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Spectrophotometric Determination of Benzoic Acid Based on Inhibitive Effect on Tyrosinase Enzyme

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A simple method for detection of benzoic acid in food products has been developed based on inhibitive effect on tyrosinase enzyme. A mixture of tyrosinase, phenol and 3-methyl-2-benzothiazolinone hydrazone (MBTH) gave a maroon coloured solution which was bleached upon addition of benzoic acid. The wavelength at maximum absorbance was determined as 504 nm. The biosensor demonstrated optimum activity at pH 7. The relative standard deviation (RSD) of the reproducibility of this method was very good with RSD value of 1.91 %. The dynamic range of benzoic acid concentration was found to be between 50-700 ppm with the detection limit of 109 ppm. The kinetic parameters Michaelis-Menten constant (K_M) and maximum absorbance (Abs_{max}) in the absence and in the presence of benzoic acid were also evaluated. The kinetic analyses show that the inhibition of benzoic acid on the tyrosinase activity is reversible and competitive with an inhibition constant of 90.9 ppm. The proposed method was compared with HPLC and satisfactory agreement was achieved.

Key Words: Benzoic acid, Inhibition, Spectrophotometric determination, Tyrosinase.

INTRODUCTION

Tyrosinase, also known as polyphenol oxidase (PPO) is a copper containing mixed function oxidase widely distributed in microorganism, animals and plants. This catalyzes two distinct reactions of melanin synthesis, the hydroxylation of a monophenol to *o*-diphenols (cresolate activity) and the conversion of an *o*-diphenol to the corresponding *o*-quinone (cathecolase activity)[1]. Besides its catalytic features, polyphenol oxidase is distinctive from the other enzymes in that it has various inhibitors and displays various inhibition patterns. Benzoic acid is one of the inhibitors of polyphenol oxidase.

Benzoic acid is a common preservative that is added to products such as foods, beverages, dentifrices, cosmetics, pharmaceuticals to prevent decomposition by microbial growth or by undesirable chemical changes[2]. However, benzoic acid at higher than permitted safety levels can do harm to human health. The maximum permitted concentrations of benzoic acid in each type of food are controlled by legislation. Concentration of naturally occurring benzoic acid in several foods did not exceed average value of 1000 mg/kg of food[3]. Maximum concentrations reported for benzoic acid added to food preservation purposes were in the range of 2000 mg/kg of food[4]. Therefore, the analytical determination of benzoic acid is not just important for quality assurance purposes but also for consumer interest and protection. Therefore, its concentration has to be controlled.

Various traditional method have been reported for the analysis of benzoic acid in foodstuffs, such as thin layer chromatography[5], high performance liquid chromatography[6,7], gas chromatography mass spectrophotometry, enzyme-linked immunosorbent assay (ELISA) and other immunochemical techniques. Nevertheless, these methods do not follow an easily continuous monitoring, because they are expensive, slow, need well trained operators and in some cases, require steps of extraction or sample pretreatment, increasing the time of analysis[8].

In enzyme-based determination, the biological element is the enzyme which reacts selectively with its substrate associated with or integrated within a physico-chemical transducer or transducing microsystem, which may be optical, electrochemical, thermometric, piezoelectric or magnetic[9]. Optical biosensor based on inhibition of mechanism of polyphenol oxidase using a novel phenol biosensor to detection of benzoic acid has not yet been reported. Amperometric biosensor based on inhibition of polyphenol oxidase using a novel phenol biosensor using polyaniline-polyacrylonitrile composite matrix has been developed by Dan Shan *et al.*[8]. Inhibition based biosensor using

mushroom tissue homogenate have been reported to by Kemal *et al.*[10] to detect benzoic acid in food products.

In this work, we had successfully developed spectrophotometric detection system for benzoic acid based on inhibition of tyrosinase. The sensing scheme is based on bleaching of maroon colour product associated with the reaction of *o*-quinone and 3-methyl-2-benzothiazolinone hydrazone in solution. The determination of benzoic acid was carried out through its inhibitory effect on tyrosinase.

