Purification, Characterization, and Molecular Cloning of Tyrosinase from *Pholiota nameko*

Yasuko KAWAMURA-KONISHI,1,2,3; Mariko TSUI,1 Seiichi HATANA,1 Masahiro ASANUMA,2
Dai KAKUTA,2 Takeshi KAWANO,2 Etsuko B. MUKUYAMA,1,2
Hideyuki GOTO,3 and Haruo SUZUKI1,2

1Division of Biosciences, Graduate School of Fundamental Life Science, Kitasato University,
Sagamihara, Kanagawa 228-8555, Japan
2Department of Biosciences, School of Science, Kitasato University,
Sagamihara, Kanagawa 228-8555, Japan
3Department of Food Science, Faculty of Bioresources and Environmental Sciences,
Ishikawa Prefectural University, Nonoichi, Ishikawa 921-8836, Japan

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Tyrosinase (monophenol, 3,4-dihydroxy L-phenylalanine (L-DOPA):oxygen oxidoreductase, EC 1.14.18.1) was isolated from fruit bodies of *Pholiota nameko* and purified to homogeneity. The purified enzyme was a monomer with a molecular weight of 42,000 and contained 1.9 copper atoms per molecule. The N-terminal of the purified enzyme could not be detected by Edman degradation, probably due to blocking, while the C-terminal sequence of the enzyme was determined to be -Ala-Ser-Val-Phe-OH. The amino acid sequence deduced by cDNA cloning was made up of 625 amino acid residues and contained two putative copper-binding sites highly conserved in tyrosinases from various organisms. The C-terminal sequence of the purified enzyme did not correspond to that of the deduced sequence, but agreed with Ala384-Ser385-Val386-Phe387 in sequence. When the encoded protein was truncated at Phe387, the molecular weight of the residual protein was calculated to be approximately 42,000. These results suggest that *P. nameko* tyrosinase is expressed as a proenzyme followed by specific cleavage to produce a mature enzyme.

Key words: tyrosinase; *Pholiota nameko*; purification; post-translational modification; proenzyme

Tyrosinase (monophenol, 3,4-dihydroxy L-phenylalanine (L-DOPA):oxygen oxidoreductase, EC 1.14.18.1) is a bifunctional copper-containing enzyme that uses molecular oxygen to catalyze the oxidation of monophenols to their corresponding o-diphenols (monophenolase activity) and their subsequent oxidation to o-quinones (catecholase activity). The resulting highly reactive o-quinones auto-polymerize to form brown polyphenolic catechol melanins.1–3 Tyrosinase is distributed in a wide range of organisms, including bacteria, fungi, plants, insects, and other animals, and is responsible for a variety of functions: skin pigmentation in mammals, browning in plants, host defense system in arthropods, and differentiation of reproductive organs and spore formation in fungi.1–7 Many studies have been carried out to determine the enzymatic and molecular characteristics of tyrosinase from various prokaryotic and eukaryotic sources.8–12 Among these, tyrosinase from *Agaricus bisporus* is the most extensively investigated because this mushroom is liable to progress to enzymatic browning and pigmentation during development and post-harvest storage, which particularly decreases the commercial value of the product,1–3 and the molecular properties of tyrosinase have been provided by information from studies of *A. bisporus* tyrosinase.

Tyrosinase contains a single type-3 copper center in its active site, such as catechol oxidase from plants and hemocyanin from mollusks and arthropods.13–15 Depending on copper ion valence and linking with molecular oxygen, the copper center consists of three forms: deoxy (Cu1–Cu1), o (Cu1–O2–Cu1), and met...
(Cu$^{II}$–Cu$^{II}$). The monophenolase and catecholase activities of tyrosinase have been explained by a proposed structural model for the active site of these three forms. Recently, crystallographic study of a bacterial tyrosinase in a complex with a helper protein has been reported, but the complex is catalytically inactive since its active center was covered by the helper protein. Hence the catalytic mechanism based on the active-center structure of tyrosinase has not been elucidated.

Tyrosinase is an attractive enzyme for industrial applications: in environmental technology for the detoxification of phenol-containing waste water and contaminated soils, the formation of antioxidant o-diphenols with beneficial properties as food additives and in pharmaceutical drugs, the formation of biopolymers with useful viscoelastic properties, and the improvement of food as to flavor, taste, and texture by polymerization of phenol derivatives and by cross-linking of protein–protein and protein–polysaccharide.

