Purification and Characterization of Tyrosinases from *Streptomyces albus*

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The bacterium *Streptomyces albus* has so far never been investigated for tyrosinase activity. The studies presented in this communication show that this bacterium may be a future source for larger production of tyrosinase. The enzyme was purified starting with 5,600 ml of culture filtrate. The crude enzyme was first purified by centrifugation, followed by ammonium sulfate precipitation and ultrafiltration. Then, melanin was removed applying a Servacell DEAE 52 resin, using the batch technique. Thereafter, the crude enzyme was loaded on a SEC Sephacryl S-100 column and, after ultrafiltration, 1.17 mg of purified tyrosinase were obtained. The molecular mass of the purified enzyme was determined by MALDI mass spectrometry to be 30,096 Da which corresponds to the obtained results from SDS-PAGE.

Using the diphenol L-DOPA and the monophenol L-tyrosine as substrates, the kinetic parameters for both substrates, K_m = 7.8 mM and 0.5 mM and k_{cat}/K_m = 157 mM^{-1} s^{-1} and 23 mM^{-1} s^{-1}, respectively, were determined. Maximal activities of the purified enzyme were recorded at pH 7.0. Long-term experiments with *Streptomyces albus* tyrosinase revealed that storage of the lyophilized enzyme sample at temperatures below zero turned out to be the best. For tyrosinase in buffer containing 20% glycerol, no loss of activity was observed at 4 °C and –60 °C.

Key words: Tyrosinase, *Streptomyces albus*, Enzyme Kinetics

Introduction

Tyrosinases (monophenol, oxygen oxidoreductase, E.C. 1.14.18.1) are nearly ubiquitously distributed in nature and are essential for pigmentation, important factors in wound healing and primary immune response. The copper pair present in their active site binds one molecule of atmospheric oxygen to catalyze two different kinds of enzymatic reactions: (I) *ortho*-hydroxylation of monophenols (cresolase activity) and (II) oxidation of *o*-diphenols to *o*-diquinones (catecholase activity). The best-known function is the formation of melanins from L-tyrosine *via* L-3,4-dihydroxyphenylalanine (L-DOPA). The complicated hydroxylation mechanism at the active centre is still not completely understood (Claus and Decker, 2006).

The monophenolhydroxylase and diphenoloxidase activities of tyrosinases are the basis for many industrial biotechnological applications (Duran and Esposito, 2000): in environmental technology for the detoxification of phenol-containing waste waters and contaminated soils (Claus and Filip, 1990), as biosensors for phenol monitoring, in pharmaceutical industries for the production of *o*-diphenols (*e.g.* L-DOPA, dopamine for the treatment of Parkinson's disease), and in cosmetic and food industries (Mayer and Harel, 1978). Synthetic melanins found applications for protection against radiation (UV, X-ray, gamma-ray), as cation exchangers, drug carriers, antioxidants, antiviral agents or immunogens (Nosanchuk and Casadevall, 2003; Wang *et al.*, 2000).

Streptomyces belongs to the family Streptomycetaceae and represents one of the most important genera of the *Actinomycetales* order. Members of this genus were intensively studied because of their capacity to produce antibiotics and enzymes

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of industrial importance, such as glucose isomerase, protease, amylase, xylanase, while their capacity to produce tyrosinase was studied to a lesser extent (Bahrim and Negoita, 2007). Bacterial tyrosinases with new features like high-temperature stability (Kong *et al.*, 2000; Liu *et al.*, 2004) or a broader substrate spectrum (Sanchez-Amat *et al.*, 2001) open further areas of application.

The tyrosinases from *Streptomyces* species are non-modified monomeric proteins with a relatively low molecular mass of ca. 30 kDa. These enzymes are secreted to the surrounding medium, where they are involved in extracellular melanin production. Advantageously, these *Streptomyces* tyrosinases can be isolated in sufficient quantities and purities for detailed structural studies.