EXPERIMENTAL

Tyrosinase form mushroom (EC 1.14.18.1, 4.741 units mg^{-1}) and phenol were purchased from Sigma. 3-Methyl-2-benzothiazolinone hydrazone was purchased from Merck. Benzoic acid was purchase from Sigma. 0.05 M phosphate buffer solution (pH 6.5) was used as buffer throughout the analysis. Tyrosinase (35000 U) was dissolved in 100 mL 0.05 M phosphate buffer pH 6.5. The tyrosinase solution was filtered with filter paper and stored at 3 ºC. 0.54 g of 3-methyl-2-benzothiazolinone hydrazone powder was dissolved in deionized water. The stock solution of 3-methyl-2-benzothiazolinone hydrazone was stored in refrigerator at 3 ºC. 0.24 g of phenol crystal was also dissolved in deionized water. Stock solution of 1000 ppm of benzoic acid was prepared by dissolving 1.00 g of benzoic acid in 100 mL of deionized water. A series of standard solution were prepared by appropriate dilution of the stock solution.

Procedure: A mixture of 2 mL tyrosinase, 6 mL of 0.01 M phenol, 3 mL of 0.01 M 3-methyl-2-benzothiazolinone hydrazone and 1.5 mL of 100 ppm benzoic acid were transferred into bottle sample. The solution was left for 0.5 h to allow a complete reaction. The measurement was carried out using UV-vis spectrophotometer (Varian-Cary Win).

Real sample analysis: 5 mL of soya sauce (purchased in a local supermarket) was accurately pipette into a 50 mL volumetric flask and diluted to the mark with deionized water. The pH was adjusted to pH 6.5 with 0.5 M of sodium phosphate dibasic. The sample was filtered to remove suspended mater. Then the whole sample was diluted with 100 mL deionized water. 5 mL aliquot of this solution was diluted with 100 mL deionized water. The same procedure was repeated with 5 mL aliquot of the previously diluted solution. 1.5 mL aliquot of this solution was added to the mixture of tyrosinase, phenol and 3-methyl-2-benzothiazolinone hydrazone solution and the absorbance was measured using Uv-vis spectrophotometer. Concentration of benzoic acid in the sample was determined using the calibration graph.

RESULTS AND DISCUSSION

Spectral study: Fig. 1 shows the absorbance spectra before (A) and after (B) addition of benzoic acid. The inhibition effect of benzoic acid on tyrosinase enzyme causes a decrease in absorbance due to the bleaching of maroon coloured solution. The reaction scheme is shown below.

$$\text{Phenol} + \text{Tyrosinase} \rightarrow \text{Dihydroxybenzene} \quad (1)$$

$$\text{Dihydroxybenzene} + \text{Tyrosinase} \rightarrow o\text{-quinone} + H_2O \quad (2)$$

$$o\text{-Quinone} + \text{MBTH (colourless)} \rightarrow o\text{-quinone-MBTH (maroon)} \quad (3)$$

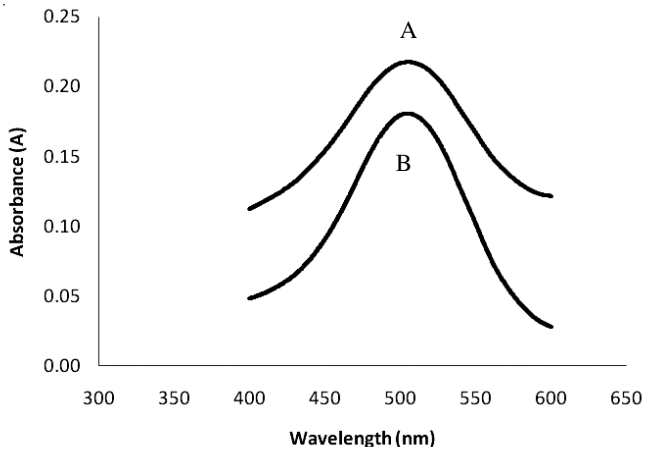

Fig. 1. Absorbance spectra for before (a) and after (b) addition of benzoic acid solution

pH Study: pH of enzyme solution can effect overall catalytic activity because enzymes like all natural proteins have a native tertiary structure that is sensitive to pH and the denaturation of enzymes can occur at extreme pH values[11]. Fig. 2 shows the effect of pH on determination of benzoic acid. pH 7 appears to be the optimum pH for the determination and was selected for further analytical procedure. This value is in good agreement with previously reported results[10-13]. This in accordance with the works reported by Shan *et al.*[8], who demonstrated that inhibitor benzoic acid does not alter the optimum pH of tyrosinase significantly.