Tyrosinase from edible mushrooms is especially expected to have biotechnological potential in food applications because the non-toxicity of the tyrosinase is certified by historical use of mushrooms as a food. In preliminary experiments, we prepared cell-free extracts of 11 kinds of commercially available mushrooms and measured tyrosinase activity using 4-tert-butylcatechol (TBC) as a substrate. The results showed that *P. nameko* has a high level of tyrosinase activity, comparable to *A. bisporus*. *P. nameko* is one of the most popular edible mushrooms in Japan and is readily obtained in large quantities inexpensively. This paper describes (i) the purification and characterization of tyrosinase from *P. nameko*, (ii) cDNA cloning of *P. nameko* tyrosinase, and (iii) nucleotide and amino acid sequence analysis of the enzyme. The results suggest that nameko tyrosinase is expressed as a proenzyme activated to form a mature enzyme by proteolysis.

**Materials and Methods**

**Materials.** Fruit bodies of *P. nameko* grown at Suzuki Farm Co. (Koriyama, Japan) were obtained from a local supermarket. 3,4-Dihydroxy-L-phenylalanine (l-DOPA), 3,4-dihydroxy-D-phenylalanine (D-DOPA), and dopamine hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO), 3-(3,4-dihydroxyphenyl) propionic acid, NADPH, p-cresol, l-Tyr, d-Tyr, and TBC from Wako Pure Chemical Industries (Osaka, Japan), and 4-tert-butylphenol and tyramine from Nacalai Tesque (Kyoto, Japan). All other reagents were of analytical grade.

**Purification of tyrosinase from *P. nameko***. All procedures were carried out at 4°C. The fruit bodies of *P. nameko* were homogenized with five volumes of 0.15 m NaCl solution using a Polytron PT10-35 (Kinematica AG, Lucerne, Switzerland), filtered through glass wool, and centrifuged. The supernatant was fractionated by 30–60% ammonium sulfate saturation. The protein precipitates were dialyzed against 50 mM Tris–HCl, pH 7.2. Tyrosinase was purified from the dialysate by the usual column chromatographic procedures, using Toyopearl DEAE-650M (Tosoh, Tokyo), Toyopearl Butyl-650M (Tosoh), Toyopearl AF-Red-650ML (Tosoh), and a Hi Load 16/60 Superdex 200 prep grade column (GE Healthcare Bio-Sciences, Boston, MA). Fractions containing tyrosinase activity were pooled and used in the present study.

**Assay of tyrosinase activity.** Tyrosinase activity in the purification procedures was assayed for 200 μM TBC at 25°C in 25 mM Tris–HCl, pH 7.2, by monitoring the absorbance at 400 nm with a spectrophotometer U-3210 (Hitachihitec, Tokyo). One unit of tyrosinase was defined as the amount of enzyme required to produce 1 μmol of t-butyl-quinone (ε$_{400}$nm = 1,150 M$^{-1}$ cm$^{-1}$) per min under the above conditions.

Monophenolase activity was determined by measuring the decrease in monophenol concentration according to the method of Sanjust et al., with some modifications. The reaction was carried out at 25°C in 10 mM Tris–HCl, pH 7.2, containing 5 μM TBC, which was added to the reaction mixture to shorten the lag period. At appropriate time intervals, aliquots of the mixture were thrown to an equal volume of acetonitrile containing 1 m perchloric acid to terminate the reaction. After centrifugation, supernatant of the mixture was loaded on a Chromolith RP-18e ODS-Hypersil column (4.6 × 100 mm, Merck, Darmstadt, Germany) and eluted with a linear gradient of acetonitrile monitoring absorbance at 280 nm using a Jasco LC-1500 apparatus (Jasco, Tokyo) equipped with an UV–Vis detector module Jasco UV-1570. Catecholase activity for TBC was obtained by monitoring the absorbance at 400 nm with a molecular absorption coefficient of t-butyl-quinone (ε$_{400}$nm = 1,150 M$^{-1}$ cm$^{-1}$). Those for the other substrates were measured in the presence of 200 μM NADPH, and were calculated from the decrease in NADPH concentration using ε$_{340}$nm = 6,220 M$^{-1}$ cm$^{-1}$ of NADPH.

