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Effect of separation on thermal stability of tyrosinase from *Agaricus bisporus*

Karolina Zynek[a], Jolanta Bryjak[a,*], Milan Polakovič[b]

[a] Department of Bioorganic Chemistry, Faculty of Chemistry, Wrocław University of Technology, Norwida 4/6, 50-373 Wrocław, Poland
[b] Department of Chemical and Biochemical Engineering, Faculty of Chemical and Food Technology, Slovak University of Technology, Radlinského 9, 812 37 Bratislava, Slovak Republic

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ABSTRACT

Modelling of inactivation kinetics was applied to assess the thermal stability of *Agaricus bisporus* tyrosinase preparations of different levels of purity. Experiments were carried out for a raw enzyme extract and preparations obtained after salting-out and ionic chromatography separation steps at six temperatures ranging from 30 to 55 °C. Each inactivation run was first fitted separately using a kinetic model derived from an irreversible one-step mechanism. The first-order model was then verified using multi-temperature evaluation in which the initial heating period was considered and temperature dependence of the rate constants was expressed through the Arrhenius equation. The modeling results showed that (i) unidentified compound(s) caused a lower thermal stability of the enzyme in the raw extract compared to that of the purified preparations; (ii) the estimated inactivation rate constants for the cresolase activity loss were two–three times higher than those for the catecholase activity loss, which indicated that the *oxy*-form of tyrosinase was more prone to thermal denaturation than the *met*-form.

1. Introduction

Tyrosinases (polyphenoloxidases, PPOs, EC 1.14.18.1) are industrially important, copper-containing oxidoreductases that catalyze both the *o*-hydroxylation of monophenols (cresolase activity) and subsequent oxidation of *o*-diphenols into reactive *o*-quinones (catecholase activity) using molecular oxygen. *o*-Quinones then undergo non-enzymatic reactions with various nucleophiles, producing intermediates, which associate spontaneously into dark brown pigments [1]. Besides well known natural substrates, mono- and diphenols, the enzyme reveals substrate specificity toward phenolic oligomers and polymers, phenolic derivatives with tyrosine residues, aminophenols and various aromatic amines [2].

Depending on the copper-ion valence and linking with oxygen, the active site of tyrosinase exists in three main oxidation states: *oxy*-(Cu^{II}–O_2–Cu^{II}), *deoxy*-(Cu^{I}–Cu^{II}), and *met*-state (Cu^{I}–Cu^{I}) [3–6]. Both the *met*- and *oxy*-state of tyrosinase enable the catecholase activity whereas the cresolase reaction requires the *oxy*-state. Tyrosinase exists mainly in the *met*-form at atmospheric pressure, room temperature, neutral pH and in the absence of any substrate. The fraction of the *met*-form at these conditions is about 85–90%, depending on the source of the enzyme, therefore it is frequently named the resting form of the enzyme [3–6].

Thermal stability is a key factor for application of any enzyme in industrial biocatalysts, biosensors or diagnostic kits. The kinetics of thermal inactivation of tyrosinases was investigated mainly for fruit enzymes from apples, avocados, grapes, pears, pineapples or plums [7,8]. It has been described by a first-order model when the obtained values of activation energy were from 111 to 364 kJ/mol [7–9]. In a single publication dealing with thermal inactivation kinetics of *Agaricus bisporus* tyrosinase, the suitability of first-order kinetics but an erroneous activation energy value of 2 kJ/mol were reported [9]. Biphasic kinetics of thermal inactivation was observed for tyrosinases isolated from palmito [10,11], sweet potato [12], kiwifruit [13] and *A. bisporus* [14] but activation energies were not estimated. None of abovementioned works dealt with the influence of enzyme purity and different structural forms on the kinetics of thermal inactivation.

The main goal of this work was to investigate the behavior of different purity preparations of tyrosinase from *A. bisporus*, which is regarded as a relatively cheap, efficient and commercially available source of the enzyme [15], in thermal inactivation experiments. To understand the kinetics of this process, a detailed analysis of the effect of temperature on the cresolase and catecholase activity loss was undertaken. Using enzyme natural substrates, L-tyrosine and L-DOPA, both activities were measured on the basis of dopaquinone formed which associate spontaneously into dark brown pigments. Multitemperature evaluation method [16–18] was used in mathematical modelling for the description of tyrosinase thermal inactivation.

