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# IDENTIFICATION AND CHARACTERIZATION OF TYROSINASE FROM STREPTOMYCES ALBUS BY MASS SPECTROMETRY

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## ABSTRACT

*The bacterium Streptomyces albus has so far never been investigated for tyrosinase activity. The studies presented in this communication show that his bacterium is maybe 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 sulphate 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 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.*

*Several isolated peptides were sequenced by MALDI MS/MS and a spectrum of the peptide with mass 1357.64 was characterized and allowing to deduce the sequence SDRQVTTGPFAYRHG. A spectrum of the peptide with molecular mass 1536.61 was characterized and deduced sequence is WVGGQMATGVSPN. Other spectrum of a peptide with molecular mass 1334.3 gave sequence DTDSGERTGHR. The identified amino acid sequences of the peptides showed very high similarity with database sequences for other tyrosinases from Streptomyces species.*

***Keywords:*** enzyme kinetics, mass spectrometry, tyrosinase, *Streptomyces albus*

## Introduction

Tyrosinases (monophenol, oxygen oxydoreductase, 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 catalyse two different kinds of enzymatic reactions: (I) ortho-hydroxylation of monophenols and (II) oxidation of o-diphenols to o-diquinones. The best-known function is the formation of melanins from L-tyrosine *via* L-dihydroxyphenylalanine (L-DOPA). The complicated hydroxylation mechanism at the active centre is still not completely understood (2).

The monophenolhydroxylase and diphenoloxidase activities of tyrosinases are the basis for many industrial biotechnological applications (4): in environmental technology for the detoxification of phenol-containing waste waters and contaminated soils (3), 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 (11). Synthetic melanins found applications for protection against radiation (UV, X-ray, gamma-ray), cation exchangers, drug carriers, antioxidants, antiviral agents or immunogens (12, 15).

*Streptomyces* belongs to the family of *Streptomycetaceae* and represents one of the most important genus of the *Actinomycetales* order. Members of this genus were intensively studied because of their capacity to produce antibiotics and enzymes of industrial importance (1). Bacterial tyrosinases with new features like high-temperature

stability (8), (10) or a broader substrate spectrum (14) 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.

## Materials 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, PIPES, copper sulfate, sodium acetate, acetic acid, guanidine hydrochloride, Servacell DEAE 52, Sephacryl S-100, Nucleosyl C18 were supplied from Sigma (Germany).

### 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 (13) 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% $MgSO_4.7H_2O$, and adjusted to pH 7.0. The cultures were incubated at 25 °C on a rotary shaker at 250 rpm. Stock cultures were maintained on RM medium solidified with 1.7% agar.

### Preparation of the crude extract

The culture supernatant was obtained by centrifugation of the culture broth at 5000 x 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 x 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 hour, the solution was centrifuged at 60,000 x *g* for 30 min at 4 °C. The pellet, containing the tyrosinase activity, 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 lyophilisate was suspended in 10 ml 25 mM PIPES buffer, pH 7.0, and dialysed in Sigma dialyzing bags against 5 liters of sterilized distilled water containing 50 μl of 10 mM $CuSO_4$ for 24 hours at 4 °C. Then, the dialysate was lyophilized once again and resuspended in the same buffer.

Then, 10 g anion-exchange material (Servacell DEAE 52) was suspended in 500 ml 25 mM sodium acetate buffer, pH 5.5, and equilibrated overnight at 4 °C. The upper layer was then decanted and the sediment was mixed with 10 ml of the sample and the mixture was adjusted to pH 7.0. Then, the sediment was centrifuged at 2 500 x 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 x 60), 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 0.4 ml/min.

For the final purification of the enzyme, a HPLC system, equipped with a Nucleosyl C18 column (100 x 2.1 mm), was applied. The sample was eluted with a linear gradient with 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. The eluted fractions were detected at a wavelength 206 nm.

### Spectrophotometric measurement of 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 diphenolase activity does not present any lag period. The dopachrome assay was performed according to (5). The increase in absorption at 475 nm, due to the formation of dopachrome ($\varepsilon_{475}$= 3 600 $M^{-1}cm^{-1}$), was monitored as a function of time. The activity is expressed as mole of L-DOPA oxidized per minute.

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 82000 $M^{-1}cm^{-1}$ according to (7).

### Electrophoresis

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 (9). Proteins separated on SDS-PAGE were

stained with 0.05% Coomassie brilliant blue R-250. Two-dimensional PAGE (2D-PAGE) was performed using a PhastSystem (Amersham Biosciences). The 2-D-gel images were digitized using a GS-710 densitometer (BioRad) and analyzed with the accompanying PDQuest 7.1 software (BioRad).