**Other analytical methods.** UV–visible absorption spectra of the purified tyrosinase were obtained with a Jasco V-560 spectrophotometer in our laboratory. MALDI-TOF MS was performed on Voyager-DE STR (Applied Biosystems, Foster City, CA) at the Peptide Institute (Osaka, Japan). The copper content of the tyrosinase was measured with a Polarized Zeeman Atomic Absorption Spectrophotometer Z-2000 (Hitachihtec) based on a standard curve determined simultaneously under identical conditions. The protein concentration was determined according to the Bradford Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin as standard. A stock solution of hydrogen peroxide was made up immediately before use, and its concentration was estimated by ε$_{240}$ =
43.6 μm−1 cm−1.33) SDS–PAGE (10%, polyacrylamide) was performed according to a modification of the method of Laemmli.34)

Analysis of amino acid sequence. The purified tyrosinase was digested with *Achromobacter* protease I (Wako Pure Chemical Industries) at a molar ratio of enzyme to the protein sample of 1:200 in 50 mM Tris–HCl, pH 9.0, containing 4 M urea at 35°C overnight. The peptide fragments in the proteolytic digests were separated using a reversed-phase HPLC column (Inertsil ODS, 4.5 × 250 mm, GL Science, Tokyo). Analyses of amino acid sequences were performed using an automated protein sequencer, PPSQ-10 (Shimadzu, Kyoto, Japan). For analysis of the C-terminal amino acid sequence, a carboxypeptidase A (183 pmol, Sigma-Aldrich) was added to 1 mg of the purified tyrosinase in a total volume of 180 μl of 0.2 M acetyl morpholine carbonate, pH 8.5, and incubated at 25°C overnight. The peptide fragments in the proteolytic digests were separated using a reversed-phase HPLC column (Inertsil C18, 4.5 mm, GL Science, Tokyo). Analyses of amino acid sequences were performed using an automated protein sequencer, PPSQ-10 (Shimadzu, Kyoto, Japan). For analysis of the C-terminal amino acid sequence, a carboxypeptidase A (183 pmol, Sigma-Aldrich) was added to 1 mg of the purified tyrosinase in a total volume of 200 μl of 0.2 M N-acetyl morpholine carbonate, pH 8.5, and incubated at 25°C overnight. The peptidic fragments in the proteolytic digests were separated using a reversed-phase HPLC column (Inertsil ODS, 4.5 × 250 mm, GL Science, Tokyo). Analyses of amino acid sequences were performed using an automated protein sequencer, PPSQ-10 (Shimadzu, Kyoto, Japan). For analysis of the C-terminal amino acid sequence, a carboxypeptidase A (183 pmol, Sigma-Aldrich) was added to 1 mg of the purified tyrosinase in a total volume of 200 μl of 0.2 M N-acetyl morpholine carbonate, pH 8.5, and incubated at 25°C overnight. The peptide fragments in the proteolytic digests were separated using a reversed-phase HPLC column (Inertsil ODS, 4.5 × 250 mm, GL Science, Tokyo). Analyses of amino acid sequences were performed using an automated protein sequencer, PPSQ-10 (Shimadzu, Kyoto, Japan). For the manufacturer's protocol.