Material and Methods

Materials

L-3,4-Dihydroxyphenylalanine (L-DOPA), L-tyrosine, L-tyrosine methyl ester, glucose, yeast extract, malt extract, glycerol, sodium glutamate, L-methionine, piperazine-*N*,*N*'-bis(2-ethanesulfonic acid) (PIPES), copper sulfate, sodium acetate, acetic acid, Servacell DEAE 52, Sephacryl S-100, Nucleosyl C18 were supplied from Sigma (Germany).

Bacterial sources

The morphological, cultural, physiological and biochemical characteristics of the investigated bacteria were compared with those of the references for similar streptomycetes species. The strain was identified as *Streptomyces albus*.

Organism and cultural conditions

Streptomyces albus (Rossi Doria) Waksman and Henrici NRRL 5778 was isolated from a local garden soil by suspending 1 g of soil in 100 ml of sterile, distilled water. An inorganic salts-agar medium (Pridham *et al.*, 1957) was used containing 0.5% D-xylose as sole carbon source.

All cultures were grown in a medium (RM) containing 0.5% yeast extract, 0.4% malt extract, 0.7% NaCl, and 0.05% $MgSO_4 \cdot 7H_2O$, and adjusted to pH 7.0. The cultures were incubated at 25 °C on a rotary shaker at 3.5 × *g*. Stock cultures were maintained on RM solidified with 1.7% agar.

For mycelium propagation, a 250-ml Erlenmeyer flask with 100 ml of RM was inoculated with spores from 2-day-old media and incubated for 24 h. The total content of the flask was subsequently added to a 2.8-l Fernbach flask containing 800 ml of RM supplemented with 0.5% D-xylose. After 24 h of incubation, the mycelium was harvested by filtration through Whatman no. 41 paper, washed twice with distilled water, and resuspended in 0.2 M potassium phosphate buffer (pH 7.0) at room temperature, maintaining a ratio of 2 g of mycelium (wet weight) per 5 ml of buffer.

Preparation of the crude extract

The culture supernatant was obtained by centrifugation of the culture broth at 5,000 × *g* for 15 min at 4 °C. The medium, containing the extracellular tyrosinase, was subjected to an aqueous two-phase system based on polyethylene glycol, PEG-8000 [5% (w/w) potassium phosphate buffer, pH 7.0]. After stirring the solution for 15 min at room temperature, it was centrifuged at 10,000 × *g* for 10 min at 25 °C. The upper black PEG-rich phase (20% of total volume) was discarded, and the clean phosphate-rich phase containing the *S. albus* tyrosinase was brought to 60% saturation with $(NH_4)_2SO_4$ under continuous stirring at 4 °C. After 1 h, the solution was centrifuged at 60,000 × *g* for 30 min at 4 °C. The pellet, containing the tyrosinase, was collected and dissolved in a minimum volume of water.

After centrifugation, the supernatant was filtered through a glass fiber filter and then through a Millipore filter of 0.45 μm pore size, and 1% (v/v) glycerol was added. Cell-free culture filtrates were concentrated by lyophilization using a freeze-dryer. The glycerol-containing lyophilizate was suspended in 10 ml 25 mM PIPES buffer, pH 7.0, and dialyzed in Sigma dialyzing bags against 5 l of sterilized, distilled water containing 50 l of 10 mM $CuSO_4$ for 24 h at 4 °C. The dialysate was lyophilized once again and resuspended in the same buffer.

Then, 10 g anion-exchange material (Servacell, DEAE 52) were suspended in 500 ml 25 mM sodium acetate buffer, pH 5.5, and equilibrated overnight at 4 °C. The upper layer was decanted, and the sediment was mixed with 10 ml of the sample and the mixture was adjusted to pH 7.0. The sediment was centrifuged at 2,500 × *g* for 15 min at 4 °C and the obtained supernatant used for further purification by size exclusion chromatography. A FPLC system, equipped with a Sephacryl

S-100 column (16 × 60 mm), was used, equilibrated with 25 mM PIPES buffer, pH 7.0, containing 150 mM NaCl. The column was eluted with the same buffer at a flow rate of 0.4 ml/min.

