyrosinase (2 μg/mL). Markers (Pharmacia, Uppsala, Sweden), 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 Material and Methods for details.

The spectrophotometric assays were recorded in an ultraviolet−visible Perkin-Elmer Lambda-2 spectrophotometer (Überlingen, Germany), on-line interfaced to a 486-DX33 microcomputer (Ede, The Netherlands). Temperature was controlled (most experiments 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 α (active tyrosinase formed in the activation process) and λ (velocity of the activation process) were calculated from triplicate measurements with 500 data points per instrumental recording for the progress curve $[P] = -\beta + \alpha t + \beta\, e^{-\lambda t}$ (Varón et al., 1993; Vázquez et al., 1993) under the assay condition of $[E]_0$ (initial protease concentration) $\gg [Z]_0$ (initial zymogen concentration). The parameter *A* (which means also the active tyrosinase formed but in these assay conditions) was also calculated from triplicate measurements of the progress curve $[P] = At^2$ (Varón et al., 1993; Vázquez et al., 1993) under the assay conditions of $[Z]_0 \gg [E]_0$. Initial estimations of this parameter were calculated by plotting the first derivative of product accumulation versus time ($[P]' = 2At$). Mean values of the appropriate kinetic parameters are shown in the figures. The different kinetic constants were calculated by fitting experimental data by linear and nonlinear regression (Leatherbarrow, 1990) using an improved Gauss−Newton algorithm (Marquardt, 1963) implemented in the Sigma Plot 2.01 program for Windows (Jandel Scientific, 1994).

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-HCl buffer (pH 7), containing 0.5 mM β-mercaptoethanol, 2% SDS, 1% bromophenol blue, and 10% glycerol and further boiled. 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). The gels were then blotted onto an Immobilon-P transfer membrane with a pore size of 0.45 μm (Millipore) for 1 h at constant voltage of 100 V in a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad, Grand Junction, CO). The ECL protocol (Amersham International, Buckinghamshire, England) was followed to develop tyrosinase bands on the gel. This method makes use of emission for the detection of immobilized specific antigens. After electroblotting, the membrane was rinsed in Tris-buffered saline (TBS) and incubated in the block solution (low-fat dried milk) for 1 h. After incubation, the membrane was washed in TBS with 0.05% Tween 20 (TBST) for 25 min and incubated with the primary antibody (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 (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 manufacturer's instructions. After 1 min of incubation, the membrane was immediately exposed to a photographic film for 1 min.

Vacuum Infiltration Experiments. Vacuum infiltration is a technique widely used to study the effect of different compounds in fresh tissues (Burmeister and Dilley, 1993; Yunxia et al., 1995; Siddiqui and Bangerth, 1996). Mushrooms were placed in six different glasses that contained the different compounds: glass 1, water as control; glass 2, 3% DMF; glass 3, 0.35 mM SDS; glass 4, 3% DMF + 10 μM trypsin; glass 5, 3% DMF + 10 μM subtilisin; and glass 6, 3% DMF + 0.3 mM aprotinin. Vacuum was applied for 5 min in every treatment by using a vacuum pump. Air inside the tissues was replaced by the solutions. Therefore, the mushrooms were homogeneously infiltrated. This experiment was carried out five times.

Color Measurement. Color was determined with a Minolta Chrome Meter (CR-200) (Minolta Camera Co., Ltd., Japan). The CR-200 chrome meter has an 8 mm diameter measuring area and uses diffuse illumination and a 0° viewing angle (spectra component included) for accurate measurements of a wide variety of subjects. A pulsed xenon arc lamp in a mixing chamber provides diffuse, even illumination over the sample surface. Six highly sensitive silicon photocells, filtered to match the CIE (Commission Internationale de l'Eclaraige) Standard Observer Response, are used by the meter's double-beam feedback system to measure both incident and reflected light (Soler-Rivas, 1998). The CIE *Lab* color system most closely represents human sensitivity to color. The *L* parameter (lightness factor) measures the light on the scale of 0 (black) to 100 (white). $+a$, $-a$, $+b$, and $-b$ parameters indicate red, green, yellow, and blue colors, respectively. Color was measured in the center of the cap in both skin and flesh tissues. The total color difference can be measured as ΔE defined by the equation

$$\Delta E = \sqrt{(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2} \qquad (1)$$

(Burton and Noble, 1993; Smith et al., 1993; Soler-Rivas, 1998). On each sample a total of five readings were taken for every infiltration experiment. Therefore, final values reported fore each parameter are the mean of 25 readings.