Cloning of *P. nameko* tyrosinase. A fruit body of *P. nameko* was powdered using a pestle in liquid nitrogen. Total RNA was extracted from 100 mg of the powder with TRizol (Life Technologies, NY). First-strand cDNA was synthesized using a First-strand cDNA Synthesis Kit (Takara Bio, Shiga, Japan). Degenerate PCR was carried out on the cDNA using forward and reverse primers (F1 and R1 respectively) and a LA Taq DNA polymerase (Takara Bio) under conditions following the manufacturer’s protocol: after holding (94°C, 2 min), 30 cycles of denaturing (1 min, 94°C), annealing (1 min, 52°C), and elongation (1.5 min, 72°C), followed by holding (68°C, 2 min), 3′-RACE was carried out using forward primer F2 and a CapFishing Full-length cDNA Premix Kit (Seegene, Seoul, ROK) with a KOD plus DNA polymerase (Toyobo, Osaka, Japan), and 5′-RACE was performed using reverse primer R3 and a 5′/3′ RACE Kit 2nd Generation (Roche Diagnostics, Basel, Switzerland) with the KOD plus DNA polymerase. The PCR fragments were cloned into pUC118 using a Mighty Cloning Kit (Blunt End, Takara Bio) according to the manufacturer’s protocol. The full-length fragments were amplified using forward primer F4 and reverse primer R4, and then cloned into pUC118. The primers used were as follows: F1, 5′-ACTGGTG-3′; R1, 5′-TTAAACAATGGCATGACGG-3′; F4, 5′-ATGTCTGCGTTGTTATC-3′; R4, 5′-TKRTNKYCN/ATAGTG-3′; F2, 5′-CGATGGAATTGATG-3′; R2, 5′-CKRTCNACR/Y-CTNGTGGACG-3′; F3, 5′-CGTTATGTCCTCGCTGACG-3′; R3, 5′-CGTTATGTCCTCGCTGACG-3′; F5, 5′-TTAAACAATGGCATGACGG-3′. All the nucleotide sequences of PCR products were determined on both strands by Hokkaido System Science (Sapporo, Japan), by the dye terminator method.

Results and Discussion

Purification of tyrosinase from *P. nameko*

To investigate the catalytic and molecular properties of tyrosinase from *P. nameko*, we purified the enzyme by ammonium-sulfate fractionation and four chromatography steps, as summarized in Table 1. The molecular weight of the purified enzyme was estimated to be approximately 42,000 by SDS–PAGE (Fig. 1) and

<table>
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<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Total activity (Units)a</th>
<th>Specific activity (Units/mg)</th>
<th>Purification (fold)</th>
<th>Recovery (%)</th>
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<td>Homogenate</td>
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<td>1.0</td>
<td>100</td>
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<td>30–60% AmSO$_2$</td>
<td>1,360</td>
<td>18,640</td>
<td>13.7</td>
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<td>29.3</td>
<td>3.5</td>
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<tr>
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<td>133</td>
<td>16</td>
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<td>287</td>
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<td>Superdex 200</td>
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<td>3,046</td>
<td>412</td>
<td>49</td>
<td>9.8</td>
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</table>

*aOne unit of enzyme was defined as the amount of tyrosinase that produces 1 μmol of quinone per min at 25°C using TBC as substrate.

![Image](385x471 to 512x617)
35,000 by gel filtration chromatography. In MALDI-TOF mass spectrometry, a parent ion signal of the enzyme was observed at \( m/z \) 42,424.39. These estimates indicate that the purified tyrosinase is a monomer. The enzyme was found to contain 1.9 copper atoms per mole enzyme of Mr 42,000 by atomic absorption analysis. The N-terminal sequence of the enzyme was not be detected by Edman degradation, probably due to blocking of it, whereas the C-terminal sequence of the enzyme was determined to be Ala-Ser-Val-Phe-OH by enzymatic digestion using carboxypeptidase A.

The purified tyrosinase exhibited a characteristic absorption spectrum with a maximum at 280 nm and a low and broad band between 300 and 400 nm (Fig. 2A). A concentrated solution of the purified enzyme clearly showed broad bands with a maximum at 343 nm (Fig. 2A, left inset) and at 656 nm (Fig. 2A, right inset). These absorption bands, in the range of 300 to 400 nm, are characteristics of the oxy-form of type-3 copper proteins and are due to an \( \text{O}_2^{2-}/\text{Cu}^{2+} \rightarrow \text{Cu}^{2+} \) charge transfer transition.\(^{35,36} \) The addition of \( \text{H}_2\text{O}_2 \) to the purified enzyme caused an increase in absorption at 343 nm (Fig. 2B). Such an absorption increase is ascribable to conversion of the met-form to the oxy-form of type-3 copper proteins.\(^{37-39} \) Therefore, \( P. \text{nameko} \) tyrosinase is thought to have been isolated in the mixture of the oxy- and met-forms.