For the final purification of the enzyme, a HPLC system, equipped with a Nucleosyl C18 column (100 × 2.1 mm), was applied. The sample was eluted with a linear gradient of buffer A [0.1% trifluoroacetic acid (TFA) in water] and buffer B (0.085% TFA in acetonitrile) for 60 min, at a flow rate of 1 ml min^{-1}. The eluted fractions were detected at a wavelength of 206 nm.

Spectrophotometric measurement of the enzymatic activity

Diphenolase and monophenolase activities were determined spectrophotometrically with the substrates L-DOPA and L-tyrosine, respectively, both at 25 °C, using an Uvikon 940 spectrophotometer. The standard reaction mixture for the monophenolase activity contained in a total volume of 1.1 ml 0.1 mg ml^{-1} tyrosinase and 1 mM L-tyrosine in 20 mM sodium phosphate buffer, pH 7.0. The steady-state rate of the monophenolase activity was calculated from the linear zone of the product accumulation curve after the lag period.

The diphenolase activity does not present any lag period. The dopachrome assay was performed according to Fling *et al*. (1963). For the measurements, a cuvette was filled with 1 ml sodium phosphate buffer (100 mM, pH 7.0) containing 5 mM L-DOPA, 0.075 to 0.225 mg of *Streptomyces albus* tyrosinase were added, and the solution was mixed for 5 s. Then the increase in absorption at 475 nm, due to the formation of dopachrome (ε_{475} = 3,600 M^{-1} cm^{-1}), was monitored as a function of time. A solution containing only 1 ml sodium phosphate buffer (100 mM, pH 7.0) with 5 mM L-DOPA was used as reference solution. The activity is expressed as mol of L-DOPA oxidized per min.

Determination of the protein concentration

The protein concentration of *S. albus* tyrosinase was determined from the optical absorption at 280 nm in 10 mM sodium phosphate buffer, pH 7.0, using a molar absorption coefficient of 82,000 M^{-1} cm^{-1} according to Jackman *et al*. (1991).

Polyacrylamide gel electrophoresis (PAGE)

The purity of the protein was checked after each step of purification by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10% polyacrylamide), according to Laemmli (1970). Plus Protein Standard (Bio-Rad, Munich, Germany) with synthetic proteins of the molecular weights 10, 15, 20, 25, 37, 50, 75, 100, 150 and 250 kDa were used as molecular weight standard. Proteins separated by SDS-PAGE were stained with 0.05% Coomassie brilliant blue R-250.

MALDI measurements

MALDI-TOF mass spectrometry experiments were performed on a Voyager DESTR instrument (Applied Biosystems, Darmstadt, Germany) according to the manufacturer's instructions. α-Cyano-4-hydroxycinnamic acid, dissolved in a 2:1 mixture of 0.1% (by volume) aqueous TFA and 0.1% (by volume) trifluoroacetic acid containing acetonitrile, was used as a matrix for the analyses. Spectrometry was performed in the positive linear ion mode.

Effect of temperature and pH value on the S. albus tyrosinase activity

The effect of the temperature on the *S. albus* tyrosinase activity was studied by incubation of the purified enzyme at −60 °C, −20 °C, 4 °C and 35 °C in 0.05 M sodium phosphate buffer, pH 7.0, for a period of 30 min. The residual *S. albus* tyrosinase activity was measured in aliquots removed at various time intervals and compared with controls incubated at 25 °C for the same periods. The effect of pH on the pure enzyme activity was examined by incubation of the enzyme in buffers with different pH values (4–10). For determination of the pH optimum of *S. albus* tyrosinase, a universal buffer (Davies buffer) instead of phosphate buffer was used. The activity of the samples was assayed under standard conditions.

Kinetic studies

Different concentrations (0.1–10 mM) of the diphenolic substrate L-DOPA and the monophenolic substrate L-tyrosine were incubated in 0.1 M sodium phosphate buffer, pH 7.0, with 0.1 mg ml^{-1} *S. albus* tyrosinase at 25 °C, and the absorption spectra were recorded at different times.

