THERMAL STABILITY OF PEROXIDASES
EXTRACTED FROM SOME FRUITS

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بسم الله الرحمن الرحيم

وَيَسْأَلُونَكَ عَنِ الرُّوحِ قَلْ الرُّوحُ مِنْ أَمْرِ رَبِّي وَمَا أُوْتِيَ مِنَ الْعَلِيمِ إِلَّاً قَليلاً

سورة الإسراء الآية 85
Dedications

To the candles that light up my life

To the flowers that decorated my life…

…Samah, Ahmed and Lamis…

To my dear loving supporting family

with love and respect.

HIND
Acknowledgement

My faithful prayers and thanks to Allah for giving me the health and strength to fulfill this work.

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The aim of this research was to study the stability of peroxidase, the indicator of the efficiency of blanching, to variable thermal treatments. Unspecified varieties of apple, orange, guava and banana were the source from which the enzyme was extracted. The level of the enzyme, in each source, was determined and the stability of the extracts, from the four fruits, on keeping under different conditions of temperatures including, freezing (-18°C), fridge (4°C) and room temperature (30°C) was investigated. The present study also has undertaken the effects of three factors temperature, heating time and different pH values on the rate of thermal inactivation of the soluble peroxidase extracted from the four fruits, and the similar trends were regarded in the extraction and the subsequent treatments.

Peroxidase enzyme was found in all of the investigated fruits, though with different levels, which were found to vary considerably with accordance to the source. Relatively, reasonable activities of the enzyme from the four fruits were detected at all investigated pH values (5.0, 6.0, 7.0 and 8.0). Higher enzymatic activities were observed at pH 6.0 and 7.0, with the highest being at pH 6.0. Lower levels were found at pH 5.0 for the fruits under study. Apple, guava and banana fruits were found to contain high levels of the enzyme, while low levels of the enzyme were detected from the orange fruit at the same pH values.

The stability of the enzyme, for the four fruits, was examined by keeping the enzyme extracts under various storage conditions, freezing temperature, fridge temperature and room temperature for one week. The enzyme extracted from the four fruits showed to retain full activity (100%) under freezing conditions at (-18°C) after one week. The enzyme extracts of the four fruits maintained activities equal to or greater than 80% of the original activity when kept for one week at (4°C), and Less than 50% of the original activity was detected after the same length of time when kept at room temperature (30°C).
To study the thermal inactivation of peroxidase from the four fruits, the crude extracts were subjected to thermal inactivation at 60°C, 70°C, 80°C and 90°C for varying length of time, 2, 4, 6, 8, and 10 min. under different pH values namely 5.0, 6.0, 7.0 and 8.0.

The thermal inactivation rate, was found to increase with both increase of temperature and time. A biphasic curve of inactivation was observed for the thermal inactivation of the enzyme and similar patterns of inactivation were observed for the enzyme extracted from the four sources. Guava and banana peroxidases, showed higher heat resistance among the four investigated fruits, while apple and orange peroxidases showed lower heat stability. Guava and banana peroxidases proved to be stable to the thermal treatments, and were not completely inactivated at the tried conditions. less heat severity was required for the inactivation of apple and orange peroxidases. Apple peroxidase has lost about 80% of its activity after 2 min. at 80°C, and 90% of the activity was lost in 2 min. at 90°C at pH 5.0, while the total inactivation was accomplished in 7 min. at 80°C, and 5 min. at 90°C, both at pH 5.0, and 6 – 8 min. at 90°C were needed for the inactivation at the other pH values investigated. Orange peroxidase was completely inactivated at 6 – 8 min. at 80°C, and 5 min. at 90°C and pH 5.0, while 4 – 8 min. were required for the inactivation at the other pH values investigated.

