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Partial purification and characterisation of two actinomycete tyrosinases and their application in cross-linking reactions☆

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ABSTRACT

Actinomycetes are a ubiquitous group of bacteria, and are hypothesised to produce tyrosinases for protection against the potential toxic effect of phenolic compounds and for the production of melanin. In this study, tyrosinase production by *Streptomyces pharetrae* CZA14^T (CZA14Tyr) and *Streptomyces polyantibioticus* SPR^T (SPRTyr) was optimised. The enzymes were partially purified and biochemically characterised to determine their suitability for industrial applications. SPRTyr was stable up to 40 °C and at pH 4.5–10.0, while CZA14Tyr was stable up to 40 °C and at pH 6.5–10.0. The enzymes showed variable stability in the presence of water-miscible organic solvents and were able to oxidize L-DOPA in the presence of these solvents. A limited inhibitory effect was observed with arbutin, EDTA, sodium chloride and sodium dodecyl sulphate, while both enzymes were strongly inhibited by the reducing agents used in this study. Inhibition of enzyme activity was observed in the presence of 1 mM Cu^{2+} and 5 mM Co^{2+} for SPRTyr, and 5 mM Fe^{2+} and 5 mM Zn^{2+} for CZA14Tyr. When applied in various cross-linking reactions both tyrosinases were able to cross-link casein and gelatine in the absence of a phenolic compound, showing potential for application in the food industry and for the production of biomaterials.

1. Introduction

Tyrosinases (EC 1.14.18.1) are Type III copper proteins that utilise molecular oxygen to catalyse two types of reactions: monophenolase and diphenolase (catechol oxidase) type reactions [1,2]. In the monophenolase reaction, the *o*-hydroxylation of a monophenol occurs to produce an *o*-diphenol. The *o*-diphenol then serves as a substrate for the subsequent catechol oxidase reaction, resulting in the formation of *o*-quinones. In addition, tyrosinases have also been shown to catalyse the *o*-hydroxylation of aromatic amines and the oxidation of *o*-aminophenols to *o*-quinoneimines [3]. Tyrosinases, along with laccases (EC 1.10.3.2) and catechol oxidases (EC 1.10.3.1), are collectively known as phenol oxidases. However, it is only tyrosinases that are able to catalyse monophenolase type reactions, which is often used as a distinguishing feature in the biochemical description of phenol oxidases [1]. The Type III copper centre of tyrosinases consists of two copper atoms (Cua and Cub) that are co-ordinated by six highly conserved histidine residues (three residues per copper co-ordination) located in the active site [4]. The signature sequence around the Cua and Cub co-ordination sites are characterised by the sequences H-X(*n*)-H-X(8)-H and H-X(3)-H-X(*n*)-H, respectively [1,5].

Tyrosinases are quite widespread in nature where they play various roles related to the health or pathogenicity of the producing organism [1]. In 1972, the first streptomycete tyrosinases were described: *Streptomyces glaucescens* [6] and *Streptomyces nigrifaciens* [7], but it is not clear whether the latter represents a true tyrosinase [2]. Very few streptomycete tyrosinases (less than twenty) have been isolated and described to date, which is quite surprising considering the fact that the genus currently consists of more than 600 validly published species (List of prokaryotic names with standing in nomenclature, 2015; http://www.bacterio.

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http://dx.doi.org/10.1016/j.molcatb.2015.10.012
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net/index.html). It has been proposed that there are three different types of tyrosinases in streptomycetes [8]. Type I or MelD (membrane bound, universal in streptomycetes, potential protective role against phenolics); Type II or MelC (secreted, sporadic occurrence in streptomycetes); and Type III (*o*-aminophenol oxidase, intracellular, rare, involved in secondary metabolite production). All three types of tyrosinases are encoded by bi-cistronic operons: one gene encodes for a chaperone or caddy protein and the downstream gene encodes for an inactive, apotyrosinase. For the streptomycete MelC extracellular tyrosinase, MelC1 is a copper chaperone involved in the binding of copper ions and the incorporation of copper into the apotyrosinase (MelC2). MelC1 is also responsible for the activation and secretion of the otherwise inactive apotyrosinase: MelC1 contains the twin arginine signal peptide sequence that allows for the export of the enzyme in its active form through the twin arginine translocation (TAT) secretion pathway [8]. In 2006, the crystal structure of the tyrosinase produced by *Streptomyces castaneoglobisporus* was elucidated, thereby providing a clearer understanding of the interaction of MelC1 with MelC2 during the formation of an active tyrosinase [9].