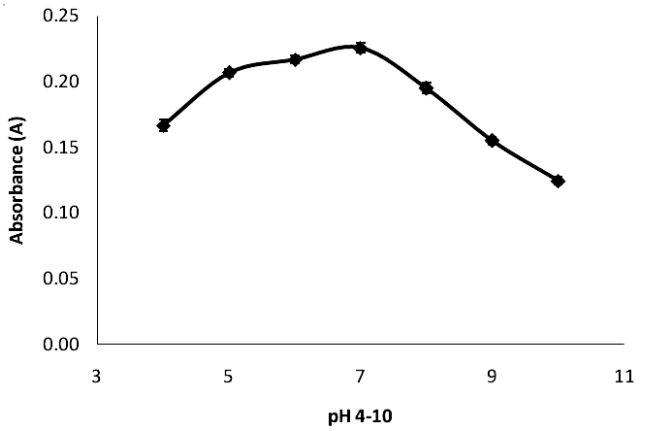

Fig. 2. Effect of different pH on the determination of benzoic acid

Reproducility: Reproducibility refers to the successive runs made by using the developed method to evaluate discrepancies in its responses. Fig. 3 showed that the reproducibility of the inhibitory action of benzoic acid on enzymatic activity of tyrosinase based on 10 measurements. The RSD for reproducibility of the developed method was calculated to 1.91 %. A small RSD values observed for this method indicate a good precision of the method being used.

Effect of amount of tyrosinase: The effect of amount of tyrosinase in mixture of solution carried out in this study is shown in Fig. 4. It can be observed that the absorbance increase until a constant value is reached. The increasing amount of tyrosinase contributes to the increasing of the enzyme-substrate complex (ES) formed and the intensity of maroon coloured solution increase depending on the amount of tyrosinase

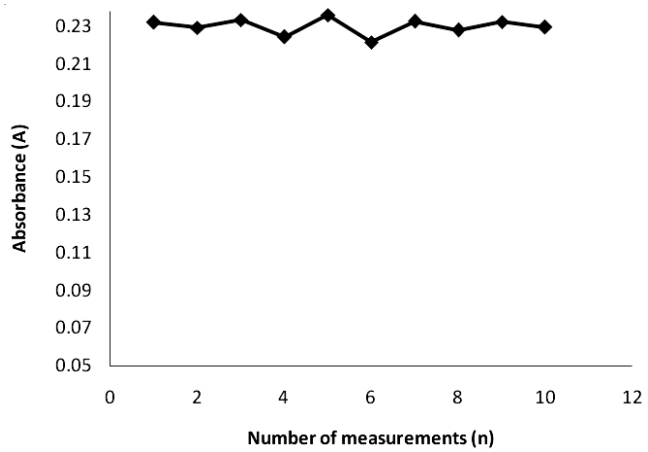

Fig. 3. Reproducibility of the determination of benzoic acid

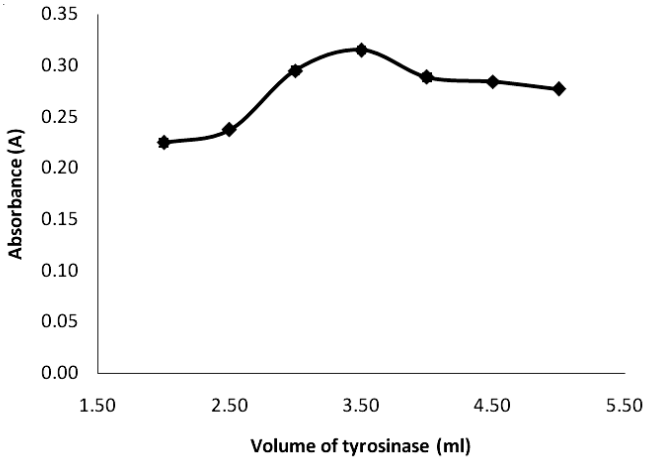

Fig. 4. Effect of volume of tyrosinase on the determination of benzoic acid

added[14]. This plateau region is reached when all available phenol has been consumed. It was observed that the addition of 3.5 mL of tyrosinase solution gives the maximum and constant absorbance for 100 ppm of benzoic acid solution. Beyond the value, addition of further tyrosinase volume will not influence the absorbance and tyrosinase enzyme is in excess.