**MALDI measurements**

A 4700 Proteomics Analyzer (Applied Biosystems, Foster City, CA) with TOF/TOF optics was used for all MALDI-MS and MS/MS applications. Samples were prepared by mixing 0.7 µL of the sample with 0.7 µL matrix solution (7 mg/ mL a-cyano-4-hydroxycinnamic acid (CHCA) in 50% ACN containing 0.1% TFA) and spotted on a stainless steel 192-well target plate. For MS/MS experiments, the instrument was externally calibrated with fragments of Glufibrinopeptide B.

**Kinetic studies**

Kinetic parameters such as Michaelis constants $K_m$, $V_{max}$ were determined for diphenolic substrate L-DOPA and monophenolic substrate L-tyrosine methyl ester from the equation of the Lineweaver-Burk plot as described by (6). All procedures in this study were carried out at 25 °C.

## Results and Discussion

From several testing bacteria only one showed tyrosinase activity. The morphological, cultural, physiological and biochemical characteristics of investigated bacterium were compared with the references for similar streptomycetes species. The strain was identified as *Streptomyces albus*.

The enzyme was purified from *Streptomyces albus* starting with 5 600 ml of the culture filtrate. As first step the crude enzyme was purified by centrifugation, following ammonium sulphate precipitation and ultrafiltration. Then melanin was removed applying a Servacell DEAE 52 resin using batch technique. Then the crude enzyme was loaded on a SEC Sephacryl S-100 column and after purification on Nucleosyl C18 column, HPLC system, 1.17 mg of purified tyrosinase with the yield of 26% was obtained **(Table 1)**.

**TABLE 1**

Summary of the purification of tyrosinase from *Streptomyces albus*

| Purification steps | 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-DEAE52 | 194 | 95.1 | 563 | 5.9 | 197 | 96.4 |
| SephacrylS-100 | 46 | 2.30 | 204 | 88.7 | 2 960 | 34.9 |
| Concentration | 4.5 | 1.17 | 152 | 130 | 4 300 | 26.0 |

The molecular mass of the purified tyrosinase was determined by MALDI mass spectrometry to be 30 096 Da **(Fig.1)** which correspond to the obtained results from SDS-PAGE

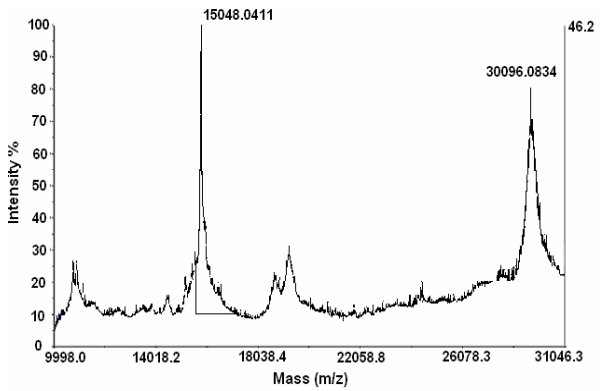


**Fig.1**. MALDI mass spectrum of the eluate, obtained after elution from the Nucleosyl C 18 column, showing a peak at 30 096 Da corresponding to the molecular weight of *S. albus* tyrosinase. The peak at 15 048 Da corresponds to exactly half the molecular weight of tyrosinase.

Using diphenol L-DOPA and 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.

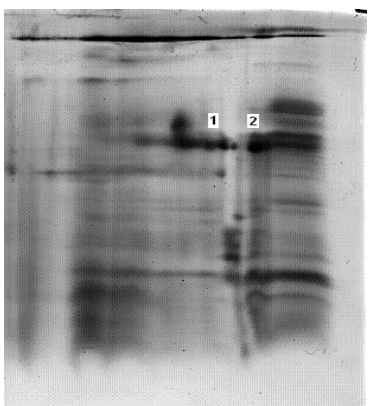


**Fig.2**. Two-dimensional polyacrylamide gel electrophoresis **(**2-D PAGE) of *Streptomyces albus* tyrosinase. Peptides were isolated from two spots 1 and 2.

Maximal activities of the purified enzyme were recorded at pH 7.0. Long-term storage of *Streptomyces albus* tyrosinase revealed the storage of a dried enzyme sample at temperatures below zero turned out to be the best and for tyrosinase in solution the storage at 4°C or at – 60°C is possible.

The total protein extract from bacterium was separated by two-dimensional polyacrylamide gel electrophoresis **(**2-D PAGE**) (Fig.2)**.

From the gel four spots were randomly selected and manually excised. The proteins were guanidinated in gel and desalted/destained in one single step.

Subsequently, the guanidinated proteins were enzymatically cleaved with trypsin and, after extraction the peptides were analysed.

For two spots, we observed a good peptide mass fingerprinting (PMF) suitable for de novo MALDI-MS/MS analysis. PMF is a technique used to identify proteins by matching their constituent fragment masses (peptide masses) to the theoretical peptide masses generated from a protein or DNA database of the peptides. For the other 2 spots, we observed none or a very weak PMF, with signal intensities that were too low for MALDI MS/MS fragmentation.