The rate of the thermal inactivation of peroxidase was found to depend on temperature, length of heating time and pH. The higher resistant to thermal inactivation was found at pH 6.0, and 7.0, with the lower resistant detected at pH 5.0, for all of the investigated fruits.
لإجراء تحليل ل thiểuة الأكسجين معة الأنسجة، تم استخدام كودكس نموذجي لدراسة التأثيرات. الأنسجة تم تربية في وسائط مكونة من 10% سائل مع 90% غاز. أجري تحليل على عدد من الأمثلة، والتي تشير إلى أن 80% من الأنسجة كانت تحت ضغط 40% منăr

10

الحمض، و20% كان تحت ضغط 40% منăr

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CHAPTER ONE
INTRODUCTION

The increase of world population was met by enlargement production of fruits and vegetable as well as other sources of food, hence, grew the need for ways and means of preservation to solve the problems of seasonality and accessibility to fruits and vegetables. In turn, the produce industry has undergone a large and growing shift from fresh commodities to minimally processed (fresh-cut) fruits and vegetables, and processed products such as juice, jam, purée and other products. When raw fruits and vegetables, being perishable in nature, are peeled, cut sliced or pressed, the resulting products would be susceptible to the deteriorative agents, the micro-organisms and the endogenous enzymes (such as peroxidase), and as response to this induced physical damage to the plant tissue, deleterious effects on the food quality occurs (Files et al., 1985).

Peroxidases are widely distributed in the plant kingdom and are present in the different plant parts. Peroxidases play an important role in the physiological functions of the plant, and play a defensive role as well in cases of injuries and microbial infection. On the other hand, these enzymes are also involved in many undesirable changes in fruit's texture, color, off-flavor and off-odor which decreases the quality of the fruits, and therefore the products.

Peroxidases are known to be the most heat tolerant enzymes. This character has frequently been used to evaluate the efficiency of the heat-treatments applied to the products (Richardson and Hyslop, 1985). The other face of this fact is disadvantageous, because the excessive heat needed to
inactivate these enzymes, lead to other quality problems, such as decreasing the bio-availability of some nutrients and diminishing other contents like vitamins, darkening the color of the product (overcook effect) and changing the organoleptic properties of the products and therefore, the acceptability of consumers.

This enzyme is in need to be paid more attention as it contributes to the quality of the food products, and to cope with its growing industrial and analytical applications.

**Objectives of the study:**

The objective of this study was to extend the knowledge concerning plant peroxidases with special emphasis on their stability to thermal treatments. This in turn, requires that the following steps be successfully taken:

1- Determination of peroxidase levels in some fruits, namely, apple, orange, guava, and banana.

2- Determination of the stability of the crude enzyme extracted from the above mentioned four fruits under various storage conditions, (Freezing, fridge and room temperature).

3- Determination of the thermo-stability of the crude enzyme extracts under different conditions of pH, temperature and length of heating time.
CHAPTER TWO
LITERATURE REVIEW

2.1. Nature and general characteristics of enzymes:

Enzymes are biochemical catalysts, of a protein nature simple or compound, present in every living system. These catalysts are capable of increasing and accelerating the velocity of biochemical reaction in a rate of $10^6$ to $10^{12}$ times compared to the same reaction velocity in the absence of these catalysts without being used up or becoming a part of the product formed (Sumner and Somers, 1947). Rodwell (1985) claimed that, essentially all biochemical reactions are enzyme catalyzed.

Enzymes are chemical substances of organic nature and elaborated by plants, animals and microorganisms. They are affected by heat and chemicals regarding their pertinacious nature.

Most of the enzymes are soluble in the aqueous media, although they may sometimes be bound to the cells and liberated only after the autolysis or other treatments such as exposure to supersonic waves. Enzymes are not soluble in the fat solvents.