Dynamic range: Fig. 5 shows the response curve of the complex towards different concentration of benzoic acid. The linear part of the response curve is re-plotted and shown in Fig. 6. The straight line obtained from this plot can be described

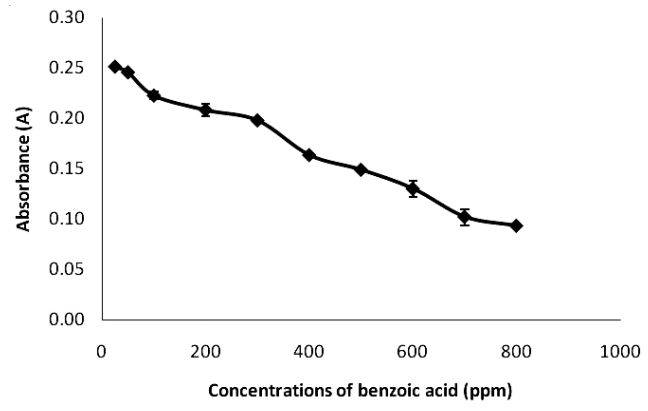

Fig. 5. Effect of different concentration of benzoic acid on the response curve

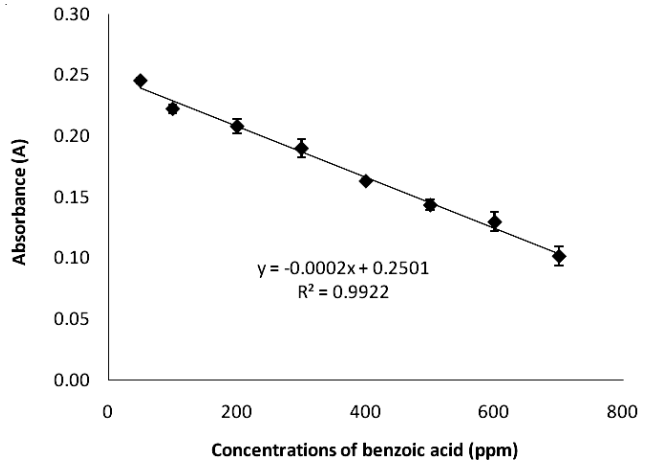

Fig. 6. Calibration plot for determination of benzoic acid

by equation y = -0.0002x + 0.2501 and the calculated correlation coefficient, R^2 was found to 0.9922. The limit of detection of the method (LOD) is defined as the concentration equivalent to a signal of blank plus three times the standard deviation of the blank was calculated to be 109 ppm.

Kinetic study: The inhibition mechanism can be studied by examining the relationship between the response of the detection system and the inhibitor[15]. In this study, the inhibition kinetics of tyrosinase by benzoic acid was investigated. The relationship of enzyme activity in the presence of different concentrations of benzoic acid using the double-reciprocal Lineweaver-Burk plots, were studied. Fig. 7, shows that the plots of 1/Abs *versus* 1/[Phenol] give a family of straight lines with different slopes that intersected one another in the Y axis. The value of Abs_{max} remains the same and the value of Michaelis-Menten constant (K_m) increases. K_m is defined as the concentration of the specific substrate at which a given enzyme yields one-half its maximum velocity[16]. The value of K_m increases (Table-1), in the presence of different benzoic acid concentration due to increasing on the concentrations of the inhibitor, which indicates that benzoic acid, is a competitive inhibitor of tyrosinase, *i.e.*, benzoic acid only binds free enzyme rather than enzyme-substrate complex $(ES)^2$. The inhibition constant for binding between benzoic acid and tyrosinase, K_i is 90.9 ppm which is obtained from the Dixon plot (Fig. 8).