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

RESULTS AND DISCUSSION

Activation of Mushroom Pro-tyrosinase. The overall reaction mechanism involved both activating and monitoring reactions. If rapid equilibrium conditions ($K_1^Z = K_m^Z$; $K_1^S = K_m^S$) are assumed, a kinetic model that fits to the latent tyrosinase activation process could be

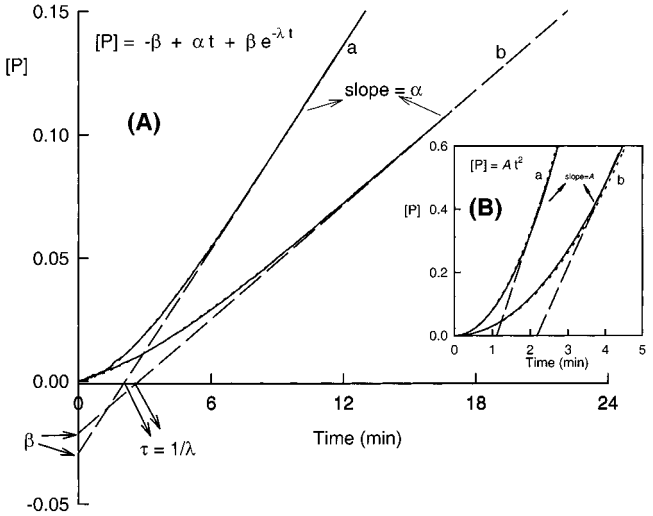
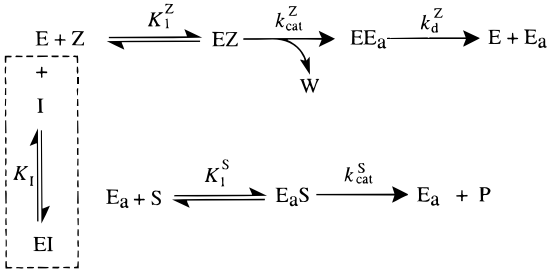


Figure 2. Progress curve for the activation of latent mushroom tyrosinase: **(**A**)** assay conditions were as follows: $[E]_0 \gg [Z]_0$, 50 mM Tris-HCl buffer (pH 7), 2.5 mM TBC, 3 nM latent tyrosinase, and (curve a) 7.4 μM subtilisin or (curve b) 8.3 μM trypsin; **(**B**)** assay conditions were as follows: $[E]_0 \ll [Z]_0$, 50 mM Tris-HCl buffer (pH 7), 2.5 mM TBC, 3 μM latent tyrosinase and (curve a) 7.4 nM subtilisin or (curve b) 8.3 nM trypsin; **(**—**)** experimental recordings; (- - -) linear regression fit to the steady state of the progress curves; (· · ·) nonlinear regression fit of the experimental recordings to eq 2 ($[E]_0 \gg [Z]_0$) and eq 6 ($[E]_0 \ll [Z]_0$) (see Materials and Methods).

Scheme 1



that shown in Scheme 1 (Varón et al., 1993; Vázquez et al., 1993), where Z is zymogen (pro-tyrosinase, latent tyrosinase), E is the activating enzyme (trypsin or subtilisin), W is one or more peptides released from Z during the formation of E_a, I is the reversible serine protease inhibitor (aprotinin), E_a is the activated enzyme (tyrosinase), S is a chromogenic tyrosinase substrate (TBC), and P is the chromophoric product (TBQ) of the E_a reaction on S.

To kinetically approach the study of zymogen activation systems by proteases (Varón et al., 1993; Vázquez et al., 1993), two assay conditions can be taken into consideration: high protease/zymogen ratio ($[E]_0 \gg [Z]_0$) or low protease/zymogen ratio ($[E]_0 \ll [Z]_0$).

High Activating Enzyme and Low Pro-tyrosinase Concentrations ($[E]_0 \gg [Z]_0$). The experimental progress curves obtained in the activation process of latent tyrosinase by trypsin and subtilisin under this assay condition were characterized by the presence of a transition phase prior to the attainment of a steady state (Figure 2A). Assuming rapid equilibrium conditions and taking into account Scheme 1, one exponential term appears. These experimental curves were fitted to the equation (Varón et al., 1993; Vázquez et al., 1993)

$$[P] = -\beta + \alpha t + \beta\, e^{-\lambda t} \qquad (2)$$

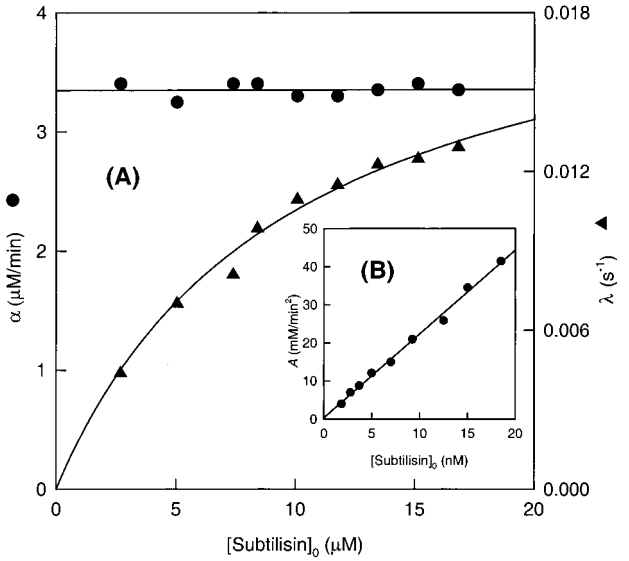


Figure 3. (A) ($[E]_0 \gg [Z]_0$) dependence of α and λ on initial subtilisin concentration. Conditions were as follows: 50 mM Tris-HCl buffer (pH 7), 2.5 mM TBC, and 3 nM latent tyrosinase. (B) ($[E]_0 \ll [Z]_0$) dependence of *A* on initial subtilisin concentration. Conditions were as follows: 50 mM Tris-HCl buffer (pH 7), 2.5 mM TBC, and 3 μM latent tyrosinase.