Enzymes accelerate the reaction velocity by lowering the activation energy for a certain reaction, this is attained through exceeding the number of the activated molecules, as reviewed by Dixon et al., (1983). Enzymes are very efficient, that one molecule of the enzyme can act on $10^2$ to $10^6$ of the substrate molecules per minute. The molecular weight of enzymes varies from one enzyme to another, some has low molecular weight of about $10^4$ dalton, but the majority of the enzymes are in the range of $1.5 \times 10^4$ to $10^6$ dalton.
2.1.1. Cofactors and Coenzymes:

As mentioned before, some enzymes are compound proteins, that imply the presence of a non-protein part attached to the protein part of the enzyme (apo protein), this conjugated protein is known as the holoenzyme. If the nature of the attached group is inorganic, it will be known as co-factor or prothetic group. Co-factors differs in the degree of binding to the apoenzyme, some can be easily hydrolyzed in the acidic medium, an example is the catalase, while some other metal ions bind strongly and do not separate from the apoenzyme unless the protein is denatured, an example is superoxide-dismutase which contains Cu++ and Zn++ ions. In some cases, these ions help in protecting the native structural conformation of the enzyme and then known as activators (Dixon et al., 1983).

Coenzymes are of organic nature, and usually dissociate and separate from the enzyme, and they should also be regarded as co-substrate as they- the coenzymes- contribute to the catalyzed reaction and be consumed with the substrate according to this equation:

\[ \text{E. C} + \text{AH}_2 \xrightarrow{\text{E. C. H}_2 + A} \]

it reacts with the substrate (AH$_2$) by another enzyme catalysis this reaction:

\[ \text{E. C H}_2 + B \xrightarrow{\text{E.C. + BH}_2} \]

Organic cofactors are exist in small and limited amounts, and a lot of them are derived from substances that mammals can not synthesize, so they are essential nutritional factors, these are vitamins, and they are very important in the metabolism reactions.
2.1.2. Specificity of enzymes:

Enzymes exhibit a markedable specificity for their substrates. The substrate binds non-covalently, sometimes covalently to the enzyme. The region of an enzyme that specifically interacts with the substrate is called the active site, which is a cleft in the tertiary structure of the enzyme. The amino acid sequence and stereo-structure of the substrate should be suitable for stereo-structure of the active site to allow this binding.

The catalysis involves formation of intermediate complex between the enzyme and the substrate before the final product is formed.

(Dixon et al., 1983)

\[
\begin{align*}
E + S & \quad \xrightarrow{K1} \quad ES \\
& \quad \xrightarrow{K2} \quad E + P \\
& \quad \xrightarrow{K3} 
\end{align*}
\]

2.1.3. Multi-enzyme forms and multifunctional complexes:

The multi-enzyme system is composed of three or more enzymes that bind tightly together with non covalent interaction, each of which catalyze a certain reaction in its original state. This grouped enzymes catalysis one accumulated reaction, an example is the pyruvate dehydrogenase. On the other hand, the multifunctional complexes, two or more enzymes are present separately in a peptide chain, each one of these enzymes catalysis one stage of the reaction whereas its product will be the substrate for the other enzyme in the complex and so on till the final product is produced, an example for it is the fatty acid synthetase (Dixon et al., 1983).
2.1.4. Classification, numbering and nomenclature of enzymes:

Initially, as reported by Rodwell (1985), enzymes were named by adding the suffix (-ase) to the substrate they act on, and many of these names remain in current use, but the expanded knowledge and the large numbers of newly discovered enzymes, necessitate the presence of a new systematic arrangements and nomenclature system, so that any given enzyme can be precisely identified. An enzyme commission, the International Union of Biochemistry (IUB, 1972) has adopted a comprehensive scheme, that classified enzymes in the bases of their chemical reaction and the reaction mechanism and so divided them into six classes, each of which containing from 4 to 13 subclasses. The first name was given to the substrate and the second, ending in (-ase) indicates the type of the reactions, these six classes are:

1- Oxidoreductases.
2- Transferases.
3- Hydrolyses.
4- Lyases.
5- Isomerases.
6- Ligases, (Synthetases).

