Research Article

Quantification of 4-amino-5-hydroxynaphthalene-2,7-disulfonic acid mono sodium salt by oxidation with tyrosinase in the presence of 3-methyl-2-benzothiazolinone hydrazone

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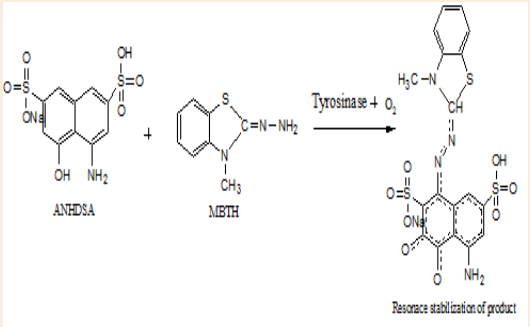
Abstract

A continuous spectrophotometric method for the rapid determination of tyrosinase activity is described. This method is based on the oxidation of 4-amino-5-hydroxynaphthalene-2,7-disulfonic acid mono sodium salt (AHNDSA) by tyrosinase form an oxidized product, latter react with the nucleophile 3-methyl-2-benzothiazolinone hydrazone (MBTH) to produce an intense colored product, that absorbs light maximally at 580 nm wavelength. The chemical reaction between MBTH and AHNDSA has been kinetically characterized. The λ_{max}, molar absorptivity coefficients, limit of detection and limit of quantification of adduct have been calculated. Kinetic parameters and catalytic parameters also calculated. The method is illustrated by measuring the enzymatic activity of mushroom tyrosinase during the hydroxylation of AHNDSA and MBTH. The proposed method can be applied to study the tyrosinase inhibition activity using mango seed kernel extract.



Keywords: Tyrosinase, 3-methyl-2-benzothiazolinonehydrazone, 4-amino-5-hydroxynaphthalene-2,7-disulfonic acid mono sodium salt, Mango seed kernel extract

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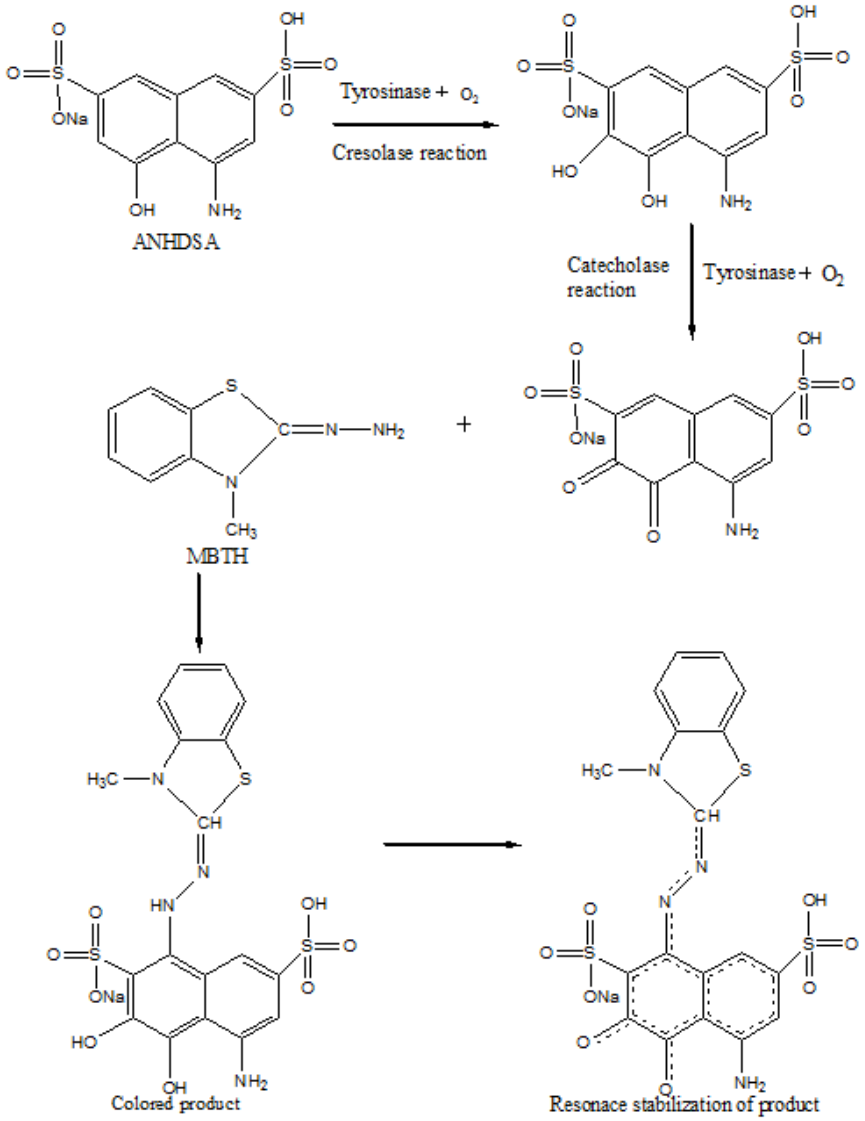
Introduction