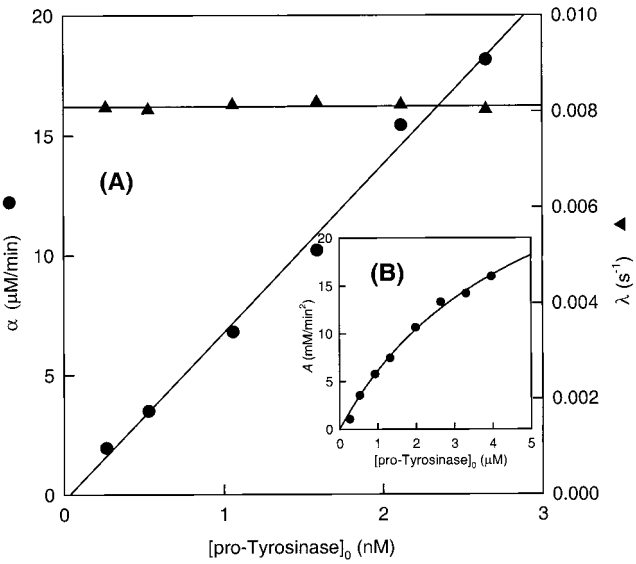


Figure 4. (A) ($[E]_0 \gg [Z]_0$) dependence of α and λ on initial latent tyrosinase concentration. Conditions were as follows: 50 mM Tris-HCl buffer (pH 7), 2.5 mM TBC, and 7.4 μM subtilisin. (B) ($[E]_0 \ll [Z]_0$) dependence of *A* on initial latent tyrosinase concentration. Conditions were as follows: 50 mM Tris-HCl buffer (pH 7), 2.5 mM TBC, and 7.4 nM subtilisin.

where

$$\beta = k^{S}_{cat} f_{zs} [Z]_0 / \lambda \quad (3)$$

$$\alpha = k^{S}_{cat} [Z]_0 [S]_0 / (K^{S}_{m} + [S]_0) \quad (4)$$

$$\lambda = -k^{Z}_{cat} [E]_0 / (K^{Z}_{m} + [E]_0) \quad (5)$$

The kinetic dependencies for the activation process of latent mushroom tyrosinase in the absence of serine protease inhibitor (Figures 3−5) (Scheme 1, without dashed box) are shown with subtilisin as activating enzyme. The same dependencies were obtained with trypsin (results not shown). Moreover, these same dependencies were also obtained with subtilisin and

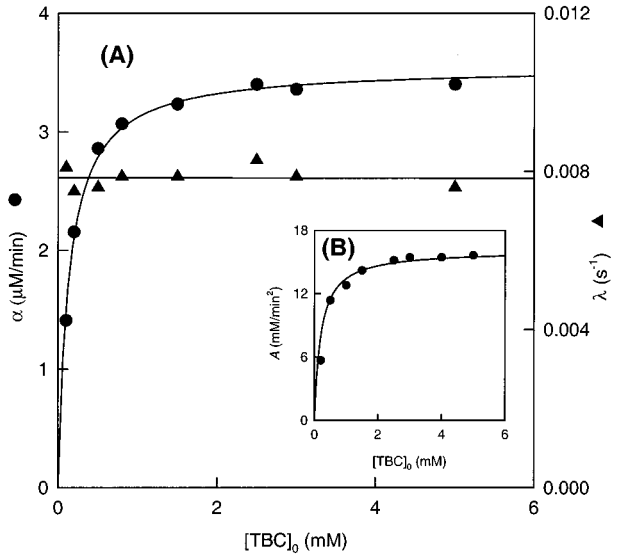


Figure 5. (A) ($[E]_0 \gg [Z]_0$) dependence of α and λ on initial TBC concentration. Conditions were as follows: 50 mM Tris-HCl buffer (pH 7), 7.4 μM subtilisin, and 3 nM latent tyrosinase. (B) ($[E]_0 \ll [Z]_0$) dependence of *A* on initial TBC concentration. Conditions were as follows: 50 mM Tris-HCl buffer (pH 7), 7.4 nM subtilisin, and 3 μM latent tyrosinase.

