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Application of *Agaricus bisporus* Extract for Benzoate Sodium Detection Based on Tyrosinase Inhibition for Biosensor Development

Vânia P. S. dos Santos[a], Lívia M.C. Silva[a], Andrea M. Salgado[a], Karen S. Pereira[b]

[a]Laboratory of Biological Sensors E-122, Biochemical Engineering Department, Chemistry School, Technology Center, Federal University of Rio de Janeiro – Horácio Macedo, 2030 Avenue – University City - Ilha do Fundão – zip code 21949-909 – Rio de Janeiro, RJ, Brazil
[b]Laboratory of Food Microbiology E-104.
paulassalviano@ufrj.br

Sodium benzoate preservative is widely used in foods and beverages. Considering the possible adverse reactions described in the literature (child hyperactivity, asthma, urticaria), there is an importance for stringent control of sodium benzoate levels in order to maintain food quality and guarantee safety for consumers. Accordingly the need for analytical methods which are quick and affordable. Nowadays, the HPLC (High-performance liquid chromatography) is the official method used for the detection and quantification of these preservatives. However, the chromatographic technique has some limitations such as the need for pretreatment of the sample and the need for highly skilled operators to perform analyses. Biosensor development may offer advantages in comparison to conventional detection techniques. The assays described here aim to validate the method of detection and quantification of sodium benzoate and will be applied in future construction of a biosensor designed to detect benzoates in food samples. The principle of the method is based on the measurement of the inhibition of tyrosinase enzymatic activity with the substrate L-tyrosine of the sodium benzoate present in samples of beverages. The concentration of the preservative is quantitatively related to the percent inhibition experienced by the enzyme. The tests were conducted using an oxygen electrode as transducer; measuring its consumption during the enzymatic reaction in the presence of the substrate. The source used was the tyrosinase enzyme extract obtained from macrofungi *Agaricus bisporus*, having enzymatic activity as determined by colorimetric method of 372 U/mL to 937 U/mL. The proportions of enzyme/substrate obtained were determined as the best ones: neat extract/solution of 1 mM L-tyrosine or extract diluted 1:1 in a buffer solution of sodium phosphate pH 6 of L-tyrosine 1.2 mM. A standard curve showed linearity of enzyme inhibition of 0.006 g to 0.014 g sodium benzoate. Tests with real samples indicated the presence of sodium benzoate below those permitted by Brazilian Law in samples of natural guaraná soft drink.

1. Introduction

Benzoic acid (E210) and its salts, benzoates In (E211) and K (E212), are among the most common preservatives used because of the most favorable cost-effective ratio. The benzoic acid undissociated is the most effective antimicrobial agent but benzoates are most commonly used due its higher solubility in water. The acceptable daily intake (ADI) according to the levels recommended by the Joint FAO/WHO Expert Additives (JECFA) is 0-5 mg/kg (WHO, 2000). Benzoic acid has an inhibitory action on the growth of fungi, yeasts and bacteria, acting more efficiently in pH ranges of 2.5 and 4.5, with the maximum permissible concentration 0.05 g/100 mL by Brazilian legislation (ANVISA, 1999).
Although its consumption is considered safe for human health (GRAS - Generally Recognized as safe), several studies indicate cases of adverse reactions due to its use even below levels set by regulatory agencies (Zengin et al.,2011). Beverage containing sodium benzoate and ascorbic acid have the potential

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for benzene formation, classified as a Group 1 carcinogen by the *International Agency for Research on Cancer* (Gardner and Lawrence 1993; Vinci et al.,2010).

Due to adverse reactions of benzoic acid and its salts, it is important to tightly control their dosages assuring the safety of consumers beyond the control of quality through rapid and affordable analytical methods.

Currently the procedure for analysis used to detect and monitor levels of benzoic acid and benzoates in foods and beverages is by HPLC. However, chromatographic methods, although reliable and established, have some limitations such as the need for extensive pretreatment of the sample, low specificity, need for highly trained technicians, and the use of greater quantities of reagents. All of these factors make the analyses often costly and expensive (Mello and Kubota 2002).

Biosensors are devices that that combine the specificity of biological reactions with suitable transducers electronics (Van Dorst et al., 2010). In general, may have advantages over the traditional techniques of analysis such as: portable, fast, selectivity, cost-effective, detection without extensive sample pretreatment (Kotanen et al., 2012).

The tyrosinase enzyme (EC 1.14.18.1) from *Agaricus bisporus*, has been used in the construction of biosensors for food and environmental analysis and provides economic advantage over the use of commercial enzymes (Sezgintürk et al., 2005; Silva et al., 2011).