Tyrosinase (monophenol monooxygenase, EC 1.14.18.1) is a multifunctional type-3 copper-containing metalloenzyme, it is a key enzyme in the undesirable browning of fruits, vegetables and in the coloring of skin, hair and eyes in animals [1]. The tyrosinase is a rate-limiting enzyme it catalyzing melanin biosynthesis which causes abnormal accumulation of melanin pigments [2]. The tyrosinase enzyme is extensively distributed in bacteria to mammals and also present different characteristics in different organs of the same organisms, such as roots and leaves of higher plants [3]. Tyrosinase shows a complex internal catalytic action mechanism involving two catalytic cycles: (a) monophenolase activity (or cresolase activity) involving the hydroxylation of monophenols in ortho position to give the corresponding o-diphenol and (b) diphenolase activity (or catecholase activity) involving oxidation of the o-diphenols to their o-quinones. Periodate like tyrosinase oxidizes o-diphenols to o-quinones but does not hydroxylate monophenols to o-diphenols [4]. This reagent has been used in several studies for comparison with tyrosinase and to characterize the o-diphenol oxidation products [5]. In all cases, the resulting quinones may undergo nonenzymatic autopolymerization to produce colored compounds [6–9]. Browning in plant materials is also associated with the enzymatic oxidation of phenolic compounds [10,11].

Several methods have been developed based on chronometric [12], manometric [13], radiometric [14], oximetric [15], electrochemical [16] and spectrophotometric [17, 18] techniques. However, the spectrophotometric technique has fascinated more attention mainly because it is convenient substrate availability, sensitive, inexpensive and allows the course of the reaction to be studied continuously [19, 20].

Spectrophotometric methods for tyrosinase assay are facing some difficulties such as fast inactivation of the enzyme in the presence of the selected substrates, product instability, low molar extinction coefficients of the substrates, and interference of the intermediates or the products absorption with the absorption of the substrates [21]. Severely limits the success of the introduced methods.

Several substrates are widely used for assaying tyrosinase activities such as L-tyrosine, L-dopa, and catechol are good examples of this type. Therefore, the reactions are usually studied either via the formation of intermediates, which are produced as a result of further chemical reactions of o-quinones, or via the depletion of an added reducing agent, which turns o-quinones back into the o-dihydroxy compounds [22]. Nevertheless, these resolutions cause indirect studies of the tyrosinase reactions.



Scheme 1

Thus, the purpose of the present work was to develop a sensitive spectrophotometric method for the quantitative analysis of tyrosinase activity, based on the kinetics of the oxidation of the 4-amino-5-hydroxynaphthalin-2,7-disulfonic acid mono sodium salt (ANHDSA) by the enzyme tyrosinase to its corresponding o-quinone which is

trapped by MBTH to yield a condensation product (**Scheme 1).** The proposed method can be applied to study the tyrosinase inhibition activity using mango seed kernel extract.

Tyrosinase activity measurements (MBTH & ANHDSA)

Materials and methods

Reagents

Mushroom Tyrosinase (MT) (EC 1.14.18.1) enzyme was purchased from Sigma (Germany). The working solution of 4-amino-5-hydroxynaphthalene-2,7-disulphonic acid mono sodium salt (AHNDSA) and 3-methyl-2-benzothiazolinone hydrazone (MBTH) solutions were prepared by dissolving a precisely weighed portion in water. All reagents were of analytical grade or higher and used without further purification. Double distilled water was used for preparing all aqueous solution.

Apparatus

Kinetic measurements and all other absorbance measurements were performed using spectrophotometer 117 (Shimadzu, Japan) with 1 cm matched quartz cells. The pH of the aqueous solution was measured using a pH - meter model (Equip-tronics, Mumbai, India, Model EQ- 614). A model 206-88950-93 thermostatic water bath (Shimadzu, Japan) was used to control the reaction temperature.