The ability of tyrosinases to oxidize mono- and diphenols has allowed for their applications in various biotechnological processes. The tyrosinase from *Agaricus bisporus* (common button mushroom) is most commonly utilized in research, largely because of its commercial availability [10]. However, the limitations of this tyrosinase (e.g., low solvent and heat stability) have encouraged researchers to look toward alternative sources of tyrosinases, including bacteria due to ease of genetic manipulation and over-expression in expression hosts such as *Escherichia coli* [10]. The biotechnological relevance of bacterial tyrosinases spans their application in bioremediation (detoxification of phenol-contaminated wastewater and soil), the production of synthetic melanins, their application in biocatalysis reactions (production of L-3,4-dihydroxyphenylalanine or L-DOPA, and application in cross-linking reactions) and biosensor technology (detection of phenols and oxygen) [10,11]. In this study, tyrosinases from two novel streptomycetes, *Streptomyces polyantibioticus* SPR^T and *Streptomyces pharetrae* CZA14^T, were isolated and biochemically characterised (solvent stability, pH stability, thermostability, effect of inhibitors) to determine their suitability for application in industrial processes. The genome sequences of the two strains were analysed for the presence of bi-cistronic tyrosinase operons and the sequences subjected to bioinformatics analyses. Finally, both tyrosinases were applied in selected cross-linking reactions to determine their potential application in industries requiring covalent protein–protein cross-linking.

2. Experimental

2.1. Materials/chemicals used in this study

All chemicals used in this study were obtained from Merck–Millipore (South Africa) and Sigma–Aldrich (South Africa), unless stated otherwise.

2.2. Tyrosinase-producing strains

S. polyantibioticus SPR^T (=DSM 44,925^T = NRRL B-24448^T) was isolated from soil collected from the banks of the Umgeni River, KwaZulu Natal, South Africa [12] and *S. pharetrae* CZA14^T (=DSM 41,856^T = JCM 13,860^T = NRRL B-24333^T) was isolated from soil collected from the base of a giant quiver tree, Karoo Desert National Botanical Gardens, Worcester, South Africa [13]. The strains were maintained on International *Streptomyces* Project (ISP) medium number 2 (ISP2 or Yeast extract-Malt Extract, YEME; g/L: 10.0 malt extract, 4.0 yeast extract, 4.0 glucose, 20.0 bacteriological agar, pH 7.3) [14] and as stock cultures in 20% (v/v) glycerol at −20 °C.