Kinetic parameters such as Michaelis constants K_m and V_{max} were determined for the diphenolic substrate L-DOPA and the monophenolic substrate L-tyrosine methyl ester from the equation of the Lineweaver-Burk plot as described by Gollas-Galvan *et al.* (1999). All procedures in this study were carried out at 25 ºC.

Results and Discussion

Purification of tyrosinase

After screening of various bacterial cultures for tyrosinase activity, the strain *Streptomyces albus* was identified as a producer of the enzyme. The best conditions for bacterium growth were with a mixture of 0.5% yeast extract, 0.4% malt extract, 0.7% NaCl, and 0.05% $MgSO_4 \cdot 7H_2O$ at pH 7.0.

After centrifugation, ammonium sulfate precipitation, and lyophilization the lyophilisate was dissolved in 25 mM PIPES buffer (pH 7.0) and then desalted and concentrated by ultrafiltration. The obtained sample was lyophilized as crude tyrosinase sample and resuspended in the same buffer. Concerning the higher isoelectric point (pI 9.5) of tyrosinase, the most suitable step for further purification was anion-exchange chromatography. Therefore, the anion-exchange batch technique using Servacell DEAE 52 resin was applied for removing melanin and other proteins from the upper layer of the culture. Thereafter the sediment was centrifuged at 2,500 × *g* for 15 min at 4 ºC, and the obtained supernatant with a specific activity of 5.9 was further used for purification.

About 3 ml of the supernatant with a specific activity of 5.9 were loaded onto a Sephacryl S-100 gel filtration column (16 × 60 mm), equilibrated with 25 mM PIPES buffer, pH 7.0, containing 150 mM NaCl, and separated at a flow rate of 0.4 ml min^{-1}. This column is suitable for the separation of proteins in the range of 1–100 kDa (Fig. 1).

The peak fractions with high tyrosinase activities were collected and lyophilized after adding 1% (v/v) glycerol. These fractions were resuspended in 2 ml distilled water and dialyzed. The

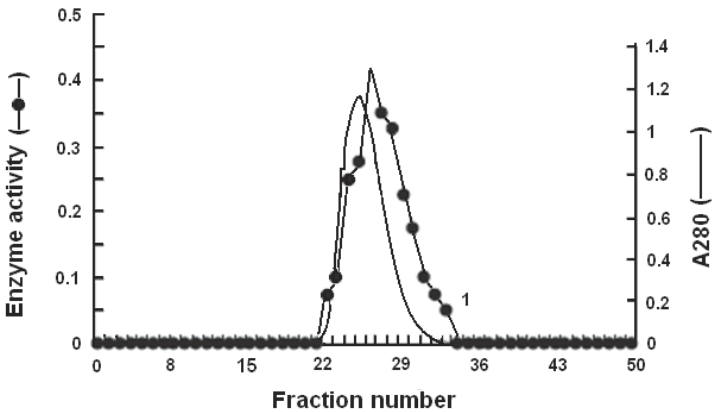

Fig. 1. Sephacryl S-100 gel filtration of crude tyrosinase from *S. albus*. Profiles correspond to 280 nm absorbance and enzymatic activity (see Material and Methods) of collected fractions. The crude tyrosinase was applied to a Sephacryl S-100 column and eluted with 25 mM PIPES buffer (pH 7.0), containing 150 mM NaCl, at a flow rate of 0.4 ml min^{-1}. Fractions 25 and 26 contained the highest enzymatic activity against 15 mM L-DOPA.

Table I. Purification steps for tyrosinase from *Streptomyces albus*.

Purification step	Total volume [ml]	Total protein [mg]	Total activity [U min^{-1}]	Specific activity [U min^{-1} mg^{-1}]	Purification (fold)	Yield (%)
Culture filtrate	5,600	18,500	584	0.03	1	100
Ammonium sulfate	146	861	331	0.38	12.7	56.7
Servacell DEAE 52	194	95.1	563	5.9	197	96.4
Sephacryl S-100	46	2.30	204	88.7	2,957	34.9
Ultrafiltration	4.5	1.17	152	130	4,333	26.0

purity of the obtained fractions was checked by SDS-PAGE (10% polyacrylamide) according to Laemmli (1970). The fractions 25 and 26 from Sephacryl S-100 chromatography showed a single protein band with a molecular mass of about 30 kDa (Fig. 2).