Preparation of enzyme solution

Freshly prepared enzyme solutions were used in this work. The stock solution of the enzyme was prepared by dissolving 0.1 mg in 100 mM phosphate buffer solution (PBS) of pH 6.8. Solid and solution of the enzyme was stored in refrigerator at $+4^0$C. In these experiments, different concentrations of MT were used in the cresolase and catecholase reactions, respectively. The best concentration of the enzyme which produced linear and fast progress of the corresponding reaction was selected (data not shown).

Preparation of mango seed kernel (MSK) extract

Fully grown unripened and disease free mango fruits (*Mangifera indica* L. cv. 'Fahlun') (Anacardiaceae) were purchased from a local market, Mysore, India. Fresh seeds were homogenized in a blender using 95% ethanol (600 ml) and shaken for 4 hours. The supernatant was passed through Whatman filter paper (no. 4). All filtrates were evaporated under reduced pressure using a rotary evaporator at 50°C and the remaining water was removed by freeze-drying and weighed to determine the yield of soluble components. The freeze-dried extract was flushed with nitrogen and stored in aluminum foil at 20°C until it was analyzed.

Results and discussion

Absorption spectra

The absorption spectrum of the colored solution produced at different concentrations of Tyrosinase was measured by the recommended general experimental protocol in a final 3 mL of reaction mixture and the spectrum was recorded at a scan rate of 2 nm/s after incubating the reaction mixture for 5 min at 25^0C on a spectrophotometer in the wavelength range 400–800 nm against the corresponding reagent blank. The optimum wavelength for maximum absorption of the colored product was 580 nm. The result is graphically represented as the in **Figure 1.**

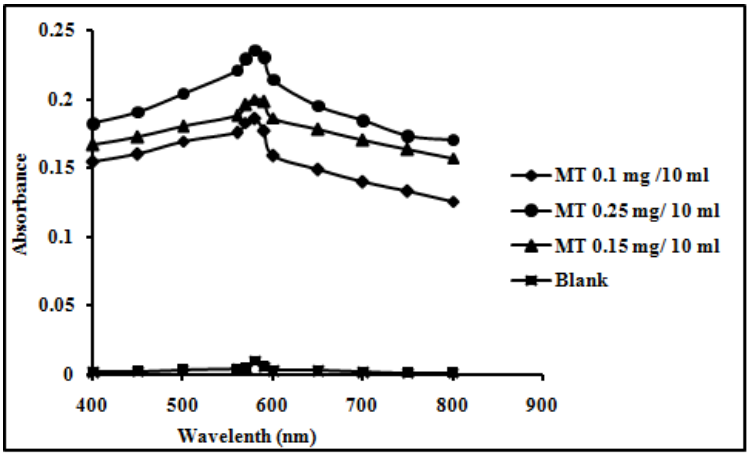


Figure 1 Absorption spectra

Temperature effect

Absorption spectra were recorded on a spectrophotometer. All tyrosinase reactions were carried out in phosphate buffer solutions. Temperature sensitivity was determined by pre-incubating 142 µM MBTH, 92 µM AHNDSA, 6.34 nM tyrosinase and phosphate buffer in a final 3 ml of reaction mixture for 10 min at temperatures ranging from 0 to 60^0C. The activity of the enzyme expressed as a function of absorbance of the colored solution. The maximum activity was found at 25^0C is as shown in **Figure 2**. Hence, further analysis was carried out at 25^0C.

