

Response of Salivary Peroxidase to Food Preservatives

Zahra Heidari¹, Reyhaneh Sariri^{1*} and Vahab Jafarian²

¹ Department of Biology, University of Guilan, Rasht, Iran

² Department of Biology, University of Zanjan, Zanjan, Iran

Summary

High performance liquid chromatography followed by ultra violet diode array was used for detection of food preservatives in some processed food samples obtained from local market. It was found that, in some cases, the concentration of common food preservatives was much higher than allowed amount recommended by food safety organizations such as Food and Drug Administration. As oral peroxidase (OPO), is of paramount importance in the oral defense mechanism, the aim of this research was to investigate the possible adverse effect of food preservatives on oral defense system. The biological activity of oral peroxidase was measured in the presence of various concentrations of some food additives *in vitro*. The results showed that, within the concentration range used in common processed foods, sodium benzoate played almost no adverse effect on the activity of oral peroxidase, while potassium sorbate acted as a mixed inhibitor on the activity of enzyme.

Keywords: Salivary peroxidase, potassium sorbate, sodium benzoate, HPLC

Introduction

It is known that saliva plays a major role in early digestion and metabolism of some foods. Salivary enzymes contribute to many important health functions including food digestion and the first line of oral defence. Human salivary peroxidase systems belong to the group of innate defense factors, whose mechanisms of action are non-specific. The antimicrobial spectrum of the salivary peroxidase systems covers both gram positive and gram negative oral and non-oral bacteria. In addition to antibacterial, it has been reported that the peroxidase system shows antiviral and antifungal activities [1]. Therefore, the perfect biological activity of peroxidase is always needed for both oral and general health maintenance.

The increasing demand of many people to use ready made and packed processed foods, has brought about more concern about the safety of such products and detection of various additives present in them. Many processed foods, such as fruit juices, soda, soya sauce, cream cheese and many others products contain various amounts of preservatives in order to prevent spoilage and increase their shelf lives.

Typically, sodium benzoate and/or potassium sorbate are added to many fruit juices and soft drinks to inhibit mold growth, prevent spoilage, and preserve freshness [2]. Under the provisions set forth by the US Food and Drug Administration (FDA) in the code of Federal Regulations, food additives can be used if they are generally recognized as safe (GRAS) and declared on the label. For instance, sodium benzoate may be used as a preservative in juices, at level of 0.1%, while potassium sorbate may be used at levels of 0.1-0.2% [2]. The present study is the first report on the inhibition by sodium benzoate and potassium sorbate of salivary peroxidase.

The peroxidase found in the oral cavity is a very important salivary antioxidant enzyme. Oral peroxidase (OPO) is composed of two peroxidase enzymes, salivary peroxidase (SPO) and myeloperoxidase (MPO). The SPO secreted from the major salivary glands, mainly the parotid gland, contributes 80% of OPO activity, while MPO, produced by leukocytes in inflammatory regions of the oral cavity forms 20% [3]. The term OPO is used here to denote the total activity of both isoforms. OPO plays a dual role, it mainly reduces the level of hydrogen peroxide excreted into the oral cavity from the salivary glands by bacteria and leukocytes, and it also increases specific antibacterial activity by inhibiting the metabolism and proliferation of various bacteria in the oral cavity [4]. Peroxidase catalyzes the oxidation of SCN^- , the detoxification product of cyanide by hydrogen peroxide:



In this reaction, SCN^- acts as the electron donating component, similar to glutathione (GSH) in other biological systems [3]. Two potent antibacterial oxidizing products evolve from this reaction: hypothiocyanous acid (HOSCN) and its conjugated anion, hypothiocyanate (OSCN^-). The cytotoxic properties of these salivary oxidants depend on the extracellular pH, and HOSCN can oxidize oxyhemoglobin into methemoglobin in erythrocytes, while both HOSCN and OSCN^- can oxidize intracellular reduced GSH. The cytotoxic antibacterial activity of HOSCN and OSCN^- stems from their ability to react with sulfhydryl groups of bacterial enzyme that are vital for glycolysis such as hexokinase, aldolase, and pyruvate kinase [4]. Under various oral stimuli, peroxidase may act as a scavenger of H_2O_2 to produce molecular oxygen, but without producing OSCN^- plus HOSCN. In any case, the accumulated antibacterial activity of the combination of peroxidase, H_2O_2 , and SCN^- is much more potent than that of H_2O_2 alone [1].