Table I summarizes the purification of *S. albus* tyrosinase, starting with 5,600 ml of culture filtrate, and yielding finally 1.17 mg of purified tyrosinase corresponding to a yield of 26%, as calculated according to:

$$\frac{\text{total enzyme activity of fraction (152)}}{\text{total enzyme activity in starting material (584)}} \cdot 100\% = \text{yield (26.0\%)}.$$

In comparison, the first bacterial tyrosinases were purified from cell extracts of *Streptomyces nigrifaciens* (Nambudiri and Bhat, 1972) in a yield of 17%, and extracellular tyrosinase from *Streptomyces michiganensis* has been isolated from 10 l fermentation broth in 1.7% yield (Philipp *et al.*, 1991).

For molecular mass determination by MALDI-TOF MS, a highly purified enzyme sample was produced by a HPLC system, equipped with a Nucleosyl C18 column (100 × 2.1 mm). A single intense peak was detected confirming the purity of the enzyme (Fig. 3).

For the determination of a potential carbohydrate content, the HPLC-purified tyrosinase was tested with orcinol/H_2SO_4 (0.1% orcinol in 20% H_2SO_4) on silica gel plates. No carbohydrates were observed, confirming that in contrast to eukaryotic organisms, bacterial tyrosinases are not glycosylated (Claus and Decker, 2006).

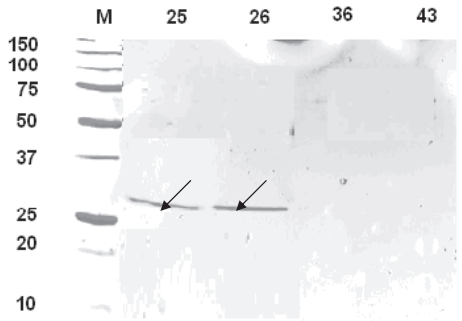

Fig. 2. Denaturating SDS-PAGE (10% polyacrylamide gel) of Sephacryl S-100 fractions 25, 26, 36, and 43. M, marker proteins (Precision Plus Protein Standard, Bio-Rad). The arrows indicate the 30-kDa tyrosinase in the active fractions 25 and 26.

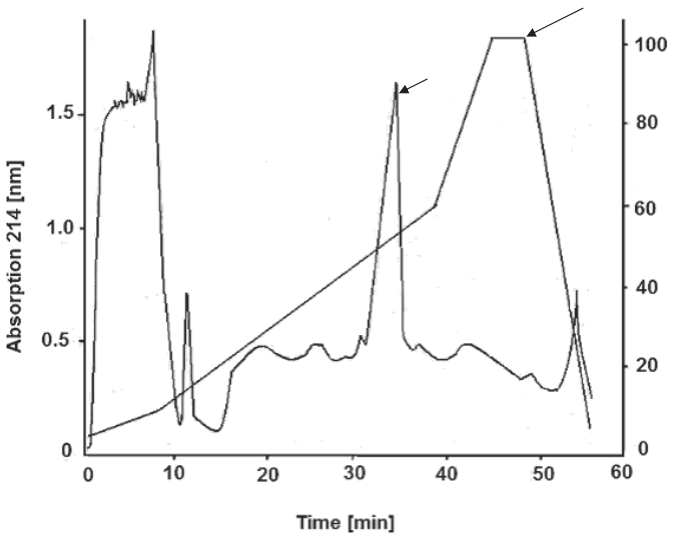

Fig. 3. HPLC purification of the *S. albus* tyrosinase using a Nucleosyl C18 (100 × 2.1 mm) column. The sample was eluted with a linear gradient of buffer A (0.1% TFA in water) and buffer B (0.085% TFA in acetonitrile) for 60 min, at a flow rate of 1 ml min^{-1}. The eluted fractions were detected at λ = 206 nm. The short arrow marks the only peak fraction containing tyrosinase, whereas the longer arrow marks the gradient.