* Corresponding author. Tel.: +48 71 320 26 77; fax: +48 71 328 04 75.
E-mail addresses: jolanta.bryjak@pwr.wroc.pl (J. Bryjak), milan.polakovic@stuba.sk (M. Polakovič).

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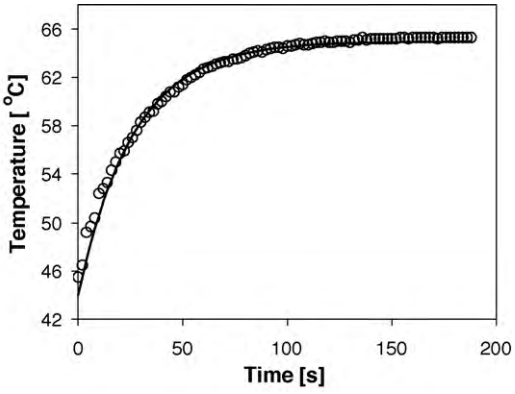

Fig. 1. An example of heating profile of tyrosinase solution at the bath temperature of 55 °C. The symbols represent experimental values and the solid line is a fitted course using Eq. (1).

2. Experimental

2.1. Materials

L-Tyrosine, L-DOPA, Lowry's reagent and bovine serum albumin were obtained from Sigma–Aldrich (Germany). Other chemicals were of analytical grade and were obtained from POCh (Poland). A non-commercial chromatographic adsorbent, DEAE-Granocel 500, was kindly donated by Prof. Jolanta Liesiene from Kaunas University (Lithuania). Fresh mushrooms for tyrosinase preparation were obtained from a local producer (Wrocław, Poland). In all isolation, purification and inactivation experiments, tyrosinase was dissolved in a 0.1 M phosphate buffer with pH 7.0.

2.2. Enzyme activity and protein concentration assays

Enzyme activity was measured in the buffer using 1 mM L-tyrosine (cresolase activity) or 1 mM L-DOPA (catecholase activity) as substrates. The reaction was monitored spectrophotometrically at 475 nm using a HELIOSα spectrophotometer (Unicam, UK). The activity was calculated from a linear absorbance increase. The enzyme activity unit (U) was defined as the amount of enzyme causing an increase of absorbance by 0.001/min at 30 °C. The mean analytical error was <2.8%. The preparations had negligible laccase, and catalase/peroxidase activities which were probed using 2.5 mM ABTS and 10 mM hydrogen peroxide, respectively, as substrates.

Protein concentration was determined by the Lowry's method [19] with bovine serum albumin as a standard. The mean analytical error was <2.0%.

2.3. Enzyme separation

Tyrosinase separation was made according to a procedure described previously [20]. Fresh mushrooms (300 g) were cut into small pieces, homogenized (IKA® EUROSTAR, Power Control-Visc 6000 Mixer, Germany) at 1200 rpm for 1–2 min in 550 mL of cold acetone (−26 °C), stirred for 30 min at 0–4 °C and centrifuged at 7000 rpm for 20 min. The separated mushroom pulp was suspended in 200 mL of the buffer and remaining acetone was removed with a vacuum pump (KNF LABOPORT® PTFE pump, Germany). The suspension was then centrifuged at 10,000 rpm for 20 min and the obtained supernatant was a crude enzyme labeled as Preparation I in the further text.

Preparation I was concentrated by precipitation. A part of proteins having negligible tyrosinase activity was salted-out with ammonium sulfate (30% saturation) during stirring at 0–4 °C for 30 min. The suspension was then centrifuged at 10,000 rpm for 20 min and the ammonium sulfate concentration in the supernatant was adjusted to 60% saturation. Protein aggregation and precipitate separation was carried out at the same stirring and centrifugation conditions as in previous precipitation step. The collected sediment was dissolved in 20 mL of the buffer and the solution was used as Preparation II in further experiments.

Preparation II was further purified by ion-exchange chromatography using DEAE-Granocel 500 as chromatographic packing (bed dimension: 16 cm × 1.2 cm). A 0.01 M phosphate buffer with pH 7.0 was used as eluent. The injected amount of Preparation II was 4 mL and the flow rate was 1 mL min^{-1}. Purification was based on selective adsorption of ballast proteins and colored impurities when tyrosinase did not bind to the adsorbent particles under these conditions. The separation process was monitored by measuring the protein concentration and catecholase and cresolase activity in the collected 3 mL fractions. The first five fractions were mixed and used as Preparation III in further experiments.

