Isolation and Characterization of Novel Tyrosinase from *Laceyella sacchari*

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Abstract: We here describe the isolation and characterization of a tyrosinase from a newly isolated soil bacterium. 16S rDNA sequence analysis revealed that the bacterium most probably belongs to the species *Laceyella sacchari* (Ls) *(>99.9 % identity).*

The tyrosinase extracellular enzymatic activity was induced in the presence of L-methionine and $CuSO_4$. The crude enzyme was first purified by centrifugation followed by ammonium sulphate precipitation and ultrafiltration. After removal of a brown pigment, probably melanin, a purified enzyme was obtained by further separation of the crude protein mixture using size exclusion chromatography. Some 10.5 mg of pure tyrosinase (LsTyr) was isolated with a molecular mass of 30 910 Da, based on MALDI mass spectrometry. Together with the observed enzymatic activity, N-terminal chemical sequence analysis confirmed that the isolated enzyme is homologous to other tyrosinases.

The kinetic parameters for the diphenol substrates L-DOPA and dopamine and for the monophenol substrate L-tyrosine were determined to be K_M = 4.5 mM , 1.5 mM and 0.055 mM, and k_{cat}/K_M = 261.5 mM^{-1} s^{-1} , 30.6 mM^{-1} s^{-1} and 56.3 mM^{-1} s^{-1}, respectively. Maximal activities of the purified enzyme were found to occur at pH 6.8.

Keywords: Tyrosinase, Laceyella sacchari, MS, MALDI.

INTRODUCTION

Tyrosinases and the related catechol oxidases (collectively termed polyphenol oxidases) comprise a family of binuclear copper enzymes found in many species of animals, plants, fungi, and bacteria that use phenol-like starting materials to produce a variety of biologically important compounds, such as melanin and other polyphenolic compounds [1, 2, 3]. These type III copper proteins are capable of two activities: monophenolase/cresolase activity (EC 1.14.18.1) and diphenolase/catecholase activity (EC 1.10.3.1). Both activities result in the formation of reactive quinones, important intermediates in the biosynthesis of compounds such as melanin.

The first crystal structure of a tyrosinase (from *Streptomyces castaneoglobisporus*) [4] offered important insights into the mechanism of phenol hydroxylation. The structure shows the enzyme in complex with its accessory caddie protein. The tyrosinase is predominately α-helical in structure and contains six histidine residues co-ordinating the two copper atoms, thus forming the active site of the enzyme. The recently published structure from another tyrosinase, isolated from *Bacillus megaterium,* reveals a similar fold [5]. With respect to its overall fold and active site architecture, the bacterial enzyme is highly similar to the related catechol oxidase from sweet potato [6]. However, the plant enzyme is only capable to perform the diphenolase reaction (EC 1.10.3.1) [7].

Recently, studies on the properties and function of tyrosinase received enormous interest due to their usefulness in numerous biotechnological applications [8, 9]. Tyrosinases from different sources have therefore been isolated and analysed [10, 11]. Due to their ability to utilize a wide variety of mono- and di-phenolic compounds, tyrosinases have been used as phenol biosensors, to cross-link proteins (including food proteins like casein), or for the removal of phenols from waste waters. Tyrosinases were also used to produce cross-linked proteins, allowing easy recycling of biocatalysts, such as lipases [1]. They were also applied to produce tailor-made melanins and other polyphenolic materials using various phenols and catechols as starting materials. Such biomaterials may have a variety of applications as organic semiconductors or in photovoltaics cells. Stamping of the tyrosinases onto plastic surfaces allows the in-situ formation of thin films of melanin which have been shown to be conductive to nerve cell growth in tissue cultures. As melanin has a bacteriostatic effect, its thin films may be useful for the prevention of bacterial contamination [12].

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1875-5305/12 $58.00+.00

Bacterial tyrosinases with new features like high-temperature stability [13, 14] or a broader substrate spectrum [15] will open further areas of application, and it is in this context that we here describe the isolation of a tyrosinase from *Laceyella sacchari* and its subsequent purification and characterization.