Determination of the molecular mass of S. albus tyrosinase

The molecular mass was determined by electrophoresis and mass spectrometry. According to MALDI mass spectrometry, the isolated protein showed mass peaks at 30,096 Da and 15,048 Da, and the latter one corresponds to half the molecular mass of the native tyrosinase (Fig. 4). This result is in good agreement with the molecular mass of 30 kDa as estimated through SDS-PAGE (Fig. 2) and is in reasonable agreement with the reported molecular mass of other tyrosinases from *Streptomyces* species.

Stability of S. albus tyrosinase

Maintaining high activity levels even after long-term storage is an important feature for a potential biotechnological application of tyrosinase. Therefore, the stability of the tyrosinase was investigated by measuring the activity as a function of time upon storage under different conditions.

The activity of *S. albus* was determined as a function of the storage time. The tyrosinase, dissolved in 50 mM phosphate buffer, pH 7.0, was stored at four different temperatures (–60 °C, –20 °C, 4 °C, and 35 °C). The enzyme activity was reduced by 25% after 40 days of storage at 4 °C, while storage of the enzyme at –20 °C or 35 °C led to an nearly complete loss of activity within the first few days (Table II). Addition of glycerol to the enzyme solution resulted in stabilization. In the presence of 20% glycerol, no loss of activity was observed after 150 days of storage of the tyrosinase at 4 °C and –60 °C, but the activity dropped already within the first days significantly at –20 °C and 35 °C. Tyrosinase solutions stored at –60 °C followed by 30 days of storage at 4 °C maintained their full activity. In addition, the storage of lyophilized tyrosinase was also studied at these four temperatures. In this case, storage at temperatures below zero, –20 °C and –60 °C, turned out to be the best conditions preserving the activity. Samples were stored for at least 4 months without any loss of activity under these conditions.

pH dependence of the S. albus tyrosinase activity

Fig. 5 shows the enzyme activity against L-DOPA (normalized with respect to the highest activity) as a function of the pH value, which is optimal at pH 7.0 and close to 6.8, the pH optimum of *Streptomyces glaucescens* tyrosinase

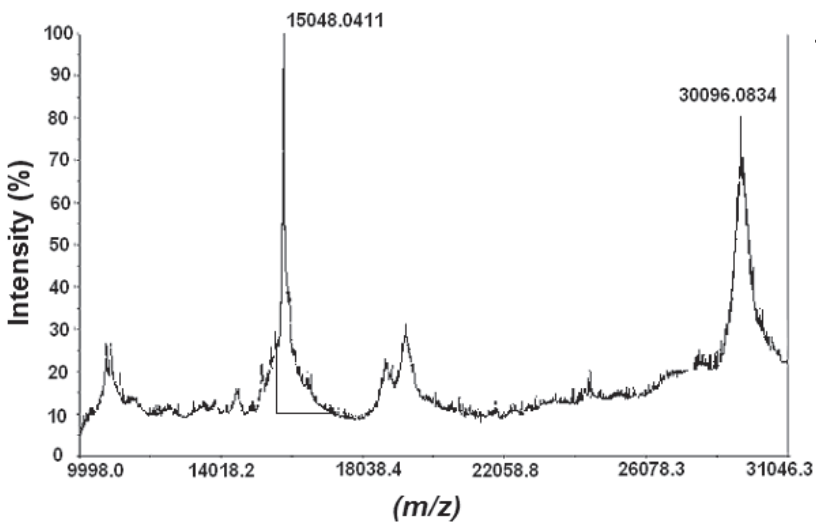

Fig. 4. MALDI mass spectrum of the eluate, obtained after elution from the Nucleosyl C18 column, showing a peak at 30,096 Da corresponding to the molecular weight of *S. albus* tyrosinase. The peak at 15,048 Da corresponds to exactly half the molecular weight of tyrosinase. Sinapinic acid, dissolved in a 2:1 mixture of 0.1% (by volume) aqueous TFA and 0.1% (by volume) TFA containing 60% acetonitrile, was used as a matrix for the analysis. Spectrometry was performed in the positive linear mode.