2.4. Inactivation experiments

Inactivation experiments were performed in a thermostated laboratory reactor equipped with a magnetic stirrer at a temperature ranging from 30 to 55 °C. The reactor was first filled with 5 mL of the buffer, preheated to a specified temperature and 5 mL of an enzyme preparation having the temperature of 25 °C were then added. A vigorous stirring (500 rpm) of the mixture was applied during the first 15 s and then, a gentle stirring at 20 rpm was used up to the end of the process. Samples were taken in certain time intervals, cooled rapidly in ice water bath and stored in it until the activity measurement. In order to study the influence of gas/liquid interfacial area on enzyme stability [21,22], thermostated reactors with internal diameters of 1.8 and 3.5 cm were used in the inactivation experiments. No effect of mechanical stirring and air–liquid interface area on the rate of inactivation was observed. Replicated measurements showed that a 95% confidence interval of the experimental error of the relative activity was 5.0%.

2.5. Heat transfer experiment

A correct analysis of the inactivation data required the knowledge of the value of heat transfer coefficient (K) because the initial heating period influenced the course of enzyme inactivation. The

Table 1
Characteristics of *A. bisporus* tyrosinase preparations.

Preparation no.	Protein mass [mg]	Protein yield[a] [%]	Protein concentration [mg mL^{-1}]	Specific activity [U mg^{-1}]		Activity yield [%]		Purification factor[b] [fold]		Catecholase/ cresolase activity ratio [–]
				Catecholase	Cresolase	Catecholase	Cresolase	Catecholase	Cresolase	
I	2786.0	–	2.78	7420	1210	–	–	–	–	6.14
II	612.9	22.0	4.55	29300	4730	86.9	86.0	3.95	3.91	6.19
III	161.6	5.8	1.55	50700	6670	39.6	32.0	6.83	5.52	7.60

[a] Related to the total protein mass in the crude extract (Preparation I).
[b] Related to the specific activity of Preparation I.