This study is intended to develop a methodology for detection of benzoic acid in foods. The method's principle is to measure the inhibition of tyrosinase enzymatic activity of the enzyme with the substrate L-tyrosine. The benzoic acid present in samples of non-alcoholic beverages and the concentration of the preservative is quantitatively related to the percentage inhibition activity suffered by the catalytic enzyme. The enzyme source used was the enzyme extract obtained from macrofungi *Agaricus bisporus*.

The tests performed in this work aims to determine the reaction conditions more favorable for the use of the enzyme extract macro fungi the future construction of a biosensor for the detection of benzoic acid.

2. Materials and methods

2.1. Obtaining the enzyme extract of *Agaricus bisporus* macro fungi and activity measurement

The process of obtaining the tyrosinase enzyme extract from *Agaricus bisporus* macrofungi follows the procedure adapted from (Kameda et al., 2006). Grounds in a blender were 340 g of mushrooms with 2 L of cold acetone; the mixture was filtered under vacuum and the residue stored at 0 °C for 24 h. Then the mushroom paste was re-suspended with distilled water and stored again 0 °C for 24 h to obtain the first enzyme extract by centrifugation at 3000 rpm for 5 minutes. The process of re-suspension and centrifuging the slurry was performed again to obtain the second extract and enzymatic activity was measured according to adapted procedure developed by Campos et al., 1996, where 1 mL of the diluted extract in the ratio of 1:10 in sodium phosphate buffer pH 6 was added to a beaker containing 1.5 mL of a substrate solution L-tyrosine 1.2 mM and 5.5 mL of sodium phosphate buffer pH 6, and taken for analysis in a spectrophotometer with absorbance set at 280 nm for 1020 s with intervals of 30 s.

2.2. Determination of conditions of use of the electrode

To perform the measurements of oxygen consumption by the reaction of tyrosinase enzyme extract with the substrate L-tyrosine we used an oxygen electrode type Clark. The procedure of saturation with air with reaction environment containing only the substrate solution before the addition of the enzyme extract, it was performed for 5 min using an aquarium pump. The tubes containing the reaction environment were sealed with PVC film during saturation to minimize gas exchange between the analysis solution and the external environment.

After this procedure, readings of variations in oxygen concentration of 1 to 5 min were taken to determine the time required for stabilization of the electrode. The time selected was determined as that in which the variations in readings were the lowest possible.

2.3. Determination of optimal concentration of enzyme and substrate

The enzyme extract (937 U/mL) was used in five different concentrations (neat and 2x, 4x, 8x and 10x diluted). In each one of these solutions were used concentrations of the substrate L-tyrosine at 4 different concentrations (1 mM, 1.2 mM, 1.6 mM and 2 mM) with the purpose of choose the lowest reaction time when the enzymatic activity of tyrosinase extract shows the slightest variation of oxygen consumption.

For the four substrate concentrations we constructed graphs of oxygen consumption as a function of the concentrations of the substrate L-tyrosine for each of the different concentrations of enzyme extract, with shorter reaction time set.

2.4. Determining the best reaction time

Assays to set the best ratio between the concentrations of enzyme and substrate resulted in reaction time range between 4 to 80 min. The shortest reaction time was chosen so that the enzymatic activity of tyrosinase extract exhibited the slightest variation of oxygen consumption for the four substrate concentrations. Then we constructed graphs of oxygen consumption in order of the concentrations of the substrate L-tyrosine for each of the different concentrations of enzyme extract, with shorter reaction times set.

2.5. Construction of the standard curve of inhibition of catalytic activity of tyrosinase by benzoic acid

To determine the inhibition percent of the catalytic activity of tyrosinase enzyme extract we tested two concentrations of enzyme extract and two L-tyrosine substrate concentrations as described in 2.3. The amounts of sodium benzoate were defined according to the maximum concentration permitted by Brazilian law for non-alcoholic beverages which is 0.05 g/100 mL (as benzoic acid) according to ANVISA. Highest and lower limits were defined based on the maximum amount allowed by law measuring a proportional 20 mL solution containing: 4 mL of enzyme extract and 16 mL of substrate L-tyrosine. The amounts of sodium benzoate used for enzymatic inhibition assays were 0.006 g, 0.008 g, 0.01 g (maximum extent permitted by law), 0.014 g, 0.018 g 0.02 g in 20 mL of the solution are proportional 0.03 g, 0.04 g, 0.05 g (maximum extent permitted by law), 0.07 g, 0.1 g 0.09 g in 100 mL of solution, respectively. Prior to testing with the addition of benzoate, the oxygen consumption was measured in a blank sample to calculate the percentage inhibition based on the equation 1:

$$\%I = \left[\frac{\Delta O_2 \text{ a/benzoate} - \Delta O_2 \text{ p/benzoate}}{\Delta O_2 \text{ a/benzoate}} \right] \text{x100} \quad (1)$$