2. MATERIALS AND METHODS

Chemicals

Servacel DEAE 52 and Sephacryl S-100 resins were purchased from Pharmacia, Freiburg. Substrates dopamin, L-dihydroxyphenylalanine (L-DOPA) and L-tyrosine were obtained from Merck, Darmstadt. All other chemicals used in this work were purchased from Serva Heidelberg and were of highest available purity and used without further purification.

Isolation of a Tyrosinase-expressing Bacterial Strain

Soil samples were collected from several different areas in Bulgaria (Sofia, Varna, V. Turnovo). Some 0.2 g of soil was dissolved in 10-ml of a saline solution and 1 ml of it was added to 20 ml of a medium known to favour bacterial growth over fungal growth (tryptone 1%, glucose 0.2% and NaCl 0.5%). The flasks were incubated overnight at 28°C and serially diluted samples were streaked on MMB selection plates containing (% w/v) KH_2PO_4 (0.035%), $MgSO_4.7H_2O$ (0.013%), $(NH_4)_2SO_4$ (0.2%), glucose (0.2%), agar (1.5%), and 50 mM Tris buffer (pH 7.0). After sterilization, L -methionine (0.1%) and a trace elements solution (0.4%) were added containing (% w/v): $CuSO_4.5H_2O$ (0.02%), $FeSO_4.7H_2O$ (0.2%), $MnSO_4.H_2O$ (0.012%), $ZnSO_4.7H_2O$ (0.013%), $CoCl_2.6H_2O$ (0.028%), $NaMoO_4.2H_2O$ (0.02%) and $CaCl_2.2H_2O$ (0.061%). The plates were incubated at 28°C for 4–5 days. Melanin production was recorded by the appearance of black or black-brown colour colonies. The selected strains were isolated and streaked again on L -methionine-containing MMB plates and on similar plates without methionine (negative control). The isolate which formed the highest number of black colonies on methionine-plates and white colonies on plates without methionine was grown in a MMB liquid medium supplemented with 0.2 mM $CuSO_4$ at 28°C for 4 days. The broth was assayed for extracellular tyrosinase activity.

The bacterium was identified by 16S rRNA sequencing, performed at the BCCM/LMG culture collection laboratory at Ghent University.

Purification of Tyrosinase

The enzyme was purified from *L. sacchari* starting with 1000 ml of the culture filtrate. The culture supernatant was obtained by centrifugation of the culture broth at 5,000 × *g* for 15 min at 4 ºC. 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 ºC. The upper black PEG-rich phase (20% of total volume) was discarded, and the clean phosphate-rich phase containing the *L. sacchari* tyrosinase was brought to 60% saturation with $(NH_4)_2SO_4$ under continuous stirring at 4 ºC. After 1 h, the solution was centrifuged at 60,000 × *g* for 30 min at 4 ºC. 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 (passing molecular masses < 10000 Da) against 5 l of sterilized, distilled water containing 50 µl of 10 mM $CuSO_4$, for 24 h at 4 ºC. The dialyzate 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 ºC. The upper layer was decanted, and the sediment was mixed with 10 ml of the sample. The mixture was then adjusted to pH 7.0. The sediment was centrifuged at 2,500 × *g* for 15 min at 4 ºC and the obtained supernatant used for further purification by size exclusion chromatography. An 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. The fraction with the highest activity was additional purified after rechromatography on the same column.

The purity of the protein was checked after each step of purification by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (10% polyacrylamide) according to Laemmli [16]. Plus Protein Standard (Bio-Rad, Munich, Germany) with proteins of the molecular masses 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.

Protein concentration was measured after each step of purification by the method of Bradford [17].

Amino Acid Sequence Determination

The fractions 17 and 18 with the highest enzymatic activity were combined and additionally purified on an HPLC Nucleosil RP C18 column using 0.1% TFA in H_2O as loading buffer and 0.085% TFA, 80% acetonitrile in H_2O as eluting solution (eluent B). The following conditions were used: 10% eluent B for 10 min followed by 10–100% eluent B within 70 min, at a flow rate of 1 ml min^{-1}. Peak fractions were dried and after dissolving in 40% methanol, 1% formic acid, they were subjected to automated N-terminal Edman sequence analysis (Procise 494A Pulsed Liquid Protein Sequencer, Applied Biosystems GmbH, Weiterstadt, Germany).