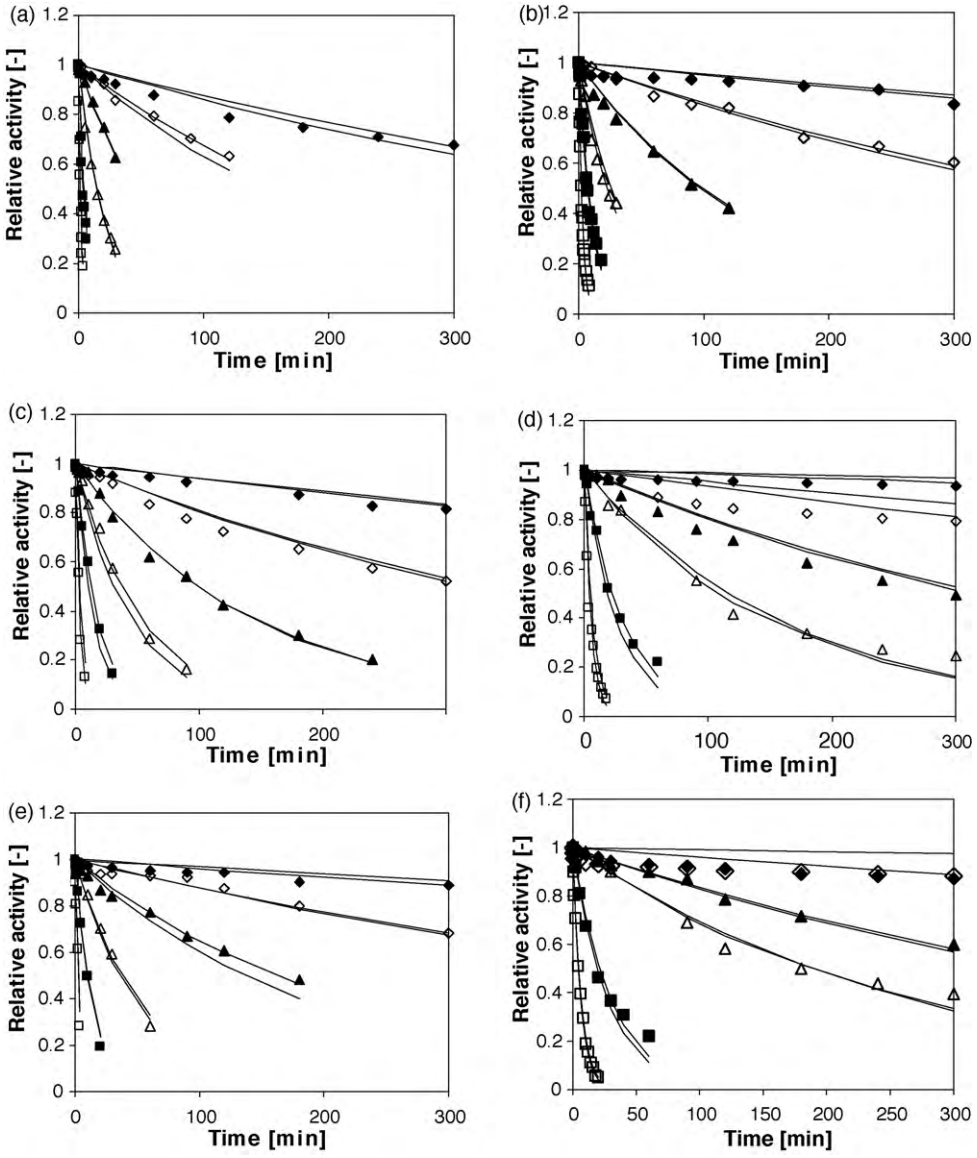

Fig. 2. Thermal inactivation of tyrosinase preparations of different purification degrees monitored by cresolase (a, c and e) or catecholase (b, d and f) activity loss, respectively: Preparation I (a and b); Preparation II (c and d); Preparation III (e and f). The symbols represent experimental data at the temperatures of 30 °C (♦), 35 °C (◊), 40 °C (▲), 45 °C (△), 50 °C (■) and 55 °C (□). The lines represent a fit with the first-order model using isothermal (dashed lines) and multitemperature (solid lines) evaluations.

experiments for the determination of heat transfer coefficient were performed at temperatures ranging from 30 to 65 °C. The temperature inside the reactor was measured using two Ni–Cr thermocouples with the diameter of 0.2 mm located in two different radial positions. It was registered by a data logger and recorded by PC [23]. The phosphate buffer and enzyme preparation were mixed in the same way as in the inactivation experiment. The temperature was measured in two-second intervals in three repeated runs for each bath temperature. It reached a steady value at about 150 s as illustrated in Fig. 1 for the measurement conducted at the bath temperature of 65 °C. It was also found that the temperature course was independent of the thermocouple position as was also noted previously [18,23]. The temperature data were fitted using a simple dynamic enthalpy balance [23]:

$$\frac{dT}{dt} = K(T_B - T) \tag{1}$$

where T_B is the bath temperature, T the solution temperature at time t and K a proportionality factor including the overall heat transfer coefficient. The coefficient K was first calculated for each run and was found to be independent of temperature. A value of $K = 2.74 \times 10^{-2}\ s^{-1}$ with a standard deviation of $6.03 \times 10^{-4}\ s^{-1}$ was obtained by the simultaneous fit of all the runs.

2.6. Mathematical modelling

Inactivation of tyrosinase was assumed to proceed according to a simple mechanism of first-order irreversible conversion of native enzyme N into an inactivated form I:

$$N \xrightarrow{k} I \tag{2}$$

The kinetic equation based on this mechanism had the form:

$$r = \frac{dC_N}{dt} = -kC_N \tag{3}$$

where r is the reaction rate, C_N the concentration of native enzyme and k the rate constant of reaction. The initial condition was $C_N = C_{N0}$ in time $t = 0$. The model also included the enthalpy balance (Eq. (1)) and an equation expressing the temperature dependence of the rate