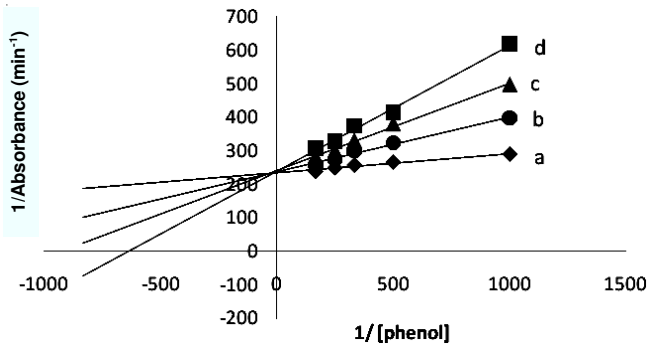

Fig. 7. Lineweaver-Burk plots for the phenol in the absence (a) and in the presence of 150 ppm (b) 250 ppm (c) and 350 ppm (d) benzoic acid

Interferences: In order to demonstrate the selectivity of the biosensor, the potential interferences from other substances,

TABLE-1
KINETIC CONSTANT FOR VARIOUS CONCENTRATION OF INHIBITOR (BENZOIC ACID)

[Benzoic acid] (ppm)	K_m	Abs_{max}
0	0.00024	0.00426
150	0.00069	0.00421
250	0.00110	0.00415
350	0.00150	0.00416

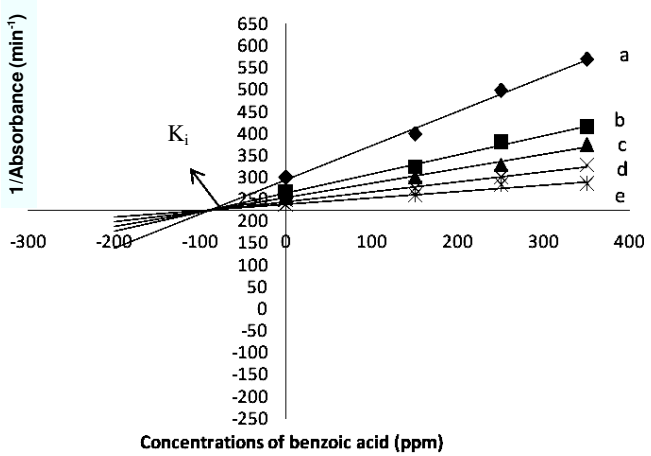

Fig. 8. Dixon plots of the benzoic acid inhibition in the presence 0.001 M (a), 0.002 M (b), 0.003 M (c), 0.004 M (d), 0.006 M (e) phenol

were studied. The effect of interferences on the detection signal was studied, using 0.01 M phenol, 100 ppm benzoic acid and 100 ppm interfering substances. The influences of sodium chloride, citric acid, ascorbic acid and boric acid on the inhibition response of benzoic acid were listed in Table-2.

TABLE-2
EFFECT OF POSSIBLE INTERFERING SUBSTANCES

Substance	Interference (%)	Substance	Interference (%)
Sodium hydroxide	5.3	Ascorbic acid	13.7
Citric acid	7.2	Boric acid	21.2

Method validation: The application of the proposed method towards detection of benzoic acid in soy sauce was investigated. The content of benzoic acid in the sample was determined from the calibration curve and the result is listed in Table-3. The result obtained was validated using HPLC.

TABLE-3
DETERMINATION OF BENZOIC ACID IN SOY SAUCE BY THE DEVELOPED METHOD AND HPLC METHOD

Sample	HPLC method (ppm)	Biosensor (ppm)	Relative errors (%)
Soya sauce	592.3	600	1.3

The relative errors are acceptable. The result determined by proposed method were in satisfactory agreement with those given by HPLC methods, indicating that is feasible to apply the developed method for determination of benzoic acid in real sample.

Conclusion

Many biosensor based on tyrosinase inhibition effect for detection of benzoic acid have been reported[2,9-12]. All the biosensor reported were based on amperometric biosensor. In this work, a spetrophotometric determination of benzoic acid based on inhibition of benzoic acid towards tyrosinase enzyme has been developed. The optimized condition for operation is at pH 7. The response range was 50-700 ppm of benzoic acid concentration with LOD value of 109 ppm. The reproducibility for detection of 100 ppm benzoic acid gave a low RSD value of 1.91 %. The inhibition constant is 90.9 ppm. The proposed method is easy to handle and a good alternative to traditional methods for benzoic acid analysis.

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