MALDI Measurements

A MALDI TOF/TOF 4800 Plus instrument (Applied Biosystems, Darmstadt, Germany) was used for MALDI-MS analysis. Samples were prepared by mixing 0.7 µL of the sample with 0.7 µL matrix solution (7 mg/mL α-cyano-4-hydroxycinnamic acid,CHCA, in 50% ACN containing 0.1%

TFA) and spotted on a stainless steel 192-well target plate. They were allowed to air-dry at room temperature, then inserted in the mass spectrometer and subjected to mass analysis. 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 analyse. The instrument was externally calibrated with a mixture of angiotensin I, glu-fibrino-peptide B, ACTH (1–17), and ACTH (18–39).

Kinetic Measurements of o-diphenoloxidase Activity

o-Diphenoloxidase activities were determined spectrophotometrically with the substrates L-DOPA and dopamine, respectively, both at 25 ºC, using a Shimadzu UV-2100 spectrophotometer. The dopachrome assay was performed according to Fling *et al.* [18]. For the measurements, a cuvette was filled with 1 ml sodium phosphate buffer (0.1 M, pH 6.8) containing different concentrations of L-DOPA, and dopamine; about 1 μg of *Laceyella sacchari* tyrosinase were then added and the solution was mixed for 5 s. 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. The activity is expressed as mole of L-DOPA, respectively dopamine, oxidized per min.

The standard reaction mixture for the monophenolase activity contained, in a total volume of 1 ml, 1 $\mu g.ml^{-1}$ tyrosinase and different concentrations of L-tyrosine in 0.1 M sodium phosphate buffer, pH 6.8. The steady-state rate of the monophenolase activity was calculated from the linear zone of the product accumulation curve occurring after the lag period.

The reaction velocity was measured from the initial quasi-linear portion of the curves (usually 0–2 min) and the Michaelis-Menten model and a Lineweaver-Burk plot were used to determine the kinetic parameters K_M, K_{cat} and V_{max} for tyrosinase.

RESULTS AND DISCUSSION

From this screening experiment, we isolated a novel melanin-producing strain from Bulgarian soil samples. The 16S rDNA sequence revealed a high similarity (99.9%) with the *Laceyella sacchari* KCTC9790^T strain, suggesting that our isolate belongs to this species. To date, there exists no report on a tyrosinase from *L. sacchari*.

Purification of Tyrosinase

The isolated tyrosinase was purified in several steps as shown in Table **1**. After centrifugation, ammonium sulfate precipitation and ultrafiltration, the supernatant was subjected to an anion-exchange batch technique using Servacell DEAE 52 resin to remove melanin and contaminating proteins from the upper layer of the culture. About 4 ml of the supernatant were then loaded onto a Sephacryl S-100 gel filtration column for additional purification Fig. (**1**). The peak fractions 17 and 18 showed the highest enzymatic activity against 5 mM L-DOPA. After rechromatography on the same column they were collected and lyophilized after having added 1% (v/v) glycerol. The purity of collected fractions was confirmed by HPLC chromatography which showed a symmetrical peak.

Using these methods, 10.5 mg of purified enzyme with high specific activity were obtained from 0.5 liter of cell culture. The protein content of tyrosinase produced from *L. sacchari* was higher compared to that from *S. castaneoglobisporus* (12 mg from 1.25 l of culture) [12], from *Streptomyces michiganensis* (1.7 mg from 2 l of culture) [19] or from *Streptomyces albus* (1.17 mg from 5.6 l of culture) [20]. However, the overall yield after purification was lower than those for the tyrosinases from *S. castaneoglobisporus* (15.4 vs. 42%) and *T. reesei* (15.4 vs. 54% respectively) [12].