%I: percentage inhibition
ΔO_2 a/benzoate: variation of oxygen in the absence of benzoate
ΔO_2 p/benzoate: variation of oxygen in the presence of benzoate

2.6. Construction of the standard curve of inhibition of catalytic activity of tyrosinase by benzoic acid in solution of organic guaraná powder

In order to verify the possible interference of guaraná in tyrosinase enzyme activity, assays were performed to determine the percent inhibition of catalytic activity of tyrosinase by benzoic acid in solution of organic guaraná extract powder. The substrate solution of L-tyrosine was added 0.02 g of guaraná extract per 100 mL of solution simulating a beverage that guaraná extract contains the minimum that must be contained in beverages guaraná based according to Brazilian law (0.02 g/100 mL).

2.7. Construction of the standard curve of inhibition of catalytic activity of tyrosinase by benzoic acid in real samples

To determine benzoate in real samples, the same procedures were carried out in section 2.4 with the substrate solution of L-tyrosine was made by diluting L-tyrosine in sample itself guaraná generic soft drink. Subsequently the pH was adjusted with sodium phosphate dibasic to obtain pH 6. We used 3 lots of 2 brands of beverage guaraná samples.

3. Results and Discussion

The enzyme extract obtained in the extraction showed activity minimum and maximum of 372 U/mL and 937 U/mL, respectively. It was determined that the settling time of the electrode will be 3 minutes based on the lowest standard deviation ± 0.08. Assays for the best ratio of the concentration of enzyme and substrate resulted in the choice of neat extract and diluted twice according to figure 1 and 2.

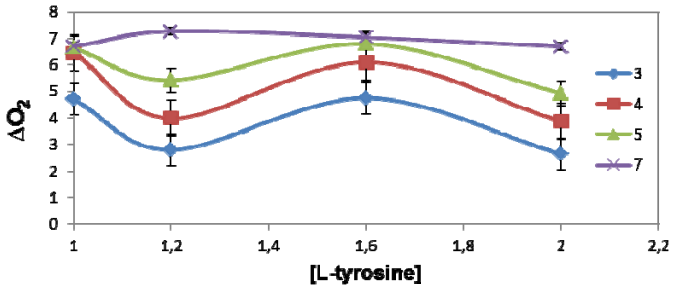

Figure 1: Graph of the oxygen consumption in the reaction times of 3, 4, 5 and 7 minutes reaction using neat enzyme extract in four concentrations of substrate solution L-tyrosine: 1 mM, 1.2 mM, 1.6 mM and 2 mM

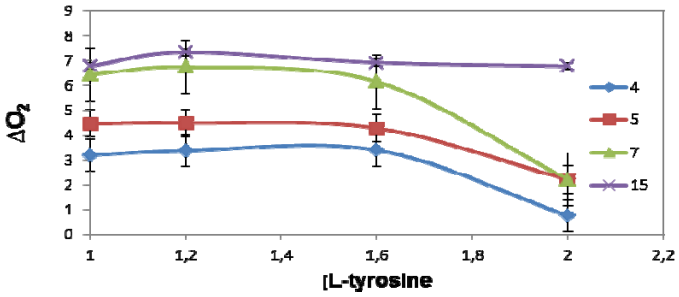

Figure 2: Graph of the oxygen consumption in the reaction times of 3, 4, 5 and 7 minutes reaction using the enzyme extract diluted two times in four concentrations of substrate solution L-tyrosine: 1 mM, 1.2 mM, 1.6 mM and 2 mM

Based on the graphs 1 and 2 it is concluded, respectively, for pure extract the reaction time at which the enzyme exhibits less fluctuation in terms of oxygen consumption is 7 minutes, while for the two times diluted extract the smallest fluctuation was obtained in reaction time of 15 minutes. Both times were chosen in view of the smaller standard deviation values, ± 0.35 for the time of 7 minutes and ± 0.25 for the time of 15 minutes.

