CHARACTERIZATION OF TYROSINASE ENZYME FROM NATIVE BACILLUS MEGATERIUM SP. STRAIN M36

Ebrahim Valipour, Burhan Arikan

Address(es): Ebrahim Valipour PhD., Molecular microbiology lab, Biotechnology Department, Institute of Basic and Applied Sciences, Cukuruva University, 01330, Adana, Turkey. Fax: 0090 3223386070, Tel No: 00905367304074.

*Corresponding author: va.cell@yahoo.com

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Abstract

Tyrosinase is a type 3 copper-containing enzyme that catalyzes the conversion of L-tyrosine to L-DOPA and finally to melamin. In this study tyrosinase enzyme from native Bacillus megaterium sp. strain M36, was produces, characterized and used to produce L-DOPA.

The M36 tyrosinase enzyme showed optimum monophenolase and diphenolase activity at pH 7.5 and conserved its maximum activity over than 95 % at pH ranging from 6.5 to 8.0. The M36 tyrosinase enzyme showed optimum monophenolase and diphenolase activity at 40 °C also, the enzyme conserved 100% of its original activity at 4-45 °C. The M36 tyrosinase enzyme was inhibited strongly by β-mercaptoethanol and about 90% by 5mM of EDTA (a chelating agent). Although the enzyme was activated at the presence of 1mM SDS, it was strongly inhibited at high concentration of SDS (above 15mM). In TLC analysis, the transformation of L-tyrosine to L-DOPA was conspicuously detected.

Keywords: Melanin, monophenolase, diphenolase, TLC

INTRODUCTION

Tyrosinase is a type 3 copper-containing enzyme that has been found widely distributed in microorganisms, plants and animals (Claud and Decker, 2006). Tyrosinase catalyses the hydroxylation of monophenol to o-diphenol (monophenolase or cresolase activity) and the oxidation of diphenol to o-quinones (diphenolase or catecholase activity). O-quinones are converted in to melamin by using nonenzymatic steps and molecular oxygen (Decker and Tuzek, 2000). Howard et al., in 1948, elucidated the biosynthetic pathway for melanin formation by tyrosinase enzyme. In mammals, tyrosinase catalyzes the biosynthesis of melanin pigments, which contributes to a fundamental part of the skin protection against UV radiation. It is also related to the browning reactions of fruit and vegetables (Seo et al., 2003). Tyrosinases have several biotechnological applications relying on the ability of the enzymes to oxidize both small phenolic molecules and protein-associated phenolic groups, i.e. the side chain of the amino acid tyrosine. Tyrosinase enzyme has very important role in bioremediation (Marino et al., 201128), production of L-DOPA, the preferred drug for treatment of Parkinson’s disease and other antioxidants (having crucial application in medical field) (Xu et al., 2012), food industry (Allouche et al., 2004), textile industry (Franciscon et al., 2012) and production of melanin (Kumar et al., 2011). Recently, because of increasing application of the tyrosinase enzyme, the interest in the isolation of new tyrosinase enzyme has been increased. Up to present, several tyrosinase enzyme from microbial strains such as Bacillus thuringiensis (El-Shora and Metwally, 2008), Pseudomonas putida F6 (McMahon et al., 2007), Ralstonia solanacearum (Hernandez-Romero, 2005), Rhizobium etli (Pino et al., 2007), Streptomyces antibioticus (Marino et al., 2010), Thermomonospora roseus (Kong et al., 2008), Streptomyces sp. REN-21Hto and Inouye, 2005, Verrucomicrobium spinosum (Fairhead and Thony-Meyer, 2010) have been isolated and characterized. Most of the strains have multicatalytic functions such as peroxidase and laccases in addition to tyrosinase activity, these characteristics make more restrictions for the strains to be used in industrial and pharmaceutical applications (Dastager et al., 2006), any way some strains which produce only tyrosinase enzyme has been isolated from soil samples (Freddi et al., 2006). These strains are appropriate for industrial applications.