(Jackman *et al.*, 1991). The enzymatic activity decreased to almost 0 at pH 4 and 9, respectively.

Kinetic studies on S. albus tyrosinase

Tyrosinases possess both, monophenol and diphenol oxidase activity. Fig. 6 illustrates the conversion of the diphenolic substrate L-DOPA and the monophenolic substrate L-tyrosine in the presence of tyrosinase.

After incubation of different concentrations of L-DOPA from 0.1 to 10 mM in 0.1 M sodium phosphate buffer, pH 7.0, with 0.1 mg ml^{-1} *S. albus* tyrosinase at 25 ºC, the absorption spectra were recorded at different times. After 15 min, the reaction with the diphenolic substrate came to an end.

After incubation of different concentrations of L-tyrosine from 0.1 to 10 mM in 0.1 M sodium

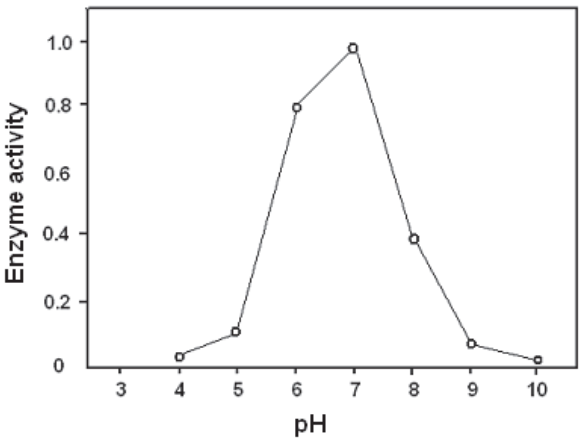

Fig. 5. Dependence of *S. albus* tyrosinase activity on the pH value. The effect of pH on the pure enzyme activity was examined by incubation of the enzyme with L-DOPA as a substrate at different pH values (4–10).

Table II. Storage stability of *Streptomyces albus* tyrosinase at different temperatures (lyophilized tyrosinase and tyrosinase dissolved in 50 mM phosphate buffer, pH 7.0, in the presence of and without 20% glycerol). Enzyme activity is shown in percentage.

Enyzme sample	Enzyme activity (%)			
	−60 ºC	−20 ºC	+4 ºC	+35 ºC
Tyrosinase dissolved in buffer	5	10	75	10
Tyrosinase dissolved in buffer with 20% glycerol	100	30	100	20
Lyophilized tyrosinase	100	100	80	40

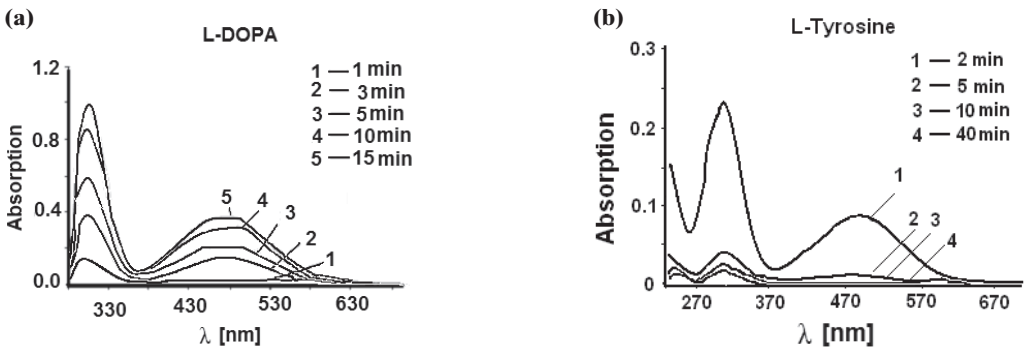

Fig. 6. (a) Absorption spectra of the products formed by conversion of different concentrations of the diphenol substrate L-DOPA from 0.1 to 10 mM in 0.1 M sodium phosphate buffer, pH 7.0, in the presence of 0.1 mg ml^{-1} *S. albus* tyrosinase at 25 ºC, at different periods of time. (b) Absorption spectra of the products formed by conversion of different concentrations of the monophenol substrate L-tyrosine from 0.1 to 10 mM in 0.1 M sodium phosphate buffer, pH 7.0, in the presence of 0.1 mg ml^{-1} *S. albus* tyrosinase at 25 ºC, at different periods of time.