The isolated peptides from one spot were sequenced by MALDI MS/MS and one spectrum of the peptide with mass 1357.64 was characterized **(Fig.3)**.

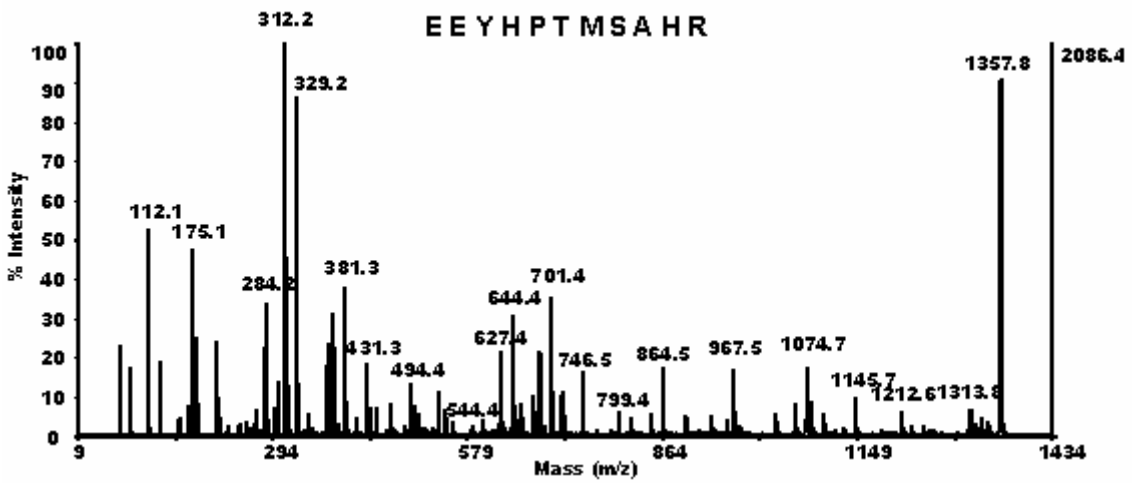


**Fig. 3** MALDI-MS/MS, spectrum of peptide at m/z 1357.64 of tyrosinase isolated from *Stremtomyces albus*.

The spectrum is dominated by peptide fragmentation, and allowing to deduce the sequence EEYHPTMSAHR. The alignment of the complete amino acid sequence of the peptides from spot 1 and 2 showed a good alignment with tyrosinases from other Streptomyces species. De novo determined peptide sequences were deduced manually and used for similarity searches using the FASTS, MS-BLAST, and the MS-Homology algorithm.

Several amino acid sequences of the peptides were identified and showed very high similarity with database sequences for other tyrosinases from Streptomyces species. A spectrum of the peptide with molecular mass 1536.61 was characterized and allowing to deduce the sequence WVGGQMATGVSPN. Other spectrum of a peptide with molecular mass 1334.3 gave sequence DTDSGERTGHR.

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## REFERENCES

1. **Bahrim G. and Negoita T. Gh.** (2007). VI simposio argentine, 10-14.09
2. **Claus H. and Decker H.** (2006) Syst. and Appl. Microbiol. **29**, 3–14.
3. **Claus H. and Filip Z.** (1990) Water Sci. Technol. **22**, 69–77.
4. **Duran N. and Esposito E.** (2000) Appl. Catal. B: Environ. **28**, 83–99.
5. **Fling M., Horovitz N. H., and Heineman S. F.** (1963) **238**, 20-45.
6. **Gollas-Galvan T., Hernandez-Lopez J., and Vargas-Albores F.** (1999) Comp. Biochem. Physiol. **122**, 77-82.

7. **Jackman P., Hajnal A., and Lerch K.** (1991) Biochem. J. **274**, 707-712.
8. **Kong K. H., Hong M. P., Choi S. S., Kim Y. T., and Cho S. H.** (2000) Biotechnol. Appl. Biochem. **31**, 113–118.
9. **Laemmli U. K.** (1970) Nature **227**, 680–685.
10. **Liu N., Zhang T., Wang Y. J., Huang J. H., Ou P., and Shen A.** (2004) Lett. Appl. Microbiol. **3**, 407–412.
11. **Mayer A. M. and Harel E.** (1978) Phytochemistry **18**, 193–215.
**Nosanchuk J. D. and Casadevall A.** (2003) Cell Microbiol. **5**, 203–223.
12. **Pridham T. G., Anderson P., Foley C., Lindenfelser L. A., Hesseltine C. W., and Benedict R. G.** (1957) Antibiot. Ann. **57**, 947-953.
13. **Sanchez-Amat P., Lucas-Elıo P., Fernandez E., Garcıa-Borron J. C., and Solano F.** (2001) Biochim. Biophys. Acta **1547**, 104–116.
14. **Wang G., Aazaz A., Peng Z., and Shen P.** (2000) FEMS Microbiol. Lett. **185**, 23–27.