For further identification, the enzyme commission, gave every enzyme a systematic code number of four digits. The first digit denotes the reaction type (main class). The second digit indicates the sub class. The third digit is for the substrate subclass while the fourth one is for the serial number of the specific enzyme. For example, the code number (E.C. 2.7.1.1.) denotes, main class 2 (Transferase), subclass 7 (Transfer of phosphate), sub class 1 (an alcohol functions as the phosphate acceptor), the fourth digit indicates hexokinase, or ATP: d- hexose-6- phosphotransferase.
2.1.5. Advantages of using enzymes:

Enzymes have several distinct advantages for use in the industrial processes:

i- They are of natural origin, therefore are nontoxic.

ii- They have a great specificity of action, hence, they can bring about reactions, not, otherwise easily carried out, specially without unwanted side reaction.

iii- They work best under mild conditions of moderate temperature and wide range of pH 2-10, thus, not requiring drastic conditions.

iv- They act rapidly at relatively low concentration and the rate of reaction can be readily controlled by adjusting temperature, pH and the amount of the enzyme employed.

v- They are easily inactivated when reactions has gone as far as desired.

2.2. Biochemistry of plant Peroxidases:

2.2.1. Nature of Peroxidase:

Peroxidases are poly-functional, heme-containing glyco-proteins, that are found in animals and plants as well as in microorganisms (Lepedus et al., 2004). Elshafie (1993) reported that, peroxidases were first discovered by Schobien (1863) and the name was given by Lionssier (1898). Peroxidases were discovered at first in the roots and sprouts of the higher plants but now they are known to be occurring in nearly all plant cells.
Peroxidase, POD E.C. 1.11.1.7, is the classic hematin peroxidase, and widely distributed in the plant kingdom (Gasper et al., 1982; Chibber and Huystee., 1984). They catalyze very specifically the reduction of \( \text{H}_2\text{O}_2 \), as an ultimate electron acceptor (Dawson, 1988), while the second substrate- the hydrogen donors- are in general not so specific, and in the reaction, the molecular oxygen is not a product of the reaction (Whitaker, 1972).

The molecular weight was reported to be a wide range (30-60000 Dalton) for the peroxidases extracted from various sources (Vămos-Vigyázó., 1981; Floris et al., 1984; Silva et al., 1991; Khan and Robison., 1993a; Clemente and Pastore., 1998).

Peroxidases are members of the oxidoreductases, which are a large group of enzymes catalyzing electron transfer through a series of mechanisms, and the catalytic mechanism involves the formation of two intermediates called compound I and II (Lepedus et al., 2004). This class includes beneficial enzymes that are added on purpose during the processing of some food products such as catalase, which eliminates the residual hydrogen peroxide after low temperature pasteurization of milk (Read, 1975). On the other hand, some members of this class, cause undesirable changes in the food products such as the enzymic browning which is caused by peroxidase and polyphenoloxidase, bleaching colors (lipoygenase), and destructing the ascorbic acid by the ascorbic acid oxidase.

2.2.2. Structure and reactions of peroxidase:

Peroxidases are heme enzymes, consisting of colorless protein (apoenzyme) combined with an iron-prophyrin. Whitaker (1972), reported that the iron in porphyrins has six co-ordination positions, four of which are taken up by porphyrin nitrogens and the fifth by a protein attachment. The sixth position can be occupied by water or some
other radical, and the enzyme appears to operate by changing the groups at this position. The enzyme is brown in color and contains one ferriprotoporphyrin \[ \text{III} \] (protohemin) group per molecule.

Peroxidase is an oxidoreductase that is capable of catalyzing oxidation reactions in the plants using either peroxides or oxygen as a hydrogen acceptor. The mechanism of peroxidase reaction is based on the formation of an enzyme-hydrogen donor complex (Walsh, 1979). Peroxidase catalysis is associated with four types of activation (Whitaker, 1972). These are peroxidatic, oxidatic, catalytic and hydroxylation reactions.