The commercial production of tyrosinase enzyme is mostly reported from the common mushroom Agaricus bisporus. Extensive research regarding this enzyme has been carried out using this mushroom tyrosinase enzyme. The mushroom’s tyrosinase enzyme exhibits relatively low pH and temperature stability and its purification is relatively hard, as compared to bacterial tyrosinases (Seo et al., 2003). To date, this is the first time that isolation and characterization of a native tyrosinase enzyme from Bacillus megaterium strain was carried out.

MATERIAL AND METHODS

In this research all material for making medium were bought from sigma and mecr. Also the substrate (l-tyrosine) was bought from sigma. According to its information wrote in sigma, L-tyrosine has the following properties; form; fine crystals and fragments, colour: white, molecular weight: 181.19 g/mol, water solubility: 0.479 g/l at 25 °c, formula: C9H11NO3

Production and partial purification of the m36 tyrosinase enzyme

Culture condition for tyrosinase enzyme production by the Bacillus megaterium sp. strain M36had been optimized previously and it was as follow: temperature (36 °C), pH (7.0), incubation time (16 hour), agitation (170rpm) , L-tyrosine (0.4mg/ml), yeast extract (0.05%), tryptone (0.423%), NaCl (3.4%) and CuSO4 (148.4µM). The native Bacillus sp:M36 was cultured at optimized culture condition and in order to enzyme extraction, to start with, the cell free extract was prepared then the extract was subjected to ammonium sulfate precipitation and dialysis.

For cell free extract preparation, the medium culture was centrifuged at 6000g for 10 min at 4°C when OD600 of medium culture was 1.3. Then the obtained supernatant was stored at +4°C and the pellets were washed twice in ice-cold 50mM potassium phosphate buffer, pH 7.0. After that the pellets were resuspended in 0.1M sodium phosphate pH 7.0 containing an inhibitory bacterial proteases cocktail (1: 4, µl: mg cell mass) and disrupted by sonication. The homogenate was centrifuged at 14000g for 15min. The supernatant achieved both by the previous centrifuge at 6000g and by centrifuge at 14000g were used as a cell free extract (Lopez-Serrano et al., 2002; McMahon et al., 2007; Michalik et al., 1976). The cell free extract was subjected to precipitation with ammonium sulfate (40, 50, 60, 70, 75, 80, 85 and 90% saturation) for 1 hour with gentle stirring. After fractionation with ammonium sulfate, the precipitated proteins are...
recovered by centrifugation at 12000g for 30 minute and dialyzed against 50mM sodium phosphate buffer, pH 6.8 with 0.02% sodium azide,  0.01mM CuSO₄. The fractions were tested to tyrosinase activity and active fractions were stored at -20 °C without loss of activity (El-Shora and Metwally, 2008). Protein concentrations of the samples were determined by Bradford method using bovine serum albumin (BSA) as the standard (Kohashi et al., 2004).

**Enzyme assay**

Tyrosinase activity is assayed by using L-tyrosine and L-DOPA as substrates. The appropriate concentration of the enzyme was determined before the enzyme activity was assayed and an aliquot of the enzyme solution is added to a 0.1mM sodium phosphate buffer (pH 6.8) containing 1mM L-tyrosine and L-DOPA, and the formation of dopachrome is monitored by measuring the absorbance at 475 nm (Rani et al., 2013). The initial rate is used for the calculation of tyrosinase activity. One international unit (IU) of tyrosinase activity is defined as the amount of enzyme required to oxidize 1µmol of L-tyrosine to dopachrome per minute under the above conditions, which was calculated using the molar extinction coefficient of dopachrome (3600 M⁻¹ cm⁻¹) by the following equation:

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\text{IU/ml} = \frac{\text{absorption } \times \text{min } \times \text{assay volume (ml)}}{e_{\text{md}} \times (1 \text{ mole }^{-1} \text{ cm }^{-1} \text{ cm }^{-3}) \times 1 \text{ cm } \times \text{enzyme volume (ml)}}
$$

**Effect of pH and temperature on enzyme activity and stability**

For this purpose, 200µl of enzyme solution (protein content, 0.05 mg/ml) was added to 1800µl buffer containing 1mM of L-Dopa for diphenolase and 1mM of L-tyrosine for monophenolase activity and incubated for 45min. The effect of pH on monophenolase activity was investigated by analyzing the activity at different pH values (pH 4, 5, 6, 7, 8, 9, 10, 11 and 12) and for diphenolase activity pH (4, 7.5) were tested to determine L-DOPA spontaneously converted to dopachrome at pH values above 7.5. pH value in which the enzyme showed maximum relative activity was determined as optimum pH for the enzyme activity (Burhan et al., 2003; McMahon et al., 2007).