Identification of the Enzyme

Several methods and techniques identified the purified active fraction as a tyrosinase, including N-terminal sequencing by Edman degradation Fig. (**2**). Within the first 21 residues of the *L. sacchari* enzyme, BLAST analysis shows that 7 of them are fully conserved among four bacterial tyrosinases, and 5 of them are conserved in three in these proteins. The active fractions of purified enzyme were also analyzed by MALDI-TOF mass spectrometry showing a single protein peak with a molecular mass of 30 910 Da Fig. (**3**) which correlates with the molecular mass of 31 kDa as estimated by **SDS-PAGE (Fig. (3) insert)** and the masses of other bacterial tyrosinases [1].

Enzymatic Activity of Tyrosinase from *L. Sacchari*

Tyrosinases possess both monophenol and diphenol oxidase activity. Figs. (**4a** and **b**) illustrate the conversion of the diphenolic substrate L-DOPA and the monophenolic sub-

Table 1. Purification Steps for Tyrosinase from *Laceyella sacchari*

Purification step	Total protein [mg]	Total activity [U min^{-1}]	Specific activity [U.min^{-1}.mg^{-1}]	Purification [fold]	Yield (%)
Culture filtrate (0.5l)	2952	21400	7.2	1	100
Ammonium sulfate	1940	18850	9.7	1.3	88.1
Ultrafiltration	201	14800	73.6	10.2	69.2
Servacell DEAE 52	42	9525	226.8	31.5	44.5
Sephacryl S-100	10.5	3305	314.8	43.7	15.4

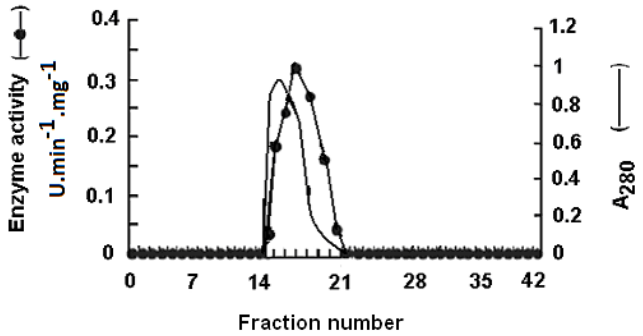

Figure 1. Sephacryl S-100 gel filtration of crude tyrosinase from *L. sacchari*. Profiles correspond to the absorbance at 280nm and the enzymatic activity of collected fractions. Fractions 17 and 18 contained the highest enzymatic activity against 5 mM L-DOPA. The activity is expressed as mole of L-DOPA, oxidized per min. The unit for specific activity is $U.min^{-1}.mg^{-1}$

	1				5				10				15				20				
TY_LS	**M**	**G**	**I**	**R**	**K**	**N**	**Q**	S	**T**	G	P	T	F	V	S	**E**	**K**	**R**	**R**	**F**	**V**
TY_BAC	**M**	**G**	**I**	**R**	**K**	**N**	**Q**	A	C	L	T	D	E	-	-	**E**	**K**	A	A	**F**	**V**
TY_SCA	**M**	Y	V	**R**	**K**	D	A	A	**T**	L	T	S	-	-	G	**E**	**K**	**R**	**R**	**F**	**V**
TY_SAV	**M**	T	V	**R**	**K**	**N**	**Q**	A	**T**	L	T	A	D	-	-	**E**	**K**	**R**	**R**	**F**	**V**
TY_MUS	V	K	N	**R**	L	**N**	I	V	D	F	V	K	N	-	-	**E**	**K**	F	F	T	L
TY_NCR	V	P	L	**R**	R	E	L	R	D	L	Q	Q	N	Y	P	**E**	Q	F	N	L	Y
TY_HUM	L	L	V	**R**	R	**N**	I	F	D	L	S	A	P	E	K	D	**K**	F	F	A	Y

Figure 2. Alignment of N-terminal sequence of the tyrosinase from *Laceyella sacchari* (TY-LS) with the N-terminal sequences of other tyrosinases deposited in GenBank. The first three enzymes are bacterial tyrosinases: TY_BAC: *Bacillus thuringiensis* tyrosinase; TY_SGH: *Streptomyces ghanaensis*; TY_SAV: *Streptomyces avermitili* tyrosinase.TY_HUM is human tyrosinase and TY_MUS is from the mushroom *Agaricus bisporus*. TY_NCR is *Neurospora crassa* tyrosinase. Identical residues are shown in bold.