Table 1. Kinetic Constants That Characterize the Activation of Mushroom Pro-tyrosinase by both Trypsin and Subtilisin Serine Proteases[a]

constant	trypsin	subtilisin
K^{Z}_{m} (μM)	3.4 ± 0.4	(1.1 ± 0.1) × 10
k^{Z}_{cat} (s^{-1})	(6.3 ± 0.5) × 10^{-3}	(2.1 ± 0.1) × 10^{-2}
k^{Z}_{cat}/K^{Z}_{m} (μM^{-1} s^{-1})	(1.8 ± 0.3) × 10^{-3}	(1.9 ± 0.3) × 10^{-3}
K^{S}_{m} (μM)	(2.0 ± 0.1) × 10^{2}	(1.3 ± 0.1) × 10^{2}
k^{S}_{cat} (s^{-1})	(7.9 ± 0.5) × 10	(1.1 ± 0.1) × 10^{2}
k^{S}_{cat}/K^{S}_{m} (μM^{-1} s^{-1})	0.39 ± 0.04	0.85 ± 0.14
A^{Z}_{max} ($\mu M/s^2$)	(8.3 ± 0.6) × 10	(3.9 ± 0.2) × 10^{2}
A^{S}_{max} ($\mu M/s^2$)	(5.0 ± 0.4) × 10	(2.8 ± 0.2) × 10^{2}

[a] Note that k^{Z}_{cat} and k^{Z}_{cat}/K^{Z}_{m} values are dependent on protease concentration and k^{S}_{cat} and k^{S}_{cat}/K^{S}_{m} values are dependent on zymogen concentration.

trypsin as activating enzymes by measuring the oxygen consumption in the coupled reaction to determine the activation of latent mushroom tyrosinase (results not shown).

Effect of Initial Serine Protease Concentration ($[E]_0$). With constant latent tyrosinase and *o*-diphenol concentration, the parameter α (steady-state rate) was not dependent on serine protease concentration (Figure 3A) (eq 4). This kinetic parameter represents the amount of active form of tyrosinase that oxidizes the *o*-diphenol coupled in the assay. This meant that the final concentration of active form of tyrosinase was always the same, independent of the protease concentration used. The kinetic constant, which describes the velocity of the activation process (λ), followed an increasing hyperbolic dependence on the activating enzyme concentration according to eq 5 (Figure 3A). From these experiments and according to eq 5, the kinetic constants K^{Z}_{m} and k^{Z}_{cat} were calculated for the activation process of mushroom pro-tyrosinase by both subtilisin and trypsin serine proteases (Table 1).

Effect of Initial Latent Tyrosinase Concentration ($[Z]_0$). With constant serine protease concentration, α was linearly dependent on latent tyrosinase concentration

(Figure 4A) (eq 4). When the assay condition $[E]_0 \ll [Z]_0$ is applied, then the velocity of the process is independent of $[Z]_0$. Thus, λ was independent of latent enzyme concentration according to eq 5 (Figure 4A).

Effect of Initial Tyrosinase Substrate Concentration ($[S]_0$). With constant latent tyrosinase and serine protease concentrations, the resulting active tyrosinase formed catalyzed the oxidation of TBC with a typical Michaelis−Menten pattern. Thus, α followed an increasing hyperbolic dependence and λ was independent of TBC (Figure 5A) (eqs 4 and 5, respectively). From these experiments, the values of k_{cat}^S and K_m^S, which characterize the action on TBC of the active tyrosinase, resulting from the activation by these proteases, could be calculated. In the case of the activation by trypsin, the values of these constants were 79 s^{-1} and 0.2 mM, respectively. The values of these kinetic constants for the activation latent tyrosinase/subtilisin system were 110 s^{-1} and 0.13 mM, respectively. In activation experiments with 0.35 mM SDS (optimum SDS concentration for activation experiments with this detergent, results not shown) the values of these constants were 160 s^{-1} and 0.4 mM, respectively. It is known that trypsin and subtilisin differ in the specificity of their proteolytic actions (Flannery et al., 1989). The catalytic efficiency (k_{cat}^S/K_m^S) of the active tyrosinase on TBC was 2 times higher after activation by subtilisin than after trypsin treatment (Table 1). Moreover, the activation by SDS involves a conformational change in the latent enzyme instead of a proteolytic cleavage (Moore and Flurkey, 1990). Therefore, in the same assay conditions (temperature, pH, $[S]_0$, and $[Z]_0$) depending on the activator used, the resulting active form shows a slightly different kinetic behavior and probably has an activation-specific molecular structure, which is currently under study. However, it is of note that both proteases showed approximately the same catalytic efficiencies (k_{cat}^Z/K_m^Z) on mushroom pro-tyrosinase, 0.0018 and 0.0019 μM^{-1} s^{-1} (trypsin and subtilisin, respectively).

Effect of Temperature. Both α and λ increased with temperature (Figure 6A). The increase of α could be interpreted by the increasing activity of active tyrosinase with temperature (Sánchez-Ferrer et al., 1995) and, therefore, the oxidation of TBC was faster. The increase of λ could be interpreted by the increasing activity of trypsin and subtilisin with temperature (Price and Johnson, 1989; Wong, 1995) and, therefore, the activation process of latent mushroom tyrosinase was faster. Moreover, it could be also possible that temperature affected K_m^Z and K_m^S values.