### 2.2.3. Classes of peroxidase:

Various types of peroxidase are exist. The properties of the enzyme depend to some extent, on its source. According to Whitaker, (1972), two main classes of peroxidase can be identified on the bases of their prothetic group, these are: The Iron containing peroxidase and the flavoprotein peroxidase.

1. The iron peroxidase is divided into two groups, the Ferriprotoporphyrin peroxidases, and this group include peroxidases from higher plants (horseradish, fig sap), from animals (Iodine peroxidase of thyroid) and from microorganisms (Cytochrome peroxidase of yeast). All these enzymes when highly purified are brown in color, and the prothetic group, the Ferriprotoporphyrin \[ \text{III} \], can be removed from the protein moiety on treatment with acidic acetone. The second group is verdo peroxidases, they are found in the milk (Lactoperoxidases) and mylocytes (myloperoxidases). The prothetic group is an ironprophyrin nucleous, but other than the Ferriprotoporphyrin \[ \text{III} \], and they are when purified, green in color.
II- The second class of peroxidases contains flavin-adenine dinucleotides (FAD), therefore, known as flavoprotein peroxidases. They are found in several animal tissues and in the streptococci.

2.2.4. Soluble and bound peroxidases:

Peroxidase enzymes are reported to be occurring in multiple molecular forms, and are present in most fruits and vegetables in soluble and bound (ionically and covalently) forms as reported by Tomáz et al., (1981); Wang and Lu.(1983); Moulding et al. (1987), (1989); Robinson et al. (1989), Silva et al. (1990), they reported that, their catalytic properties are influenced by variety and/or cultivar, growth and the physiological stage. Haard, (1973) found that the bound and soluble enzymes isolated from the pulp of ripening banana fruits were increased at the onset of the respiration climacteric. In contrast, the soluble peroxidase extracted from tomato (Kokkinakis and Brooks, 1979) and babaya fruits (Silva et al., 1991) as well as in peach fruits (Neves, 2002), reach a maximum peak followed by a marked decrease at the initial levels.

Civello et al. (1995) stated that, generally, peroxidase enzyme is found in glycosylated form and associated to the membrane, though soluble isoenzymes were also encountered in banana fruit (Haard and Tobin, 1971) and in tomato fruit (Thomáz et al., 1981). In strawberry fruit, peroxidase is mainly found associated to the membrane and in other systems, enzymes in both soluble and associated forms were found in comparable proportions (Civello et al., 1995). Some authors have described this enzyme as associated to internal membranes of chloroplast (Huff, 1982), to cellular walls, and to internal membrane of the tonoplast (Thomáz et al., 1981). Furthermore, an important activity of soluble peroxidase were encountered in orange flavedo (Chamarro and Molina., 1989), rice leaf (Kar and Mishra., 1976), pineapple fruit
(Van Lelyveld et al., 1991), tomato (Thomáz et al., 1981), apple (Gorin and Heidema, 1976) and banana (Haard and Tobin., 1971).

The soluble peroxidase form is extracted with low ionic strength buffer from the macerated plants, while buffers of greater ionic strength are needed to extract the ionically bound form (Gkinis and Fennema, 1978).

To solubilize the ionically bound form, the additions of various salts, such as CaCl2 and MgCl2, to the buffer are needed to release the enzyme from the cell components (Neves, 2002). The treatment of the macerated cells with cellulase and/or pectinase has been claimed to separate the covalently bound peroxidase (Gkinis and Fennema., 1978).

2.2.5. Intracellular distribution of peroxidase:

The presence of peroxidase has been described in different plant parts, including climacteric and non-climacteric fruits (Haard and Tobin, 1971; Gorin and Heidema., 1976; Marangoni et al., 1989; Chamarro and Molina, 1989; Biles and Martyn, 1993).