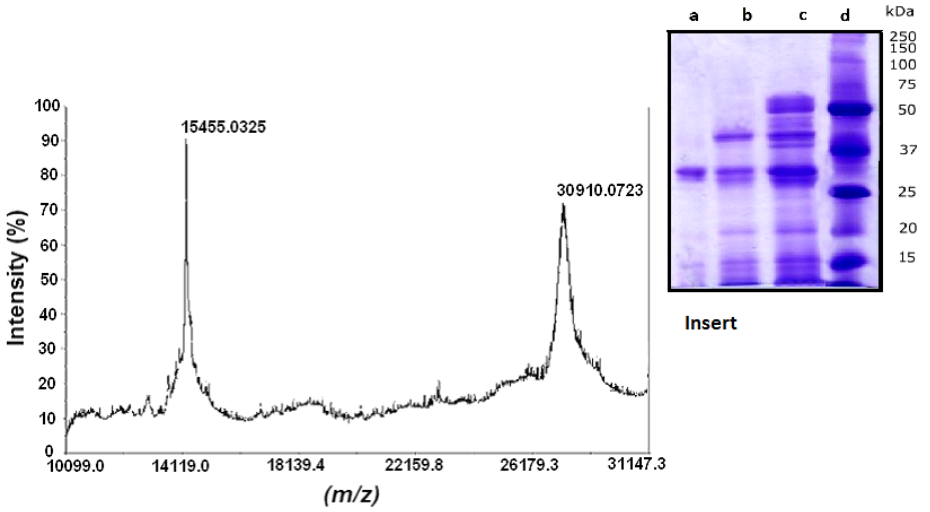

Figure 3. MALDI-TOF mass spectrum showing the molecular mass peak of *L. sacchari* tyrosinase at 30,910 Da. The mass at 15,455 Da corresponds to the doubly charged species. The analysis was performed in the positive linear mode. **Insert:** SDS-PAGE (10% polyacrylamide). Lane a: Purified tyrosinase after Sephacryl S-100; lane b: after Servacell DEAE 52; lane c: after ultrafiltration; lane d: Plus Protein Standard. Proteins separated by SDS-PAGE were stained with 0.05% Coomassie Brilliant Blue R-250.

strate L-tyrosine in the presence of tyrosinase. After incubation of different concentrations of L-DOPA (0.1 to 5 mM in 0.1 M sodium phosphate buffer, pH 7.0) with **1 µg.ml^{-1}** *L. sacchari* tyrosinase at 25 ºC, the absorption spectra recorded at 2 min time intervals showed that the reaction to convert diphenolic substrate into product almost stops after 20 min Fig. (**4a**). The kinetic parameters of tyrosinase for this substrate, based on the Michaelis-Menten model Fig. (**5a**) were determined from the Lineweaver-Burk plot, as described by Gollas-Galvan *et al.* [21], to be *K*m = 4.5 mM and *k*cat/*K*m = 261.5 $mM^{-1}\,s^{-1}$ Table **2**. Using different concentrations of the diphenol dopamine as substrate (5-30 mM), the kinetic parameters of the tyrosinase were found to be *K*m = 1.5 mM and *k*cat/*K*m = 30.6 $mM^{-1}\,s^{-1}$ Table **2**.