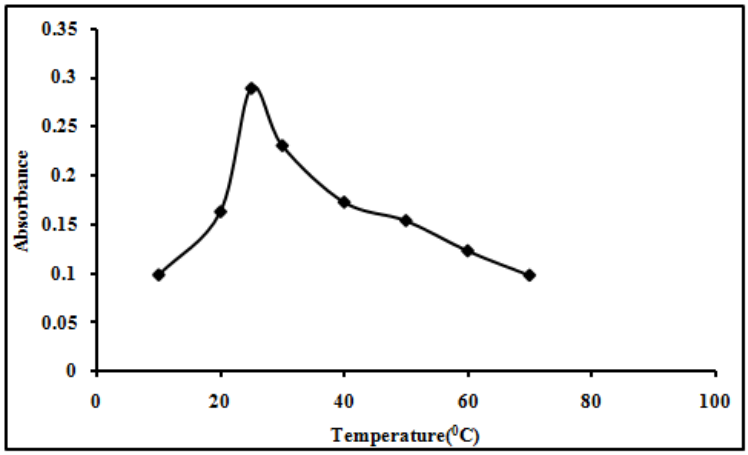


Figure 2 Effect of temperature on reaction

Determination of molar extinction coefficients

The incubation of MBTH and AHNDSA with tyrosinase resulted in the formation of a colored product. The relationship between optical density at lambda maximum (λ_{max}) and tyrosinase concentration (molar extinction coefficient) was determined as follows. A series of 3 ml spectrophotometric reactions were carried out in triplicate at different concentrations of AHNDSA. All spectrophotometric assays were carried out using a UVIDEC-610 ultraviolet-visible (UV-Vis) spectrophotometer. Temperature was controlled at 25 °C using a temperature controlled water bath. The standard 3 ml of the reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 142 µM MBTH and tyrosinase. All reactions were started with the addition of 100 µl of different concentration of AHNDSA. Measure the absorbance against the corresponding control contained all reaction components except substrates, which

was replaced with 50 mM potassium phosphate buffer (pH 7.0). Negative controls containing all assay components without substrate or enzyme failed to either produce a color reaction or show substrate consumption by specrophptometer. Two different concentration of commercial mushroom tyrosinase was used as the source of enzyme for molar extinction coefficient value determination. The molar extinction coefficient values for the product of tyrosinase activity with MBTH and AHNDSA was calculated within the linear part of the curve using the Beer-Lambert law, $A=\varepsilon cl$, where A is absorbance, ε is the molar extinction coefficient, c is the concentration and l is the path length.

The incubation of different concentration of AHNDSA with mushroom tyrosinase and MBTH, a dark violet color is obtained in the reaction medium. Various concentration of AHNDSA (185.28-2.89 µM) were incubated with mushroom tyrosinase (0.1 mg/10 ml) in the presence of 142 µM MBTH and the absorbance was measured at 580 nm. The linearity is obtained between 5.79 - 92.7 µM of AHNDSA with R^2 value 0.9979 (**Figure 3**). The molar extinction coefficient value of the o-quinone–MBTH adduct was determined from the Beer–Lambert law and value was found to be 0.53×10^4 L/mol/cm. The limit of detection and limit of quantification were also being calculated and values were found to be 0.27 µM and 0.84 µM respectively. The whole assay was repeated with 0.25 mg/10ml concentration of tyrosinase. The molar extinction coefficient value, limit of detection and limit of quantification values were calculated and values were found to be 0.89×10^4 L/mol/cm, 0.3 µM and 0.92 µM respectively.

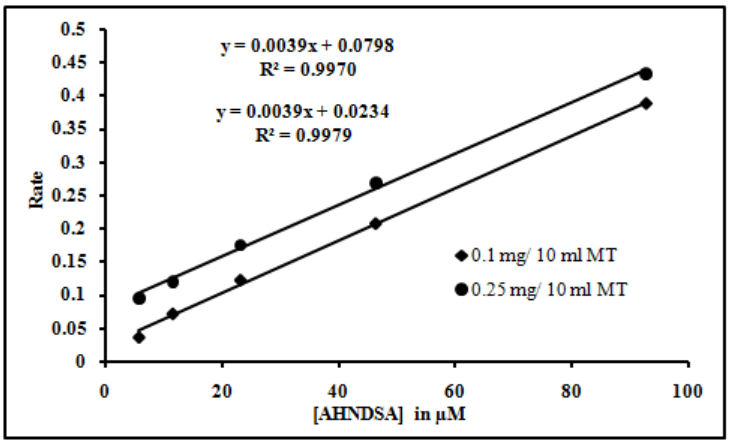


Figure 3 Linearity plot, (♦) with 0.1 mg/10ml concentration of tyrosinase, (●) with 0.25 mg/10ml concentration of tyrosinase.