Read (1975) stated that, unlike many of the digestive systems, peroxidase is usually intracellular as are the other oxidoreductases. Peroxidases are also located in the ribosomes (Darimont and Baxter, 1973), in the mitochondria (Haard, 1973), cell wall (Jansen et al., 1960; Ranadive and Haard., 1972; Bircka and Miller, 1974), intracellular space (Haard and Timple, 1973), protoplast (Bircka and Miller, 1974), endosperm (Laberge and Kruger, 1976), parenchyma cell (Ronadiv and Haard, 1972; Sergeeva et al., 1984), and in the vacules of fleshy fruit epidermal tissues (Perrey et al., 1989; Blom et al., 1991; and Ferrer et al., 1992).
2.2.6. Diversity of plant peroxidases:

Isoenzymes are the result of different gene expressions, which provide products of different chemical composition but are having, to some extent, the same characteristics and activity. Plants POD are reputed for their large and variable number of isozymes (Gaspar et al., 1982). The isozymic profiles are often flexible and can reflect genetic diversity, posttranslational modifications, physiological conditions, and extraction artifacts. Furthermore, this apparent intra-species diversity is complicated by intra-species variations (Simon, 1993). Lately, more than forty gene and c-DNA sequences, entire or partial, have been reported from 17 different plants, and their number is still increasing, and by completing the analysis of peroxidase structure-function relationships with primary structure data of cloned POD. Welinder (1992), has developed the concept of a peroxidase super-family, constituted of plant, fungal and bacterial heme-containing enzymes, two types of plant peroxidases have been established:

1- The classical secretory enzyme, guaiacol peroxidase, routed through secretory pathway to the vacules or to the extra-cellular space, showing low substrate specificity.

2- The enzyme of the prokaryotic lineage, ascorbate peroxidase, residing in the cytosol and apparently also in the chloroplast, showing relatively high affinity for ascorbate.

The expression of distinct isoenzyme is specific to particular organs, tissues or cell types. The relative and absolute abundance of the POD protein displays the same kind of variation. Thus, the profile of isozymes and the total amount of activity in the leaf may be vastly
different from that in the stem or flower of the same plant (Gijzen, 1997). Roots and seeds, the epidermal cells of all tissues, and the tissues responding to stress or pathogen attack, are often found to be rich sources of the enzyme. The differential expression of peroxidase isozymes is possible because these enzymes are typically represented by large gene families in most plant species (Thordal-Christensen et al., 1992; Gadea et al., 1996). The expression of discrete peroxidase genes is therefore directed by regulatory sequences that may be highly divergent among the various members of the gene family. Another consequence of multi-gene families is that, the individual members are free to evolve independently and may be recruited to perform new functions under favorable selective pressures. The seed peroxidases are regarded in this context. The accumulation of peroxidases in the seed tissues most likely reflects an adaptation that serves different purposes depending on the plant species under consideration (Gijzen, 1997). The peroxidases that found in higher plants, include isoenzymes with basic, neutral, or acidic isoelectric points (Gijzen, 1997).

The chemical composition of the different isoenzymes is different. Welinder, (1992) stated that, the amino acid composition varies distinctively between the isoenzymes, and a large number of peroxidases differ by more than 50% in the amino acid sequence.

2.2.7. Factors influencing the expression of the isoenzymes and affecting peroxidase activity:

It is well known that, enzymes are sensitive to some factors such as enzyme concentration, substrate concentration, temperature, pH, presence and absence of inhibitors and activators. Many reports showed the stimulation of guaiacol peroxidase activity and the expression of new isoenzymes in different plant species (Lepedus et al., 2004), in response to various influences such as the treatment with ozone and simulated acid rain (Scalet, 1995), elevated deposition of fluorides
(Keller, 1974), SO₂ treatments (Fanz et al., 1993), or high sulfur and heavy metal loads (Van Assche and Clijster., 1990; Roitto, et al., 1999). During tissue aging, the activity observed to be increased as reported by Gaspar et al. (1991). The mechanical stimulation (rubbing), was also found to affect the evolution of peroxidase activity and vessels lignification in tomato plants.