The two times diluted extract was elected to perform the inhibition tests due to having lesser standard deviation value and to give an economic advantage implying a greater volume of extract. However, inhibition assays using the two times diluted extract result in non-satisfactory results since inhibition values were very close for the various tested concentrations of benzoate according to figure 3 and are therefore not suitable for the method since this makes it impossible to quantify the preservative.

Moreover, the extract pure allowed variation in enzyme inhibition was greatest among the tests with different concentrations of the inhibitor, featuring a linear increase of variation of the inhibition with increasing concentration of sodium benzoate in concentration range benzoate 0.006 g 0.014 g, giving a linear curve with the equation y = 3169x + 29.99 and R ² = 0.93, above these concentrations the inhibition occurred so that the variation in oxygen consumption was not sufficiently separate so to quantify it.

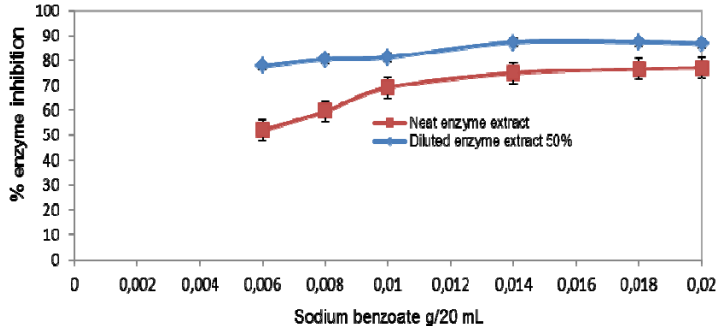

Figure 3: Graph of the inhibition curve pattern of the enzyme tyrosinase by sodium benzoate at a concentration of 0.006 g, 0.008 g, 0.01g, 0.014 g, 0.018 g and 0.02 g. using the extract neat enzyme extract and diluted 50% with a substrate solution of 1 mM L-tyrosine.

Tests to check the possible interference of guaraná extract, the enzymatic activity of tyrosinase, are shown in Figure 4.

Comparing the curves of inhibition in the absence and presence of organic guaraná, it can be concluded that guaraná did not affect the linearity of inhibition which remained at concentrations of benzoate 0.006 g 0.014 g with linear curve with the equation y = 4959,3x + 22,279 and R^2 = 0,9109.

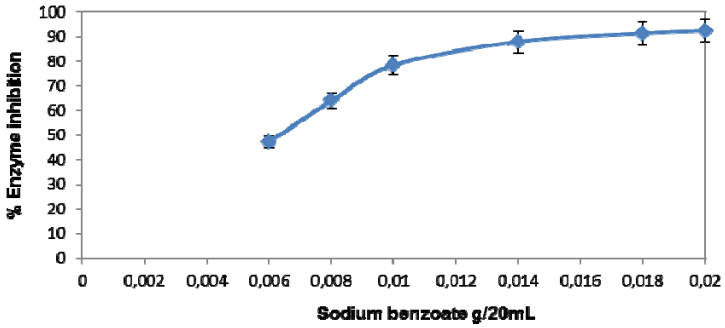

Figure 4: Graph of the percentage of enzyme inhibition in the presence of concentrations of sodium benzoate: 0.006 g, 0.008 g, 0.01 g, 0.014 g, 0.018 g and 0.02 g. using extract enzyme neat with a substrate solution of L-tyrosine 1.2 mM containing 0.02 g of guaraná extract powder.

The tests on commercial samples of iced guaraná are summarized in Table 1.

Table 1: The tests on commercial samples of beverage guaraná using the enzyme tyrosinase enzyme inhibition

Samples of refreshment guaraná	Batch	Concentration of sodium benzoate g/100 mL
A	1	0.032
A	2	0.020
B	1	0.026

According to the results in Table 1, all samples showed concentrations of sodium benzoate below permitted by Brazilian law. However, these concentrations exhibited values below the range of detection/quantification method.

4. Conclusion

The method developed in work, has potential for use for application in the construction of a biosensor for detection benzoate in beverages. The use of the enzymatic extracts of *Agaricus bisporus* macrofungi as a source tyrosinase enzyme provides an economic advantage over the use of commercial enzymes. Aiming to improve the detection method next steps include: test concentrations of sodium benzoate below 0.006 g; test methods suitable for immobilizing the enzyme tyrosinase present in the extract, to improve the linearity of the standard curve, increasing the detection range and sensitivity of the method and to use the standard method of detection in foods benzoates (HPLC), for comparison with results achieved by the methodology employed in such studies.

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