Kinetic constant measurement

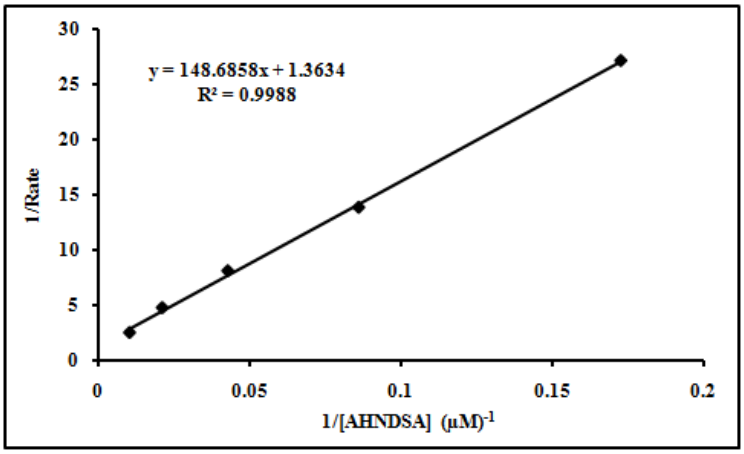


Figure 4 Lineweaver–Burk plot

A typical Michaelis–Menten plot and its linearization by the Lineweaver–Burk plot **(Figure 4)** (y=148.68 + 1.3634) was obtained for AHNDSA between 5.79- 92.7 µM in the above described optimal working conditions of the tyrosinase based enzyme catalytic reaction, namely: concentration of tyrosinase is 6.34 nm, phosphate buffer pH 7. The kinetic parameters Michaelies-Menten constant (K_m) and V_{max} were calculated as the average of 3 consecutive determinations: K_m = 109 µM and V_{max} = 0.7334 µM min^{-1}. The catalytic constant ($K_{cat}=V_{max} / [E_o]$) and specificity constant (K_{cat}/ K_m) of AHNDSA was found to be 0.1155 min^{-1} and 1.059 × 10^{-3} μM^{-1} min^{-1}, respectively. The catalytic efficiency (K_{eff} = 1/slope × $[E_o]$, where slope = 148.68 min^{-1} and $[E_o]$ = 6.34 nM) and catalytic power ($K_{pow} =V_{max} / K_m$) of the proposed method are 1.0592 ×10^6 M^{-1} min^{-1} and 6.720×10^{-3} min^{-1}, respectively.

Determination of mushroom tyrosinase inhibition by MSK extract

The proposed method was used to study the tyrosinase inhibition by using MSK extract. A series of 3ml spectrophotometric reactions containing a 50 mM potassium phosphate buffer (pH 7.0), 142 µM MBTH and 6.34 nM tyrosinase and various sample concentrations (100 µL) with or without enzyme were prepared and incubated for 5 min at 25°C, then add 46 µM AHNDSA. The amount of product formed was measured using spectrophotometer at 580 nm. kojic acid (KA) was used as a positive tyrosinase inhibitor control. The extent of inhibition by the extract was expressed as the percentage of concentration necessary to achieve 50% inhibition (IC50).

Table 1 shows the effect of MSK extract and well known tyrosinase inhibitor kojic acid (KA) on inhibition of tyrosinase activity. kojic acid having more half inhibition concentration (3.42± 0.1 µg/mL) than the MSK extract (87 ± 0.23 µg/mL). This is because of anti-tyrosinase potency of MSK extract may be attributed to its major principle and other unidentified constituents. MSK extract have been demonstrated to possess chelating activity when compared to EDTA [23], a possible mechanism for their anti-tyrosinase activity may involve the chelation of copper atoms which are required for the catalytic activity of tyrosinase [24, 25].

Table 1 Inhibition effect of MSK extract on tyrosinase activity

Test compounds	Anti-tyrosinase IC_{50} ± SEM (µg/mL)
MSK extract	87 ± 0.23
Positive reference: kojic acid	3.42± 0.1

Conclusion

The aim of the present study are an attempt to develop a new, simple spectrophotometric approach for the quantification of tyrosinase activity using AHNDSA and MBTH as chromogenic co-substrates; provide a plausible mechanism of the reaction and study the feasibility of the assay for the tyrosinase inhibition by the MSK extract. The proposed method having linearity range 5.79 - 92.7 µM with R^2 value 0.9979 for the AHNDSA substrates also having lower value of the Michaelies-Menten (K_m= 109 µM) constant from Lineweaver-Burk plot indicates stronger affinity between substrates and active site of enzyme, which signifies high selectivity and specificity of the proposed method. The molar extension co-efficient obtained for this method was high which indicates that the violet colored product is highly stable and this method can therefore be adopted in the routine analysis for the quantification of tyrosinase. Further, this method was applied for the study the tyrosinase inhibition by using MSK extract.

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