Also, the enzyme activity was analyzed at a range of temperatures from 10 to 70°C (10, 20, 30, 40, 50, 60 and 70) and the temperature showing maximum relative activity was determined as optimum temperature for the enzyme activity. In order to ascertain the temperature stability, the enzyme solutions in different tubes are incubated at various temperatures in the range from 0°C to 70°C for 2 hour then residual activity is assayed in enzyme assay condition (Liu et al., 2004).

**Effect of detergents on enzyme activity**

To examine the effects of sodium dodecyl sulphate (SDS), ethylenediamine tetraacetic acid (EDTA), Urea, Tween-80, TritonX-100, β-Mercaptoethanol and PMSF are incubated the enzyme in the presence of these detergents and substrate (Aygan et al., 2009; Cafl et al., 2012).

**Kinetic study of M36 tyrosinase enzyme**

The initial rate of enzyme reaction for L-tyrosine and L-DOPA was determined at various concentrations. The resulting data was analyzed and the $K_m$ and $V_{max}$ values are calculated by Michaelis–Menten and Hill equation $v_i = \frac{V_{max}}{K_m + [S]}$ and Lineweaver-burk equation

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\frac{1}{v_i} = \frac{1}{V_{max}} \times \frac{1}{[S]} + \frac{1}{V_{max}}$$(Mc Mahon et al., 2007; 45) Zanjani et al., 2009). After addition of 200µl of enzyme solution (protein content, 0.05mg/ml) to potassium phosphate (50mM, pH 7) containing various concentrations ranging from 0.02 to 0.8 for tyrosine and 0.06 to 2.0 for L-DOPA, the reaction medium with L-DOPA and with L-tyrosine was incubated at room temperature for 30min and 45 min, respectively. After that the reaction medium with L-tyrosine was diluted 5 times and reaction medium with L-DOPA was diluted 10 time and both of them was subjected to study of OD₄₇₅ by spectrophotometer. The obtained data was used to calculation of velocity.

**Electrophoretic study**

The enzyme solution was loaded in several well of Non-denaturing PAG (8% w/v) and after separating protein bands, a single lane of the gel was sliced out of the gel using a clean scalpel. The tyrosinase enzyme related band was stained by placing the gel slice in substrate (L-tyrosine (0.1mg/ml) and CuSO₄ (50µM) in phosphate buffer (0.1M, pH 7)) for 60 min. The formation of a dark-brown band indicated the position of the tyrosinase enzyme. The remaining lanes of the gel were placed in 50mM phosphate buffer, pH 7.0. Using the activity stained lane as a guide to the location of tyrosinase, the corresponding band was sliced out of the unstained lanes. The gel slice was homogenized and resuspended in a 50mM phosphate buffer and kept overnight at 4 °C. The gel suspension was centrifuged at 12000 g for 10 min to remove remaining gel fragments and the obtained supernatant was subjected to SDS-PAGE (12%) analysis for determination of the tyrosinase enzyme molecular weight (Arikan, 2008).

**Thin layer chromatography analysis of the reaction mixture**

The conversion of L-tyrosine to L-DOPA by M36 tyrosinase enzyme was analyzed by thin layer chromatography. For this purpose, phenol-water system (75:25) (v/v) was used as a mobile phase and 3% ninhydrin in n-butanol as spray and staining reagent. Besides of TLC analysis, (Rani et al., 2007; Raval et al., 2012).

### Statistical analysis

All experiments were conducted in three replicates; data generated were subjected to statistical analysis using Microsoft Excel and presented as mean±SE.

### RESULT AND DISCUSSION

**Preparation of the M36 tyrosinase enzyme**

The enzyme was precipitated by ammonium Sulfate 85% and centrifugation at 13000g and dialyzed against 50mM sodium phosphate buffer (pH 6.8 containing 0.02% sodium azide and 0.01mM CuSO₄).