Effect of Serine Protease Inhibitor. Aprotinin is a not very selective protein inhibitor of serine proteases. It belongs to the Kunitz-type trypsin inhibitors and forms very tight, but reversible, complexes with a number of proteases (Powers and Harper, 1988). The effect of the inhibition of trypsin and subtilisin by aprotinin in the activation process of latent mushroom tyrosinase was studied (Scheme 1 with dashed box). Both parameters α and λ decreased in the presence of aprotinin (Figure 7A). The inhibition of trypsin was stronger than that of subtilisin. The inhibition of the activation process by trypsin was almost complete in the range of the aprotinin concentration used. In the activation process by subtilisin, complete inhibition was not reached (Figure 7A), which agrees with the literature (Powers and Harper, 1988; Salvesen and Nagase, 1989).

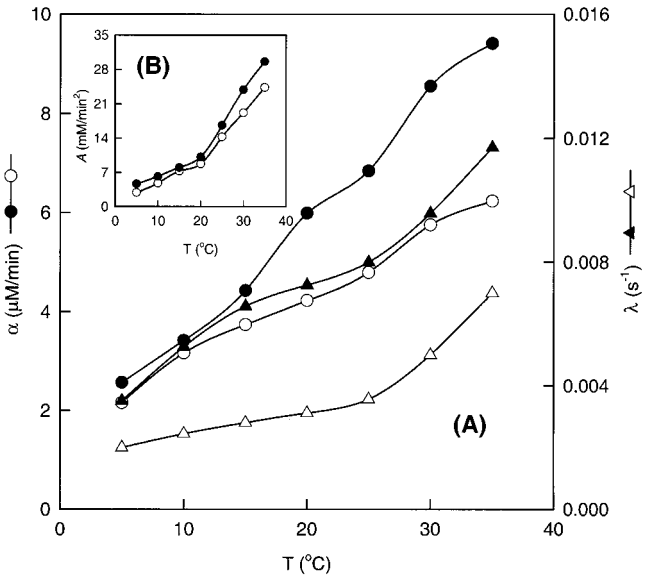


Figure 6. **(**A**)** ($[E]_0 \gg [Z]_0$) dependence of α and λ on temperature. Conditions were as follows: 50 mM Tris-HCl buffer (pH 7), 2.5 mM TBC, (●, ▲) 7.4 μM subtilisin or (○, △) 8.3 μM trypsin, and 6 nM latent tyrosinase. **(**B**)** ($[E]_0 \ll [Z]_0$) dependence of *A* on temperature. Conditions were as follows: 50 mM Tris-HCl buffer (pH 7), 2.5 mM TBC, (●) 7.4 nM subtilisin or (○) 8.3 nM trypsin, and 3 μM latent tyrosinase.

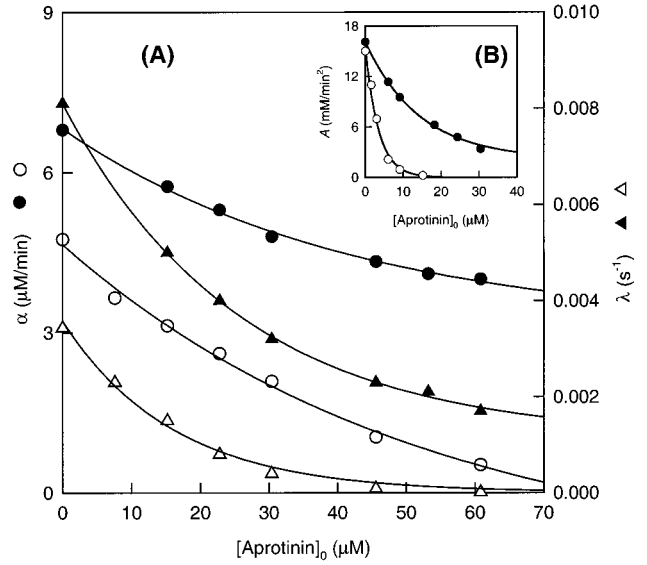


Figure 7. **(**A**)** ($[E]_0 \gg [Z]_0$) dependence of α and λ on initial aprotinin concentration. Conditions were as follows: 50 mM Tris-HCl buffer (pH 7), 2.5 mM TBC, (●, ▲) 7.4 μM subtilisin or (○, △) 8.3 μM trypsin, and 6 nM latent tyrosinase. **(**B**)** ($[E]_0 \ll [Z]_0$) dependence of *A* on initial aprotinin concentration. Conditions were as follows: 50 mM Tris-HCl buffer (pH 7), 2.5 mM TBC, (●) 7.4 nM subtilisin or (○) 8.3 nM trypsin, and 3 μM latent tyrosinase.