Also for the determination of the monophenolase activity with L-tyrosine as substrate the absorption spectra were recorded at different times Fig. (**4b**). Due to the lag phase the

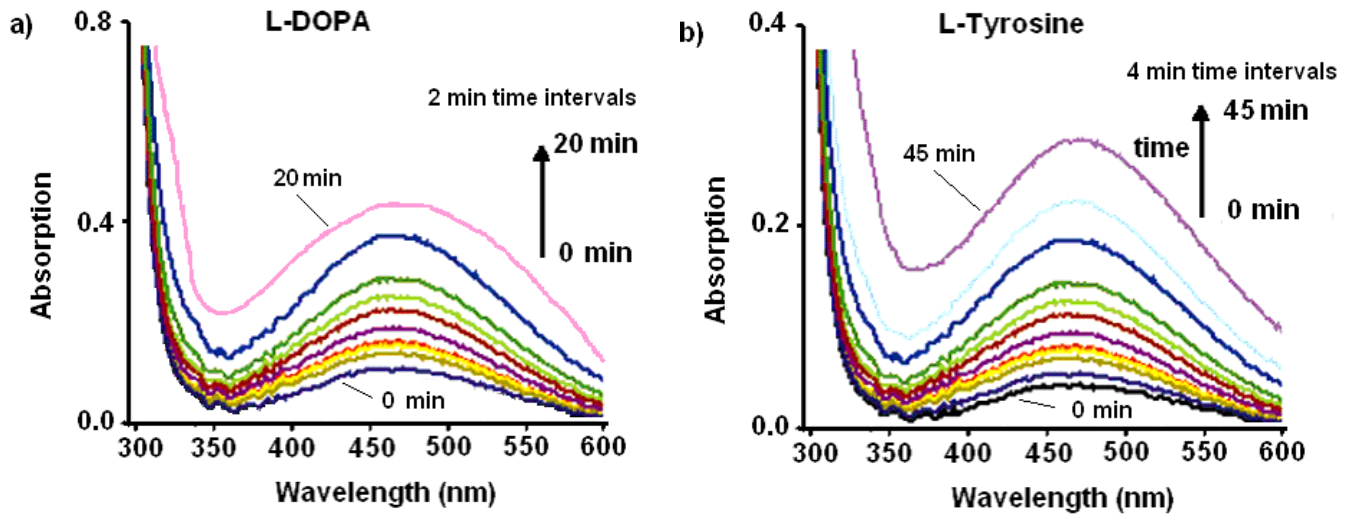

Figure 4. (**a**) Absorption spectra of the products formed by conversion of different concentrations of the diphenol substrate L-DOPA from 0.1 to 5 mM in 0.1 M sodium phosphate buffer, pH 7.0, in the presence of 0.1 μg ml^{-1} *L. sacchari* tyrosinase at 25 ºC, at 2 min time intervals. (**b**) Absorption spectra of the products formed by conversion of different concentrations of the monophenol substrate L-tyrosine from 0.1 to 2 mM in 0.1 M sodium phosphate buffer, pH 7.0, in the presence of 0.1 μg ml^{-1} *L. sacchari* tyrosinase at 25 ºC, at 4 min time intervals.

Table 2. Summary of the enzymatic parameters of *Lacceyela sacchari* tyrosinase for mono- and di-phenoloxidase activity in comparison with data for other tyrosinases. Kinetic parameters were determined from Lineweaver-Burk plots described by Gollas-Galvan *et al.* (21) using the Michaelis-Menten model

Tyrosinases from:	Substrates								
	Dopamine			L-Dopa			L-Tyrosine		
	K_M (mM)	k_{cat}	k_{cat}/K_M	K_M (mM)	k_{cat}	k_{cat}/K_M	K_M (mM)	k_{cat}	k_{cat}/K_M
Lacceyela sacchari	1.5	46	30.6	4.5	1177	261.5	0.055	3.1	56.3
Bacillus megaterium				0.35	10.1	289	0.075	2.5	329
Illex argentinus native (ST94)	9.3	2000	215						
Ipomoea batatas	2.5	138.0	55.0						
Streptomyces albus				7.8	1263	157			

Reaction conditions: an amount of 1.02 μg purified enzyme was assayed in 1 ml 0.1 M sodium phosphate buffer, pH 6.8, containing substrate concentrations ranging from 0.1 to 5.0 mM L-DOPA, from 5 mM to 30 mM dopamine and from 0.1 to 2.0 mM L-tyrosine.

recording time of this reaction was extended to 45 min, whereby even then the reaction kept converting the substrate into product.