**Catalytic properties of the purified tyrosinase**

The monophenolase and catecholase activities of the purified tyrosinase were determined using five monophenols and five \( o \)-diphenols respectively as substrates. Though the tyrosinase reaction is complicated and the exact reaction mechanism has not been elucidated, it is generally accepted that it is simply analyzed by Michaelis–Menten kinetics.\(^{40} \) Hence we obtained kinetic parameters, \( K_m \) and \( k_{cat} \), from the double reciprocal plots, which are summarized in Table 2. As shown in this table, the \( k_{cat}/K_m \) value for dopamine was much higher than those for the other substrates, suggesting that it is a preferable substrate for \( P. \text{nameko} \) tyrosinase. When \( l \)-Tyr, tyramine, and \( d \)-DOPA were used as substrates, the reaction rate increased linearly with an increase in the substrate concentration and we could not determine the \( K_m \) and \( k_{cat} \) values from the double reciprocal plots. In these cases, we obtained tentative \( k_{cat}/K_m \) values, assuming that the \( K_m \) values were much larger than the substrate concentration used and that the \( V_{max}/K_m \) value followed to the slope of the line in the plots of the reaction rate vs. the substrate concentration.
D-Tyr showed an interesting behavior, a sigmoid relationship between the reaction rate vs. the substrate concentration. We are studying this behavior in detail, but here we do not show the results.

Molecular cloning of tyrosinase cDNA
cDNA cloning of tyrosinase from *P. nameko* was carried out by degenerate RT-PCR and RACE using the first strand cDNA of poly(A)⁺ RNA extracted from a fruit body of *P. nameko* as a template. RT-PCR was carried out using degenerate primers constructed by Wichers et al. 41) on the basis of sequence homologies in the copper-binding domains for a number of plant and fungal tyrosinases. The DNA sequence analysis of the PCR products revealed that the DNA fragment corresponding to nucleotide 268–856 in the cDNA sequence determined in the present work (Fig. 3) was amplified. 5' RACE was performed using a specific reverse primer (nucleotide 670–689 in the cDNA) that was designed from the internal sequence of the PCR products. Amplified DNA fragments, of about 700 bp, which contained the sequences of nucleotide 669 in the cDNA, were obtained. On 3' RACE, DNA fragments of about 1.3 kbp were amplified using a forward primer corresponding to nucleotide 774–803 in the cDNA. Finally, DNA fragments which contained the full-length cDNA, of about 1.9 kbp, were obtained using a pair of forward and reverse primers (nucleotides 1–25 and 1854–1878 respectively in the cDNA). The complete nucleotide sequences were determined for 10 plasmid clones of the full-length cDNA. Two distinct cDNAs for *P. nameko* tyrosinase, cDNA-1 (AB275646) and cDNA-2 (AB275647), represented in Fig. 3, were obtained from the fruit body of *P. nameko*. Six and four clones carried cDNA-1 and cDNA-2 respectively. The nucleotide sequence of cDNA-2 was different from that of cDNA-1 at 12 positions, but the nucleotide sequences of cDNA-1 and cDNA-2 were almost identical, since the calculated homology was 99.4%, except for the poly(A)-tails.

Fig. 3. Nucleotide and Deduced Protein Sequences of *P. nameko* Tyrosinase cDNA-1.
Base substitution at 12 positions and amino acid substitutions at 5 positions observed in cDNA-2 are shown in italics. The sequences of fragments of the purified enzyme obtained by *Achromobacter protease* I digestion are underlined. The putative copper ligands are squared. Cleavage site of *P. nameko* tyrosinase is indicated by an arrow.
Deduced amino acid sequences

The amino acid sequences deduced from the nucleotide sequences of cDNA-1 and cDNA-2 are also presented in Fig. 3. The difference in the sequences between cDNA-1 and cDNA-2 resulted in amino acid substitutions at five positions. We term the tyrosinases encoded by cDNA-1 and cDNA-2, TYR-1 and TYR-2, respectively. TYR-1 and TYR-2 consisted of 625 amino acid residues, and their molecular weights were calculated to be 67,498.7 and 67,487.6, respectively. These values are quite different from the size of the purified enzyme, estimated from SDS–PAGE as 42,000. This is discussed in the following section.