**Effect of pH and temperature on enzyme activity and stability**

The result of this research showed that the M36 tyrosinase enzyme had maximum monophenolase and diphenolase activity at pH, 7.5 (Figure 1). This result was in accordance with tyrosinase enzyme originated from Streptomyces sp. REN-21 (pH 7.0) (ito and Inouye, 2005), Rhizobium etli CFN42 (pH 7.5) (Pimero et al. 2007) and Pseudomonas putida F6 (pH 7.0) (McMahon et al., 2007). Notwithstanding, the tyrosinase enzyme from B. thuringiensis (Liu et al., 2004) and T. roseum (Kong et al., 2000) have shown to have maximum activity at 9.0 and 9.5, respectively. The M36 tyrosinase enzyme could conserve its maximum activity over than 95 % at pH (6.5-8.0). Before pH (6.5) and above pH (8.0) the activity and stability of the enzyme was dropped. These findings are similar to the finding of Shuster and Fishman (2009).

The tyrosinase enzymes have two copper in its active site and each of the two metal atoms; Cuα and Cuβ from the active site are coordinated by three conserved histidines which are located in a four α-helix bundle (Claus and Decker, 2006). The α-helix is structured by hydrogen bonds. Generally changing of pH value (extremely basic or acidic) causes changes in the charge of B-hond donor and acceptor groups, it can rearrange the H-bonds and change the conformation/folding of the protein.

**Figure 1** Effect of pH on activity and stability of the Bacillus megaterium M36 tyrosinase enzyme (monophenolase). The enzyme showed maximum activity (0.52 IU) at pH=7.5 and 97.5% of its maximum activity at pH=8.0. At pH lower than 6.5 and higher than 8.0 the activity of the enzyme was steeply decreased.

The M36 tyrosinase enzyme showed optimum monophenolase and diphenolase activity at 40 °C also, the enzyme conserved 100% of its original activity at 4-45 °C (Figure 2). The monophenolase and diphenolase activity of the enzyme was deeply decreased at temperature below 30 °C and above 55 °C, probably this result was related to that, the tyrosinase enzyme has mostly composed from α-helix, on the other hand α-helix is more flexible than the others structures. This result was more or less closed to other investigations.

The M36 tyrosinase enzyme showed up to 95% activity at temperature ranges 45°C, in contrast to this, the activity of tyrosinase enzyme from P. putida F6 (McMahons et al., 2007) has been decreased dramatically at temperature above 30°C and the enzyme of Streptomyces michiganensis DSM (Philipp et al., 1991) has showed optimum activity at 33°C. Moreover there is some reported tyrosinase enzymes wht higher optimum temperature. Also the
M36 tyrosinase enzyme was different with the tyrosinase from Rhizobium etli CFN42 (50°C) (Pinero et al., 2007), Bacillus (HR03) (55°C) (Dalfard et al., 2006), Bacillus thuringiensis (75°C) (El-Shora, Metwally, 2008) and Thermomicrobium roseum (70°C) (Kong et al., 2000). The M36 tyrosinase enzyme conserved its original activity at 45°C, contrary to this Trichoderma reesei (Cura et al., 2010) tyrosinase started to lose its activity relatively quickly at temperature above 30°C.