Processed foods, such as fruit juice, soda, soy sauce, cream cheese, and many other products are often sold with food additives to prevent spoilage. Typically, sodium benzoate and /or potassium sorbate are the preservatives that are used in juices to inhibit mold growth, prevent spoilage, and preserve freshness [2]. Under the provisions set forth by the US Food and Drug Administration (FDA) in the code of Federal Regulations, food additives can be used if they are generally recognized as safe (GRAS) and declared on label. For instance, sodium benzoate may be used as a preservative in juices, however, its usage should not result in levels exceeding 0.1% in the beverage, while potassium sorbate may be used at levels of 0.1 -0.2% [2].

An extensive investigation on the level of some food preservatives in processed foods found in Rasht market, showed that in many cases sodium benzoate and potassium sorbate were much higher than the recommended dose. The aim of this study was, therefore, investigating the effect of food preservatives on biological activity of salivary peroxidase. Therefore, any food additives should be selected so that they do not affect the salivary enzymes.

Experimental

Samples

Samples were obtained from food stores and chosen to be representative of what a consumer would find in a market – basket study. Sample sizes ranged from 10 to 60 g. Each collected sample was tested for the presence and amount of both preservatives, sodium benzoate and potassium sorbate [2].

Reagent

HPLC grade sodium benzoate and potassium sorbate were purchased from Sigma, acetonitrile, HPLC grade water, ammonium acetate and glacial acetic acid were obtained from Merck, and filters Machery-Nagal membrane filter prorafile 0.45 µm used for samples. Reagents used for enzyme assay were of analytical grade and used as supplied by manufacturers without further purification. 4-Amino Antipyrine, phenol and hydrogen peroxide were purchased from Merck chemical company. All buffers were prepared freshly within our laboratory and their pH was double checked.

High Performance Liquid Chromatography (HPLC)

Mobile phase preparation

The mobile phase was prepared according to the data given in Table 1.

Solvent	TFA	Methanol %	Acetonitrile %	H ₂ O %
A	0.01	5	5	90
B	0.1	5	90	5

Standard preparation

Exactly 50 mg of either sodium benzoate or potassium sorbate were added to a 100 ml volumetric flask and brought to volume with HPLC-grade distilled water. 2.5, 5.0, 10, 20, and 50 mg/l Dilutions from the stock solution were made using mobile phase. Standard solutions were not filtered prior to HPLC analysis [2].

Sample preparation

Beverage samples were prepared by diluting 1.0 ml of each sample with 10 ml of mobile phase and solid samples by blending 10 g of each with 50 ml of mobile phase for 2 min. The samples were then allowed to settle for 5 min and 1.0 ml of the supernatant were used later [2]. After dilution, all samples, solid or liquid, were filtered through Machery-Nagal membrane filter (0.45 μm) to remove particle matter from the samples and to prevent these particles from damaging the pumping or injection system, or clogging the column.

HPLC Instrument

A high-performance liquid chromatography instrument (Waters, USA) equipped with a binary pump, dual wavelength absorbance detector and Breeze data station was used. The HPLC operating mode was gradient with injection volume of 10 μl and the column temperature 20°C. The chromatographic column was a C18, 4.6 \times 250 mm, 5 μm , 300A° Supelco, Bellefonte PA, USA. Sample data collection was optimized to 12 min per sample with UV detection at 225 nm for sodium benzoate and 255 nm for potassium sorbate and the detector wavelength switched between analytes during each run. The optimal flow rate (pump speed) was determined to be 0.7 ml/min.

Time (min)	Flow rate (ml/min)	Solvent A (%)	Solvent B (%)
0	0.7	95	5
15	0.7	30	70

Collection of saliva samples

30 female students (mean age 22-24 years, $s_x = 2.34$) from the University of Guilan volunteered to donate their saliva samples. Timed un-stimulated whole saliva samples (3 ml) were collected in clean, dry in sterile pre-weighted tubes. The duration of saliva sampling was altered among individuals depending on their flow rate (2.0-5.0 minutes). The flow rate was calculated by measuring the time required to collect one ml of saliva in minutes. The saliva samples were immediately centrifuged at 800 \times g for 10 min at 4°C to remove squamous cells and cell debris. The resulting supernatant was stored at -18°C until used for determination of peroxidase activity.