Low Activating Enzyme and High Pro-tyrosinase Concentrations ($[E]_0 \ll [Z]_0$). The experimental progress curves obtained in the activation process of latent tyrosinase by trypsin and subtilisin under these assay conditions showed a parabolic profile where the steady state was not strictly reached (Figure 2B). These experimental curves were fitted to the equation (Varón et al., 1993; Vázquez et al., 1993)

$$[P] = \frac{k_{cat}^S[S]_0}{K_m^S + [S]_0} \frac{k_{cat}^Z[Z]_0}{K_m^Z + [Z]_0} \frac{[E]_0}{2} t^2 = At^2 \qquad (6)$$

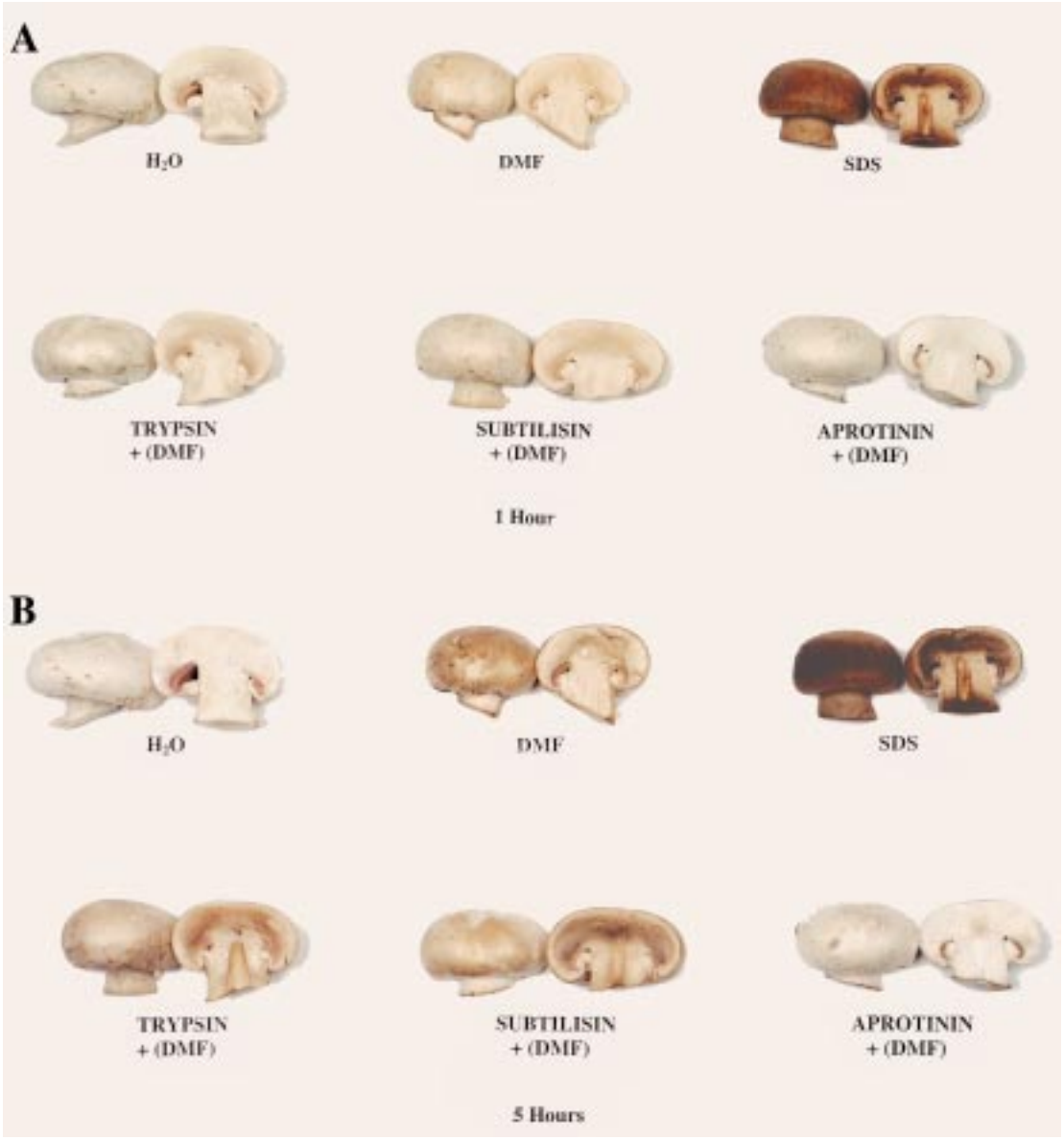


Figure 8. Vacuum infiltration of mushrooms with different compounds. Pictures were taken after (A) 1 h and (B) 5 h of the infiltration. Conditions are detailed under Material and Methods.