**Effect of detergents on enzyme activity**

The M36 tyrosinase enzyme was studied in presence of various inhibitors (Figure 3a). The enzyme was inhibited strongly by β-mercaptoethanol. β-mercaptoethanol is a reducing agent which inhibit dopachrom and melanin synthesis by reducing quinones (an intermediate) to L-DOPA. Similar results were obtained for Bacillus megaterium tyrosinase (Shuster and Fishman, 2009), and Thermomicrobium roseum tyrosinase (Kong et al., 2000) that was completely inhibited by β-mercaptoethanol (1nmol). The M36 tyrosinase was inhibited about 90% by 5mM EDTA (a chelating agent). The agent can inhibit the enzyme by chelating of Cu from its active site. Similarly Bacillus megaterium tyrosinase was inhibited up to 27% by 1mM EDTA (Shuster and Fishman, 2009) and Bacillus (HR03) tyrosinase enzyme was partially inhibited by 1mM EDTA (Dalfard et al., 2006). In contrast to the result of this research tyrosinase enzyme from Bacillus thuringiensis (El-Shora and Metwally, 2008) was activated at high concentration EDTA from 200 to 400mM. Effect of different concentration of SDS (0.2-30mM) on the M36 tyrosinase enzyme was studied. Although the enzyme was activated at the presence of 1mM SDS, it was strongly inhibited at high concentration of (above 15mM) SDS (Figure 5b). Previously, activating effect of SDS on tyrosinase enzyme from Xenopus laevis (Wittenberg and Trippett, 1985), A. bisporus (Espin and Wichers, 1999), Bacillus sp. (Dalfard et al., 2006) and Bacillus megaterium (Shuster and Fishman, 2009) has been reported which was in agreement with our result. According to the paper published by Gandia-Herrero, although, active site of enzyme is not affected by SDS, a stepwise conformational change affected the enzyme activity by increasing accessibility of its active site to the substrate (Gandia-Herrero et al., 2005).

**Production of L-DOPA from L-tyrosine**

In TLC analysis, the transformation of L-tyrosine to L-DOPA was conspicuously detected. Ascorbic acid, used to prevent further oxidation of L-DOPA, did not give interfering spots (Figure 4).

**Kinetic study of M36 tyrosinase enzyme**

The M36 tyrosinase enzyme was shown to obey Michaelis-Menten kinetics when L-tyrosine and L-DOPA was used as a substrate. The $K_v$ value of M36 tyrosinase for L-tyrosine (0.15mM) was lower than L-DOPA (0.58mM). The obtained $V_v$ was 1.7μM.min⁻¹.mL⁻¹ for L-tyrosine and 6.2μM.min⁻¹.mL⁻¹ for L-DOPA. $K_v$ value of M36 tyrosinase enzyme was similar to the previously reported $K_v$ values with the L-tyrosine, for example ; 0.2mM for Agaricus bisporus (Selinheimo et al., 2009) and 0.19mM for Rhizobium etli CFN42 (Cabrera-Valladares et al., 2006), also it is higher than the value (0.075mM) reported for Bacillus megaterium (Shuster and Fishman, 2009) and it is less than the values 0.563mM, 1mM, 0.421mM reported for Bacillus thuringiensis (El-Shora and Metwally, 2008), Streptomyces sp. REN-21 (Itou and Inouye, 2005) and Verrucomicrobiobium spinosum (Fairhead and Thony-Meyer, 2010), respectively. The $K_v$ value of M36 tyrosinase enzyme for L-DOPA was higher than $K_v$ value of tyrosinase from Agaricus bisporus (0.17mM) (Selinheimo et al., 2009), P. patula F6 (0.33) (McMahon et al., 2007) and Bacillus megaterium (0.35mM) (Shuster and Fishman, 2009) for the same substrate, but it was lower than $K_v$ values of tyrosinase from Trichoderma reesei (7.5mM) (Selinheimo et al., 2009), Rhizobium etli CFN42 (2.44mM) (Cabrera-Valladares et al., 2006), Streptomyces antibioticus (8.9mM) (Marino et al., 2011), Streptomyces castaneoglobisporus (8mM) (Kohashi et al., 2004) and Verrucomicrobiobium spinosum (7mM) (Fairhead and Thony-Meyer, 2010).
Analysis of pigment (melanin) in rheology and microstructure of Bacillus megaterium M36 tyrosinase enzyme

Electrophoresis and enzymatic activities in gel

After dialysis, tyrosinase M36 was electrophoresed by using native PAGE (12%) analysis. This analysis showed almost 34kDa bond of the enzyme (Figure 5). This result was similar to the result of Shuster and Fishman (2009) who have demonstrated the tyrosinase from Bacillus megaterium to be almost 35kDa.

Electrophoresis analysis of the Bacillus megaterium M36 tyrosinase Enzyme. Lane (A) shows tyrosinase activity, lane (T) shows the enzyme molecular weight almost 34KDa, almost 15μg of protein was loaded, lane (M) shows protein marker.

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