The kinetic parameters of tyrosinase for L-tyrosine, based on the Michaelis-Menten Fig. (**5b**) model were found to be *K*m = 0.055 mM and *k*cat/*K*m = 56.3 mM^{-1} s^{-1} Fig. (**5b**).

The calculated kinetic parameters of *L. sacchari* tyrosinase in comparison with other tyrosinases Table **2** show that the enzyme has lower activity against dopamine than have tyrosinases from *Ilex argentinus* native (ST94), *Ipomoea batatas*, and much lower activity against L-tyrosine than the tyrosinase from *Bacillus megaterium*. In contrast, the activity of *L. sacchari* tyrosinase is higher against L-DOPA in comparison with tyrosinase from *Streptomyces albus*.

As a general conclusion the enzyme isolated from *Laceyella sacchari* has both monophenolase and diphenolase activities, which confirms that it is a real tyrosinase.

Its biochemical characteristics make *Laceyella sacchari* tyrosinase an interesting candidate for future analytical and preparative biotechnological applications.

ACKNOWLEDGMENTS

These studies were supported by research grants from the Bulgarian Ministry of Education (POST-DOC-06), the FWO-Flanders (VS.026-10N), and the BELSPO/ESA-PRODEX programme.

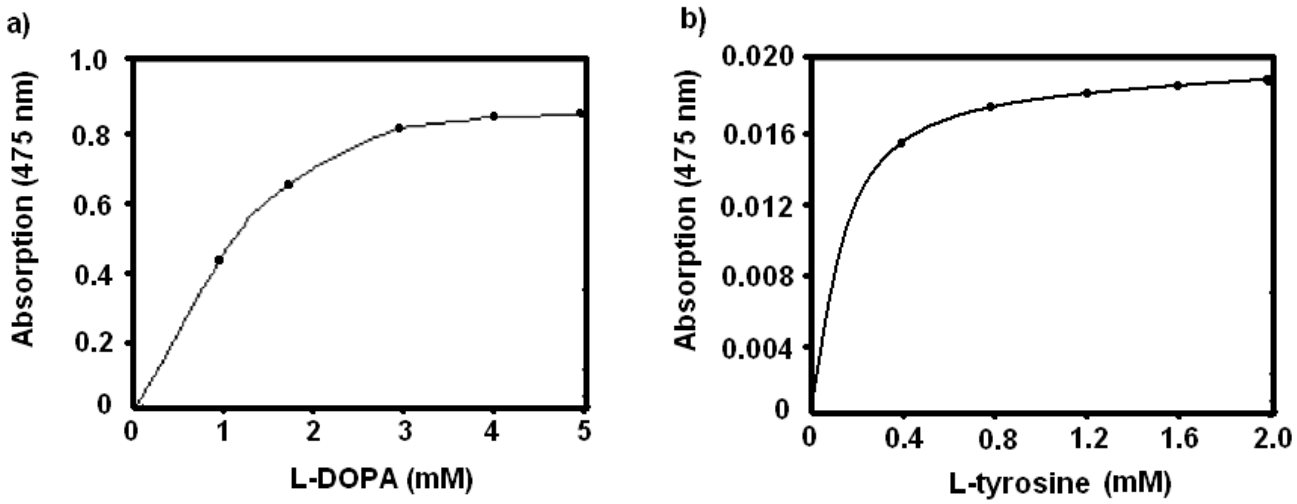

Figure 5. (a) Michaelis-Menten kinetics of LsTyr as a function of the concentration of the substrates. The *K*m value, determined using L-DOPA (0.1 – 5 mM) as a substrate, was calculated to be 4.5 mM. (**b**) Enzyme kinetics as a function of the concentration of substrate. The *K*m value, determined using L-tyrosine (0.1 – 2 mM) as a substrate, was calculated to be 0.055 mM.

ABBREVIATIONS

LsTyr = Tyrosinase from *Lacceyela sacchari*

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Received: September 30, 2011 Revised: January 17, 2012 Accepted: January 18, 2012