2.2.8. Physiological role of plant peroxidase:

Peroxidase enzyme has been implicated in a variety of physiological processes such as ethylene biosynthesis (which is involved in many processes in plant growth and senescence), cell development, membrane integrity, response to injury, and disease resistance (Vàmos-Vigyàzo.,1981;Hammer Schmidt.,1984;Abeles and Biles., 1991; Gillikin and Graham., 1991; Haard., 1997), the participation of POD in the late stages of lignin forming process (Mäder and Füssi.,1982; Wakamatsu and Takahama.,1993), the relationship with respiratory control, gene control and hormone metabolism are reported by Haard (1977). The enzyme was found to participate in various oxidative processes and in the degradation of auxins (Normanly et al., 1995).

2.2.9. Thermal inactivation and regeneration of peroxidase:

Thermal treatment is the most utilized method for stabilizing foods because of its capacity to destroy microorganisms and to inactivate enzymes. The optimum temperature for most of the enzyme reactions usually lies between 30 °C and 50 °C, and being pertinacious in nature, enzymes are therefore thermo liabile. Braverman (1974) had stated that it is sufficient to apply temperature of 70 °C to 80 °C for two to five minutes in order to destroy most of the enzymes. This fact had been widely used in food industry, but the study
of peroxidase thermal inactivation has proved that peroxidase is a stable enzyme when undergoing thermal treatments, (Gibriel et al., 1979; Baardseth and Slinde, 1980). Halpin et al. (1989) and Polyák-Fehér (1992) stated that, this character can be used as a measure of adequate blanching process. Both blanching and dehydration are thermal treatments commonly applied primarily for fruits and vegetables as a mean of food preservation, however, while blanching is considered a preprocessing treatment, dehydration is considered to be the terminal heat process in itself. Blanching is a fairly mild heat treatment which involves exposing the plant tissue to some form of heat, usually steam or hot water, for a prescribed time at a specific temperature, and it is done prior to canning, freezing and dehydration operations to inactivate the undesirable enzymes present in fruits and vegetables and to reduce the microbial load. The target enzyme for blanching is the peroxidase as it is reputed to be the most heat resistant enzyme and was reputed to have a relationship with food deterioration specially for color and off-flavor, although, Williams et al. (1986) had reported that lipoxygenase rather than peroxidase is the primary causative enzyme in developing off-flavor in English green peas, and that this enzyme is more heat sensitive than peroxidase, while Ganthavoran et al. (1991) compared the thermal stability of peroxidase and lipoxygenase in the asparagus and concluded that lipoxygenase in asparagus is more heat stable than peroxidase, and therefore, the heat treatment sufficient to inactivate peroxidase in the asparagus may not be sufficient to inactivate the lipoxygenase in the asparagus which assures the importance of the independent evaluation of the enzyme stability from different sources. Civélllo et al. (1995) also stated that, peroxidase is considered a stable enzyme when undergoing thermal treatment. However, the resistance to treatments depends on the
source of enzyme. Moreover, in a given source it varies from one isoenzyme to another. Pomer et al. (1997) has stated the same result, they declared that, the different isoenzymes showed different resistances to heat treatment making it necessary to evaluate the thermo-stability of each one separately. Haplin et al. (1989) claimed that, the basic isoenzymes in the green peas were much more thermally resistant than other neutral isoenzymes, also present in the system. Civéllo et al. (1995), who studied the thermal stability of the peroxidase from the strawberry fruit stated that, two peroxidase isoenzymes were detected and they are basic ones, and that their thermal stability was greater than that described for cauliflower (Lee et al., 1984) and than those reported for two basic isoenzymes present in green peas (Halpin et al., 1989).

Agostini et al. (1997) had reported that, the anionic isoenzyme (A₂) which was isolated from the turnip roots has exhibited great thermo-stability like the commercial horseradish (HRPC) and the extracellular isoenzyme from cell suspension of cowpea. The author has also claimed this high thermo-stability is advantageous in number of application.