Peroxidase activity

The biological activity of the enzyme on 4-amino antipyrine was measured spectrophotometrically. The oxidation of 4-amino antipyrine was measured at 25°C in 3 ml of 0.3 M phosphate buffer, pH 7.4, containing 0.001 M hydrogen peroxide, 0.002 M 4-aminoantipyrine and 0.15 M phenol. 40µl of diluted saliva was then added and the change in absorption at 510 nm ($\Delta A/\text{min}$) was recorded [5]. The change in absorption at 510 nm is due to the formation of a chromogen product with a λ_{max} at 510 nm. One unit of activity was defined as the amount of enzyme that caused an absorbance change of 0.001 per minute under standard conditions [5].

Enzyme activity in the presence of food preservative

The concentrations of all compounds, i.e. H₂O₂ and 4-amino antipyrine and phenol were kept constant (0.001, 0.002 and 0.15 M respectively) and the food preservative concentration varied from 300-2000 ppm for benzoate sodium and 300-8000 ppm for potassium sorbate that calculated from food samples by HPLC. The decrease in activity (%) was then plotted against concentration of food preservatives in µM. The reaction rate was measured at various concentrations of 4-amino antipyrine [5].

Results and discussion***Analysis of food items***

All of the samples were analyzed according to the optimized extraction procedure described earlier, peak identification of the potassium sorbate and sodium benzoate were based on the comparison between the retention times of standard compounds and were confirmed by spiking known standards to the sample. Quantification was based on the external standard method using calibration curves fitted by linear regression analysis [6].

A total of 60 samples were tested in this study. Liquid products tested were apple, grape, orange, and grapefruit, The samples selected for this study were chosen to fall under three categories, products that declared the preservatives sodium benzoate and /or potassium sorbate on their labels, samples which directly stated "no preservatives" on the label, and samples without labels. The results obtained showed that food samples had concentration ranging from 300-2000 ppm for sodium benzoate and 300-8000 ppm for potassium sorbate.

The effect of preservative concentration on rate of peroxidase activity was measured and it was shown that food preservatives, sodium benzoate and potassium sorbate affected the rate in way proportional to their concentration (Fig. 1). In order to obtain the amount of inhibitor causing 50% rate reduction (IC₅₀), V_I/V_0 was plotted against log [I]. The value of IC₅₀ in each case was obtained from the resulting curves (Fig. 2). It was found that the value of IC₅₀ for potassium sorbate was 29.85 mM, while in the case of sodium benzoate it was almost 10 times higher.

It can be suggested that the activity of peroxidase was decreased in the presence of potassium sorbate, while sodium benzoate had almost no direct effect on enzyme activity.

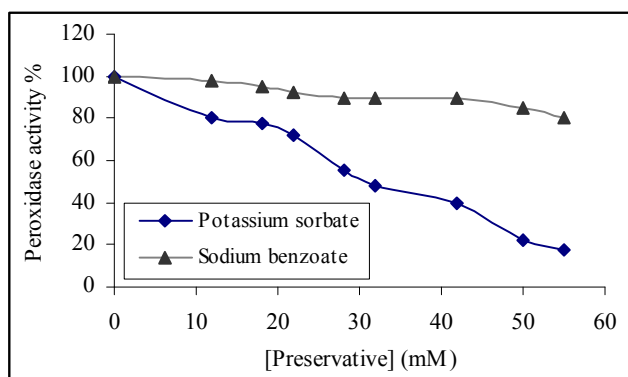


Figure 1. In-vitro effect of two common food preservatives on peroxidase activity.

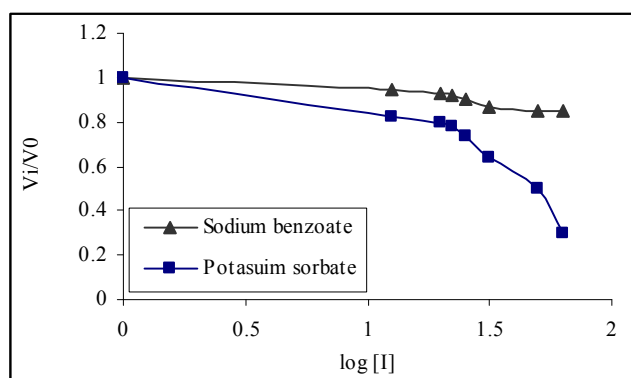


Figure 2. The IC_{50} curve of tyrosinase in the presence of food preservatives.

The results indicated that, in the allowed concentration range, sodium benzoate does affect adversely the biological activity of salivary peroxidase *in vitro*, while potassium sorbate was a mixed type non-competitive inhibitor of the enzyme.