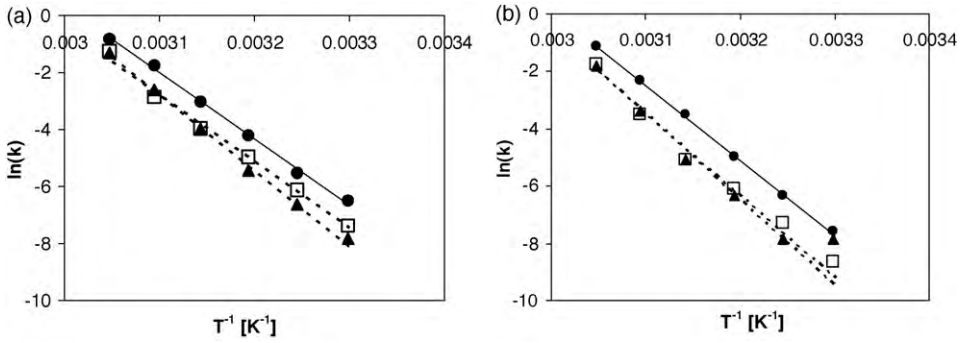

Fig. 3. Arrhenius plots for the first-order rate constants of tyrosinase inactivation monitored by (a) cresolase and (b) catecholase activity loss, respectively. The symbols represent the values obtained by isothermal evaluation (Table 2) for individual tyrosinase preparations: I (●), II (□), and III (▲). The dashed lines were calculated from the coefficients of Arrhenius equation obtained by multitemperature evaluation and presented in Tables 2 and 3, respectively.

Table 2
Results of multitemperature evaluation of thermal inactivation of tyrosinase preparations monitored by cresolase activity loss.

Preparation	k_0 [min^{-1}]	Ea [kJ mol^{-1}]	SD[a] [%]
I	0.0277 ± 0.0014	193.2 ± 4.75	3.51
II	0.0126 ± 0.0005	193.6 ± 4.66	3.74
III	0.0100 ± 0.0004	221.4 ± 4.40	2.88

[a] SD; standard deviation of activity data fit.

constant through Arrhenius equation [17]:

$$k = \exp(\ln\, k_0)\exp\left[E_a/RT_0(1 - T_0/T)\right] \quad (4)$$

where E_a is the activation energy, k_0 the rate constant at the reference temperature T_0 = 315.65 K and R the universal gas constant.

The parameters of the model, the rate constant at the reference temperature and activation energy were estimated by a simultaneous fit of data sets obtained at different bath temperatures using the parameter estimation software Athena Visual Workbench (Stewart & Associates Engineering Software, Madison, WI, USA; http://www.athenavisual.com).

3. Results and discussion

The most advantageous method of disruption *A. bisporus* cells is homogenization of mushrooms in cold acetone [20]. It was applied also in this study to obtain a crude enzyme extract (Preparation I). Further concentration and purification steps of tyrosinase included double salting-out with ammonium sulfate and anion-exchange chromatography on a cellulose bead adsorbent with DEAE functional groups. These two separation procedures provided Preparations II and III, respectively, which were further used in the stability investigation.

The efficiencies of the isolation and purification steps are presented in Table 1. The salting-out procedure allowed the removal of a significant part of ballast proteins as the specific tyrosinase activity increased about four times. The further purification by chromatography enabled to get the enzyme with the specific tyrosinase activity 5–7 times higher than that of the crude extract. It was also found that the specific catecholase activity of the enzyme preparations was several times higher than the corresponding cresolase activity. The ratio of the activities was essentially the same (6.14 and 6.19, respectively) in Preparations I and II. It however slightly increased to 7.6 in Preparation III. This may be explained by a higher susceptibility of tyrosinase *oxy*-form toward various destabilizing factors [9,24].

Tyrosinase is a cytosolic enzyme therefore isolation and purification processes for this target protein can remove potential stabilizers or destabilizers of the enzyme. For that reason, three tyrosinase preparations of different degrees of purity were probed in inactivation experiments. Preliminary inactivation experiments showed that the decrease of relative enzyme activity was protein concentration-dependent when the rate of inactivation was faster at lower enzyme concentrations (data not shown). In order to assess unequivocally the influence of purification degree on enzyme stability, the same value of protein concentration was adjusted in all preparations for inactivation experiments. For that reason, Preparations I and II were diluted with the buffer to the protein concentration value of 1.54 mg mL^{-1}.

Thermal stability of the tyrosinase preparations was examined in the temperature range of 30–55 °C. Fig. 2 shows that a relative activity decrease was considerably higher for the crude enzyme extract. Some species in the extract probably destabilized the enzyme and their removal improved the thermal stability of tyrosinase preparations. Fig. 2 further shows that the catecholase activity loss was always slower than the corresponding cresolase activity loss. This implies that the resting form of tyrosinase, *met*-form, may be considerably more stable than the *oxy*-form.