The slope of the parabola is represented by the parameter *A*, which is used to differentiate it from the slope α in the previous assay conditions ($[E]_0 \gg [Z]_0$). When $[E]_0 \ll [Z]_0$, the steady state was not strictly reached, and therefore only the dependencies of *A* on $[E]_0$, $[Z]_0$, $[S]_0$, and temperature could be studied. Initial estimations of *A* were taken from the plot of the first derivative of the accumulation product versus time ($[P]' = 2At$) (Varón et al., 1993; Vázquez et al., 1993).

Effect of Initial Serine Protease Concentration ($[E]_0$). The steady state was not reached because there was a high latent tyrosinase concentration. The low activating enzyme concentration could not complete the conversion of the latent to the active form of tyrosinase. Thus, because a proportional amount of latent tyrosinase was converted to active tyrosinase, the parameter *A* was linearly dependent on the initial trypsin or subtilisin concentration (Figure 3B) (eq 6).

Effect of Initial Latent Tyrosinase Concentration ($[Z]_0$). When $[Z]_0 \gg [E]_0$, the parameter *A* showed a typical Michaelis dependence on $[Z]_0$, and therefore *A* increased with a hyperbolic pattern when latent tyrosinase concentration was increased (Figure 4B) (eq 6). The value of the kinetic constant A^Z_{max} (maximum rate of the protease on mushroom pro-tyrosinase in this assay condition) was calculated according to eq 6 (Table 1).

Effect of Initial Tyrosinase Substrate Concentration ($[S]_0$). The parameter *A* increased with a hyperbolic pattern when the tyrosinase substrate (TBC) concentration was increased (Figure 5B) (eq 6). The value of the kinetic constant A^S_{max} (maximum rate of the active tyrosinase on the substrate in this assay condition) was calculated according to eq 6 (Table 1).

Effect of Temperature. As in the case of $[E] \gg [Z]$, the parameter *A* increased in the activation of latent tyrosinase by both subtilisin and trypsin serine proteases (Figure 6B). With increasing temperature the parabolic curves obtained (results not shown) were narrower and with higher slope *A*, resulting from the increase of the protease and tyrosinase activities, respectively. In this case it could be also possible that temperature affected the binding processes in both the zymogen−protease and active tyrosinase−TBC interactions.

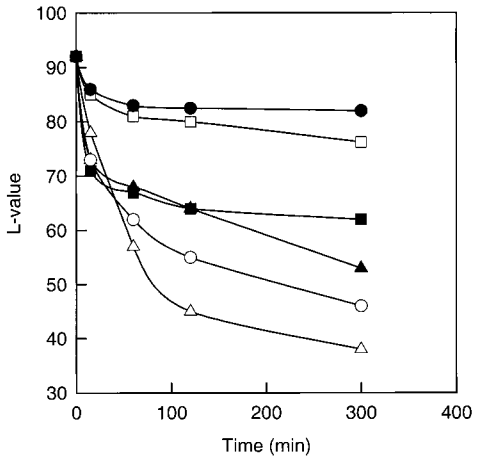


Figure 9. *L* values (degree of discoloration) for vacuum infiltration experiment: (□) H_2O; (■) DMF; (△) SDS; (○) subtilisin + DMF; (▲) trypsin + DMF; (●) aprotinin + DMF. Conditions were the same as in Figure 8.

Effect of Serine Protease Inhibitor. Under these assay conditions ($[E]_0 \ll [Z]_0$) aprotinin was in excess with respect to serine protease, and therefore the inhibition of trypsin and subtilisin was higher than when $[E]_0 \gg [Z]_0$. The parameter *A* decreased in the activation process by both trypsin and subtilisin proteases. The inhibition was stronger in the activation process by trypsin (complete inhibition of the activation process with 0.05 mg/mL aprotinin) than for subtilisin activation (Figure 7B).

VACUUM INFILTRATION EXPERIMENTS

These experiments tried to establish a possible correlation between in vitro assays and a possible in vivo situation. Mushrooms were vacuum infiltrated with trypsin, subtilisin, and aprotinin. Other compounds were also used such as water (as control), DMF [which breaks membranes but does not activate the enzyme (in vitro assays)], and SDS [which is a well-known activator of latent tyrosinases (Moore and Flurkey, 1990; Chazarra et al., 1997; Escribano et al., 1997)]. DMF was also added in mushrooms infiltrated with both serine proteases and aprotinin to break membranes and therefore to release endogenous proteases and phenolic substrates. The evolution of mushroom discoloration was followed at different times after treatment (Figure 8). *L*, *a*, and *b* values were measured. *L* values are the most representative for the increase of browning because they reflect the brightness (see Materials and Methods) (Figure 9). After 5 h, SDS (0.35 mM) proved to be the most potent activator. Mushrooms infiltrated with 3% (v/v) DMF also turned brown compared with the control (water). As DMF does not activate the latent enzyme (results not shown), endogenous activators (probably serine proteases; Burton et al., 1993, 1997) might be responsible for activation of the enzyme. Mushrooms infiltrated with both trypsin and subtilisin serine proteases (10 μM) also turned brown. However, mushrooms infiltrated with 0.3 mM aprotinin remained even whiter than mushrooms infiltrated only with water (Figures 8 and 9). The total degree of discoloration (ΔE) (the higher ΔE value, the higher the degree of discoloration) in mushrooms according to the eq 1 (Materials and Methods) was the following (mushrooms infiltrated with): H_2O = 16.36; DMF = 29; SDS = 54; subtilisin = 46; trypsin = 41; aprotinin = 10.7. Therefore, aprotinin was able to inhibit mushroom serine proteases, thus preventing melanin formation.