2.3. Screening for tyrosinase activity: solid media

S. polyantibioticus SPR^T and *S. pharetrae* CZA14^T were streaked onto various solid media for the detection of melanin production (which served as an indicator of tyrosinase production). The following media were used: YEME (ISP2); ISP medium number 6 (ISP6; g/L: 15.0 peptone, 5.0 proteose peptone, 0.5 ferric ammonium citrate, 1.0 K_2HPO_4, 0.08 $Na_2S_2O_3$, 1.0 yeast extract, 15.0 bacteriological agar, pH 7.0) [14], ISP medium number 7 (ISP7; g/L: 15.0 glycerol, 0.5 L-tyrosine, 1.0 L-asparagine monohydrate, 0.5 K_2HPO_4, 0.5 $MgSO_4{\cdot}7H_2O$, 0.5 NaCl, 0.01 $FeSO_4{\cdot}7H_2O$, 1.0 ml trace salts solution, 20.0 bacteriological agar, pH 7.2; Trace salts solution, g/100 ml: 0.1 $FeSO_4{\cdot}7H_2O$, 0.1 $MnCl_2{\cdot}4H_2O$, 0.1 $ZnSO_4{\cdot}7H_2O$, filter sterilize) [14]; DSMZ medium 553 (GPHF-medium, g/L: 10.0 glucose, 5.0 peptone, 5.0 yeast extract, 5.0 beef extract, 0.74$CaCl_2{\cdot}2H_2O$, 15.0 bacteriological agar, pH 7.2); modified phenoxazinone production medium (MPPM; g/L: 10.0 glycerol, 10.0 glucose, 10.0 soya flour, 5.0 casamino acids, 5.0 yeast extract, 4.0 $CaCO_3$, 1 ml trace salts solution, 15.0 bacteriological agar, pH 7.0; Trace salts solution, g/100 ml: 1.0 FeSO4; 0.9 ZnSO4, 0.2 $MnSO_4$) [15]; and Czapek solution agar (CZ; g/L: 30.0 sucrose, 2.0 $NaNO_3$, 1.0 K_2HPO_4, 0.5 KCl, 0.5 $MgSO_4{\cdot}7H_2O$, 0.01 $FeSO_4{\cdot}7H_2O$, 15.0 bacteriological agar, pH to 7.3 ± 0.2) [16]. *Streptomyces antibioticus* NRRL B-2770^T, a known tyrosinase producer, was included in all experiments as a positive control. Plates were incubated at 30 °C for 7 days and monitored for the production of a dark brown to black pigment.

2.4. Enzyme production

For the production of the tyrosinases, 10 ml pre-cultures of the two tyrosinase-producing strains were prepared in MPPM: 100 μl of the stock cultures was used to inoculate every 10 ml volume preculture media. *S. polyantibioticus* SPR^T was inoculated into MPPM, pH 5.5, while *S. pharetrae* CZA14^T was inoculated into MPPM, pH 6.5. The *S. polyantibioticus* SPR^T and *S. pharetrae* CZA14^T precultures were incubated for 48 h at 22 °C and 30 °C, respectively, shaking at 160 rpm. For both strains, a 5% pre-culture inoculum (20 ml) was used to inoculate a 400 ml volume of MPPM (in a 2 L shake flask), which was supplemented with 4 ml 100 mM filter sterilised $CuSO_4{\cdot}5H_2O$ (final concentration of 1 mM). The 400 ml volume cultures were incubated as before, but only for 24 h. The cultures were centrifuged at 10,000 × *g* for 5 min at 4 °C and the culture supernatants used for the purification of the extracellular tyrosinases.

2.4.1. Partial purification of the tyrosinases: ammonium sulphate precipitation, dialysis and anion exchange

The culture supernatants from both strains were subjected to ammonium sulphate precipitation. The culture supernatant was initially brought to a 40% saturation level, stirred at 22 °C for 1 h and centrifuged at 10,000 × *g* for 10 min at 4 °C. The resultant supernatant was brought to a 56% saturation level, stirred at 22 °C for 1 h and centrifuged at 10,000 × *g* for 10 min at 4 °C. The pellets were resuspended in 50 mM potassium phosphate buffer (pH 6.5) and dialysed at 4 °C for 24 h against ice-cold 50 mM potassium phosphate buffer, pH 6.5 (buffer replaced after the first 3 h and after 20 h). After 24 h, the dialysis buffer was replaced with a 20% (w/v) polyethylene glycol 8000 solution (prepared in 50 mM potassium phosphate buffer, pH 6.5) to concentrate the enzyme solution (incubated at 4 °C for 24 h). The concentrate was redissolved in 50 mM potassium phosphate buffer, pH 6.5, and applied to a DEAE Sephadex™ A-50 (GE Healthcare) column. The enzymes were eluted with 50 mM potassium phosphate buffer, pH 6.5. The