In order to assess the inactivation behaviour of the tyrosinase preparations with different levels of purity quantitatively, all inactivation runs were fitted simultaneously with the first-order model (Eqs. (1), (3) and (4)) using the method of multitemperature evaluation. A good agreement of experimental and model activity values (solid lines) is demonstrated in Fig. 2. Obtained parameters, the reaction rate constants at the reference temperature and activation energies, as well as the activity standard deviations are presented in Tables 2 and 3, respectively. The activity standard deviations for tyrosinase inactivation monitored by cresolase test (Table 2) fulfilled the adequacy criterion for all preparations. On the other hand, the limit value of the 95% confidence interval of activity deviation was slightly exceeded for inactivation of Preparations II and III (Table 3), monitored by catecholase test. The discrepancy between the experimental and model data is visible in Fig. 2d and f at the two lowest temperatures of 30 and 35 °C. The model values are higher here than the corresponding experimental values. At these conditions, the rate of thermal inactivation was by far the slowest so a non-thermal effect could have affected the observed rate of activity loss.

Table 3
Results of multitemperature evaluation of thermal inactivation of tyrosinase preparations monitored by catecholase activity loss.

Preparation	k_0 [min^{-1}]	Ea [kJ mol^{-1}]	SD[a] [%]
I	0.0139 ± 0.0004	216.7 ± 3.08	3.14
II	0.0044 ± 0.0003	237.0 ± 7.87	5.56
III	0.0040 ± 0.0003	249.2 ± 4.40	5.50

[a] SD, standard deviation of activity data fit.

Tables 2 and 3 show that the estimated activation energies of the enzyme preparations with different purities were similar, from 193 to 221 kJ mol^{-1} for cresolase and from 217 to 249 kJ·mol^{-1} for catecholase, which is also demonstrated by the similarity of the slopes of Arrhenius plots in Fig. 3. These values are in the middle of the very broad range of 111–364 kJ mol^{-1} reported in literature for different fruit tyrosinases [7,8].

The values of k_0 for Preparation II were about twice (cresolase) or three times (catecholase) lower than those for Preparation I whereas they were only about 20% and 10% higher than those for Preparation III (Tables 2 and 3), This means that most components destabilizing the enzyme were removed in the salting-out step. The results presented in Tables 2 and 3 provide another important comparison between the k_0-values of cresolase and catecholase activity loss. The latter values were 2.0–2.9 times lower. The values of k_0 and E_a may be interpreted so that the activation entropies of the inactivation of *oxy*- and *met*-forms are different whereas their activation entropies are the same. This would further imply that the denaturation pathways of both forms are very similar but proceed at different rates.

4. Conclusions

The investigation of the thermal inactivation kinetics of tyrosinase from *A. bisporus* was conducted in a broad range of temperatures. This range corresponded to enzyme half-live times from about 3 days to <2 min. Since tyrosinase is able to express its catecholase activity via *oxy*- and *met*-forms of the protein whereas expressing cresolase activity requires the *oxy*-form, the catecholase and cresolase activities were probed using L-tyrosine and L-DOPA as substrates.

The analysis of inactivation curves monitored by both substrates and at six temperatures indicated a monophasic inactivation. Multitemperature evaluation was applied and the reaction rates at the reference temperature and activation energies of the first-order model were estimated for three tyrosinase preparations of different purities. A comparative analysis of the results revealed a higher thermal stability of purified tyrosinase and a lower thermal resistance of the *oxy*-form of the enzyme.

Understanding the relation between the tyrosinase purity and thermal stability as well as distinguishing the stability of different forms can have a wide impact on its practical applications. First, the knowledge of thermal stability of crude enzyme extracts is indispensable to control mushroom browning under thermal processing in food industry. Secondly, tyrosinases have a great potential in bioorganic chemistry which is so far limited by a demand for purer enzyme preparations that exclude side reactions with other oxidative enzymes. Thus, the knowledge about higher thermal stability of purer preparations and discrimination of *oxy*- and *met*-form stabilities comes close to solving many practical questions. Thirdly, both catecholase and cresolase activities should be monitored in any processes of tyrosinase thermal inactivation.

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