sequence alignments of tyrosinases from variousorganisms have shown that two putative copper-binding sites, CuA and CuB, are highly conserved. Each of these contains three His residues, named HA1, HA2, and HA3 for CuA, and HB1, HB2, and HB3 for CuB. The CuA- and CuB-binding sites consist of HA1-x(n)-HA2-x(8)-HA3 and HB1-x(3)-HB2-x(n)-HB3, respectively, where n is a variable number of residues. Figure 4A shows the amino acid sequences at these CuA and CuB sites in fungal tyrosinases, indicating that the deduced amino acid sequence of the P. nameko tyrosinase complies with this general scheme. Fungal tyrosinases have a highly conserved cysteine residue at position 2 from HA2. This residue is thought to contribute to the structure and function of tyrosinase, because the residue is covalently linked to HA2 via a thioether bridge in the N. crassa tyrosinase, and is perhaps bound to the copper A atom in A. oryzae tyrosinase. In the case of P. nameko tyrosinase, the N-terminal residue of the enzyme is an acetylated Ser, as reported for N. crassa tyrosinase.

Cleavage of C-terminal region of P. nameko tyrosinase

The C-terminal amino acid sequence of the purified enzyme, A-Ala-Ser-Val-Phe-OH, was not found in the C-terminal region of TYR-1 or TYR-2, but corresponded to residue 384–387 of them (Fig. 3). Though the N-terminal sequence of the purified enzyme was not determined by Edman degradation, as described above, our preliminary experiments suggest that the N-terminal residue of the enzyme is an acetylated Ser, as reported for N. crassa tyrosinase. Furthermore, the purified enzyme did not show any properties of glycosylated proteins (data not shown), and if we assume that the encoded tyrosinase is processed from the C-terminus by a cleavage at Phe387 and undergoes acetylation of Ser2 after truncation of Met1, the molecular weight of the
resulting tyrosinase is calculated to be 42,453.3 and 42,425.3 for TYR-1 and TYR-2 respectively. These values agree well with the mass of the purified tyrosinase ($m/z$ 42424.39). Hence we propose that $P$. nameko tyrosinase is expressed as a proenzyme followed by C-terminal cleavage at the specific site and N-terminal acetylation to produce a mature enzyme.

C-terminal cleavages of fungal tyrosinases have been reported as in tyrosinases from Neurospora crassa,\textsuperscript{47} A. bisporus $AbPPO1$ and $AbPPO2$ cDNAs,\textsuperscript{41} Pycnoporus sanguineus,\textsuperscript{49} and Trichoderma reesei.\textsuperscript{11} The sizes of the polypeptides cleaved ranged from 20k to 29k. In these studies, the cleavage site is clearly identified as Phe407 in $N$. crassa tyrosinase.\textsuperscript{47} Tyr at position 381 of $A$. bisporus tyrosinase $AbPPO1$ and Tyr at position 384 of $P$. sanguineus tyrosinase are postulated to be the putative cleavage site by a chymotrypsin-like serine protease as well as $N$. crassa tyrosinase.\textsuperscript{47} since these Tyr residues are situated in a highly conserved sequence, Leu-Tyr-Gly, among fungal tyrosinases, as shown in Fig. 4B. The present study indicates that $P$. nameko tyrosinase is not cleaved at Tyr382 of the putative cleavage site, but at Phe387, which corresponds to Phe at position 407 of the cleavage site of $N$. crassa tyrosinase (Fig. 4B), indicating that the Tyr residue in the conserved sequence of Leu-Tyr-Gly is not a common cleavage site of fungal tyrosinases.\textsuperscript{39}

Espín et al.\textsuperscript{49} reported that a latent (inactive) form of $A$. bisporus tyrosinase, with a molecular size of 67,000, isolated from fruit bodies, was partially digested in vitro by trypsin and subtilisin Carlsberg, and the resulting enzyme was active. The molecular size of the activated enzyme was about 43,000, and activation was inhibited by a protease inhibitor, aprotinin.\textsuperscript{50} Hence it has been accepted that similar activation processes occur in many fungal tyrosinases by endogenous protease such as serine protease.\textsuperscript{13} In the case of $P$. nameko tyrosinase, the activation process is unclear, and several problems remain to be resolved: (i) whether the encoded tyrosinase consisting of 625 amino acid residues is active, (ii) whether the encoded tyrosinase can be specifically cleaved at Phe387 by specific proteases, and (iii) the role of the C-terminal peptide 388–625 in the encoded tyrosinase. To answer these questions, construction of an expression system for $P$. nameko tyrosinase is required.

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References

(1994).