phosphate buffer, pH 7.0, with 0.1 mg ml^{-1} *S. albus* tyrosinase at 25 ºC, the absorption spectra were recorded at different times. Due to the lag phase, characteristic for monophenolic substrates, the recording time of this reaction was extended to 40 min, whereby even then the reaction did not come to an end.

The kinetic properties of tyrosinase from *S. albus* were studied using different concentrations of the diphenolic substrate L-DOPA and the monophenolic substrate L-tyrosine methyl ester. The purified tyrosinase (0.1 mg ml^{-1}) was incubated with different concentrations of L-DOPA from 0.1 to 10 mM and its enzymatic activity was measured specrophotometrically. Kinetic parameters, such as Michaelis constants K_m and V_{max}, were determined from the equation of the Lineweaver-Burk plot as described by Gollas-Galvan *et al.* (1999). K_m and V_{max} values of *S. albus* tyrosinase for L-DOPA were calculated to be 7.8 mM and 1.7 mM s^{-1}, respectively (Fig. 7a).

The enzymatic activity of purified tyrosinase for different concentrations of L-tyrosine methyl ester (0.1 to 10 mM) was also studied, and the K_m and V_{max} values were calculated to be 0.5 mM and 0.013 mM s^{-1} respectively (Fig. 7b). The calculated parameters of *S. albus* tyrosinase are summarized in Table III.

The enzyme isolated from *Streptomyces albus* shows both, monophenolase and diphenolase activities, confirming that it is a real tyrosinase. These characteristics make *Streptomyces albus* tyrosinase an interesting candidate for future analytical and biotechnological applications.

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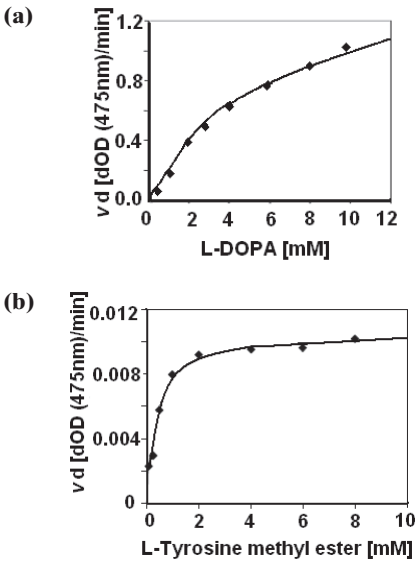

Fig. 7. (a) Enzyme kinetics as a function of the concentration of substrate. The K_m values were determined using L-DOPA (0.1–10 mM) as a substrate. According to the Lineweaver-Burk model, the K_m value was calculated to be 7.8 mM. (b) Enzyme kinetics as a function of the concentration of substrate. The K_m values were determined using L-tyrosine methyl ester (0.1–10 mM) as a substrate. According to the Lineweaver-Burk model, the K_m value was calculated to be 0.5 mM.

Table III. Summary of the enzymatic parameters of *Streptomyces albus* tyrosinase for different mono- and diphenol substrates. Kinetic parameters were determined from the equation of the Lineweaver-Burk plot as described by Gollas-Galvan *et al.* (1999).

Parameter	Substrate	
	L-Dopa	L-Tyrosine methyl ester
K_m [mM]	7.8 ± 0.4	0.5 ± 0.1
k_{cat} [s^{-1}]	1,263 ± 59	9 ± 2
k_{cat}/K_m [mM^{-1} s^{-1}]	157 ± 15	23 ± 5

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