Conclusion

Recently there has been increasing interest between researcher to use salivary biomarkers for various diagnostic purposes. This is, in part, due to the fact that saliva analysis is relatively fast, simple and non-invasive method. Oral fluid sampling is safe for the operator and the patient having advantage of easy and low cost storage conditions. These characteristics have made it possible to monitor several biomarkers in infants, children, elderly and non-collaborative subjects [7].

On the other hand, the use of food preservatives in order to prevent microorganism growth and reduce quality loss is inevitable. Many of these chemical compounds not only prevent the growth of moulds, yeasts and bacteria but they also protect foods from rancidity, browning, or developing black spots. They are also aimed to reduce any damage to some essential amino acids and the loss of some vitamins [6].

The salivary antioxidant system, in which the peroxidase is the pivotal enzyme, has been drawing increased attention in recent years. Salivary peroxidase seems to be of paramount importance in the oral defense mechanism, especially against the attack of free radicals. In addition, it has been demonstrated that saliva can inhibit the production of reactive oxygen species (ROS), such as the superoxide free radical and H_2O_2 , the most potent inducer of oral cancer [8].

Salivary defence mechanisms are the first line protection against harmful exogenous microorganisms entering the human body via the gastrointestinal tract [9]. In addition to the exogenous pathogens, these factors inhibit the over-growth of endogenous microflora by various mechanisms. Human salivary peroxidase systems belong to the group of innate defence factors, whose mechanisms of action are non-specific. The antimicrobial spectrum of the salivary peroxidase system covers both gram-positive and – negative oral and non-oral bacteria. In addition to antibacterial, the peroxidase systems have been reported to exhibit antiviral as well as antifungal activity [8].

It has been demonstrated that the peroxidase mediated oxidation products display significant inhibitory activity against such pathogens as salmonella typhimurium, pseudomonas aeruginosa, staphylococcus aureus, and porphyromonas gingivalis [11-14]. Furthermore, candida albicans and C. krusei and many viruses, including HIV, Herpes simplex type 1 and respiratory syncytial virus, have been shown to be sensitive to the peroxidase system [11-14]. Many other salivary antimicrobial agents have recently been shown to display antiviral activity. Based on these observations, it can be concluded that salivary antimicrobial agents not only protect the oral cavity, but they are also able to play a role in the protection of the digestive tract. Therefore, the importance of salivary peroxidase systems has been spread not only to oral but also to a subject's general health.

Among oral microorganisms, facultative anaerobes are the major class of bacteria that produce and release large amounts of H_2O_2 . The accumulation of higher concentrations is prevented by the dismutation of H_2O_2 by salivary peroxidase. Consumption of H_2O_2 is biologically and clinically significant because H_2O_2 is highly toxic to many mammalian cells, including fibroblasts and epithelial cells isolated from oral mucosal and gingival tissues [3].

Human saliva is known to exert various mechanisms to inactivate some mutagenic and carcinogenic compounds, such as food derived aflatoxin B1. One of these inactivating mechanisms is peroxidation by salivary peroxidase [3].

Oral squamous cell carcinoma (OSCC) is the sixth most common human cancer, free radicals. Such as reactive oxygen and nitrogen species (ROS and RNS), which induce oxidative and nitrative stress, are principal inducers of OSCC. Accordingly, it is of no surprise that evolution armed the oral cavity with advanced salivary antioxidant system that also contains antinitrosamine inhibitory agents. This salivary antioxidant system is based on peroxidase system [15].

The pharmacokinetic studies in human subject reported by several workers indicated that after oral and dermal, benzoate is metabolized in the liver by conjugation with glycine, resulting in the formation of hippuric acid. Sodium benzoate at a general optimum concentration of 0.1% could be used for preservation of such products as soft drinks, fruit drinks, margarine and certain fish products. On the other hand, it has been shown that activity of several enzymes could serve as a useful tool in clinical diagnosis, especially those enzymes known to be preferentially associated with certain cells and tissues of an organism [16]. On the other hand, reported changes in serum levels of cholesterol, albumin, and total protein with enlargement of hepatocytes with glassy cytoplasm in peripheral area of the liver of rats and mouse fed sodium benzoate of varying concentrations also, atrophy of the spleen and lymph following short-term feeding of rats with sodium benzoate. The finding report of increased level of the two enzymes agree with possible damages to the liver of the experimental animals [16].

Acknowledgements:

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