CONCLUSIONS

A theoretical model for zymogen activation has been applied to kinetically describe the activation process of latent *A. bisporus* tyrosinase by the serine proteases trypsin and subtilisin. All kinetic dependencies under two sets of assay conditions (respectively, high and low ratios of latent tyrosinase/serine protease concentration) fulfilled the predictions of the theoretical model (Figures 2−5). Low temperature proved to be a good way to prevent *o*-quinone formation by decreasing both protease (lower λ values) and tyrosinase (lower α and *A* values) activities (Figure 6). A serine protease inhibitor (aprotinin) was included in the activation process to either completely avoid or to slow the final browning reaction (Figures 7 and 9).

Trypsin and subtilisin Carlsberg were used as serine proteases in these activation assays: trypsin as standard and widely studied serine protease and subtilisin Carlsberg because it has properties close to those previously described for a purified *A. bisporus* serine protease (Burton et al., 1993). This together with vacuum infiltration experiments (Figures 8 and 9) could confirm the role of mushroom serine proteases in the activation of latent mushroom tyrosinase. This possible in vivo approach by using vacuum infiltration proved to be a good link between in vitro activation assays and a possible physiological situation in mushrooms.

The increase of protease activity (because of storage, senescence, pathogen attack, etc.) (Burton, 1988; Burton et al., 1993, 1994, 1997; Gea et al., 1995; Soler-Rivas et al., 1997) could involve a cascade of events including the activation of the latent tyrosinase and the decompartmentalization of phenols (because of tissue lysis; Villanueva, 1966). Either the endogenous proteases of mushroom (most of them serine proteases; Burton et al., 1993, 1997) or pathogen proteases (Kalberer, 1984; Leger et al., 1997) could activate the latent enzyme to render the active form of tyrosinase, which in turn could catalyze the oxidation of the phenolic compounds to give rise to the reactive *o*-quinones. The latter nonenzymatically polymerize to melanins (Rodríguez-López et al., 1992; Ros et al., 1994b; Espín et al., 1997a,b, 1998). The result of this cascade of processes is a loss of quality of the mushroom, which turns brown and develops a modified texture. The study reported in this paper strongly encourages the use of protease inhibitors as a novel and alternative tool combined with traditional methods such as low temperature to minimize this enzymatic browning.

ABBREVIATIONS USED

A, slope of the progress curve of [P] vs time when $[E]_0 \ll [Z]_0$ (*A* represents the amount of active tyrosinase formed); AbPPO, primary antibody (polyclonal antibody anti-tyrosinase developed in mouse); α, slope (steady-state rate) of the progress curve of [P] vs time when $[E]_0 \gg [Z]_0$ [α represents the amount of active tyrosinase formed (α and *A* represent the same concept under different assay conditions)]; E, activating enzyme (serine protease: trypsin or subtilisin Carlsberg); Bis-Tris, bis-[2-hydroxyethyl]iminotris[hydroxymethyl]methane; DMF, *N*,*N*′-dimethylformamide; $[E]_0$, initial serine protease concentration; HRP-Ab, secondary antibody (goat anti-

mouse IgG conjugated peroxidase antibody); k_{cat}^{S}, catalytic constant of active tyrosinase toward TBC; k_{cat}^{Z}, Catalytic constant of serine protease toward pro-tyrosinase; k_{d}^{Z}, deacylation constant of the complex EE_a; K_I, inhibition constant; K_m^S, Michaelis constant of tyrosinase toward TBC; K_m^Z, Michaelis constant of serine protease toward latent tyrosinase; λ, apparent constant to describe the time that the system needs to reach the steady state when [E] $\gg$ [Z] (λ represents the rate of active tyrosinase formation in the activation process); M_r, molecular mass; [P], product (TBQ) concentration; PB, sodium phosphate buffer; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel; subtilisin, subtilisin Carlsberg from *Bacillus licheniformis* (type VIII); TB, transfer buffer, pH 8.3; TBC, 4-*tert*-butylcatechol; TBQ; 4-(*tert*-butyl)benzo-1,2-quinone; TBS, Tris-buffered saline (pH 7.5); TPCK, tosylamido-2-phenylethylchloromethyl ketone; Tris, tris[hydroxymethyl]aminomethane; TBST, Tris-HCl buffer saline (pH 7.5) with 0.05% Tween 20; Z, zymogen (protyrosinase, latent tyrosinase); $[Z]_0$, initial zymogen concentration.

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