Cano et al. (1990) studied the effect of some thermal treatments on the activity of the peroxidase and the polyphenoloxidase of banana fruit. The authors have reported that, the blanching of peeled banana in boiling water produced significant inactivation of both isoenzymes (96% - 100%), and that this effect was apparently not related to fruit maturity. The treatment conditions were drastic and produced a slight over-cooking of the final product, but this treatment is necessary to inhibit color deterioration of frozen banana slices if the simultaneous of chemical preservative is to be avoided. This thermal treatment was employed by Garcia et al. (1985) as a pretreatment in the processing of
banana pulp stored at tropical temperatures. The thermal inactivation was greater for the polyphenoloxidase than for peroxidase and this effect was constant with the accepted idea that the peroxidase is more thermally stable enzyme in vegetables (Read, 1975).

Some heat treatment other than boiling water was used by the same authors, and they stated that the microwave treatment showed different effects on the two enzymes activities depending on banana maturity level at the processing date. The thermal inactivation of peroxidase with this method produced greater inactivation of peroxidase reaching smaller peroxidase values after 20 days of storage and produced non-enzymic darkening due to Millard reaction mainly in green and full yellow bananas. These results agree with the hypothesis reported by Garcia et al. (1985) which concluded that the greater starch content will reduce heat transfer rates during thermal treatments diminishing its effectiveness.

The heat, being beneficial in destroying enzymes and microorganisms may alter as well, many organolyptic properties of the food and diminishing the bioavailability of some nutrients, so there is a growing interest in searching for methods able to reduce the intensity of the heat treatments needed for food preservation. Garcia et al. (1991) had proved that the simultaneous use of heat and ultrasonic waves could reduce substantially, at the atmospheric pressure and temperature in the range of 70°C to 90°C, the heat resistance of two strains of Bacillus subtilis. This combined treatment so called mono-thermo-sonication (MTS) was used by López et al. (1994) in the inactivation of peroxidase, lipoxygenase and polyphenoloxidase. A synergistic effect which can substantially reduce the enzyme resistance and the heat treatment required for inactivation was observed in each case, and the enzyme destruction efficiency of the combined process greatly increases with
ultrasonic wave amplitude. They also stated that this combined treatment could help to solve, in milk, and other drinks, problems caused by the thermo stable enzymes.

Several authors claimed that the heat inactivation of peroxidase is biphasic, that the heat inactivation curves showed two almost linear sections of different gradient corresponded to an initially rapid inactivation followed by a second phase of a smaller rate of decent. Among these authors are: Winter, (1971); Ling and Lund, (1978); Delincee et al., (1979); Henley and Sadana, (1985); Chang et al., (1988); Ganthavoran, (1991). This phenomenon was generally accepted to be due to the presence of isoenzymes of different heat stabilities (Winter, 1971; Henley and Sadana, 1985). On the other hand, Winter, (1971) explained the deviation of peroxidase thermal inactivation from the first order Kinetics were due to formation of enzyme aggregates with different heat stabilities.

Lu and Whitaker (1974) suggested that for peroxidase heat inactivation, the mechanism involves the release of the heme moiety previous to the denaturation of the libranted apoprotein. Following heat inactivation of peroxidase, regeneration of activity can occur after sometime. This phenomenon as explained by Braverman (1974) the regeneration was attributed to a reversible denaturation of protein. The deterioration of the processed food was related partially to this phenomenon. Hemeda and Klein (1991) studied the inactivation and regeneration of peroxidase activity in vegetable extracts treated with some antioxidants. They used quercetin, rutinic acid, chlorogenic acid and tocopherol with and without heat treatment. The results of the combined treatment were almost complete inhibition of the peroxidase. The regeneration in both heated and treated with antioxidants was less than those only heated or antioxidants- treated and the
regeneration after the frozen storage was zero in heated and antioxidant treated extracts. The authors have related the effectiveness of this combined treatment to two factors. First, the solubility of the antioxidants in general was greater in the heated solutions, which may have increased the inhibitory effect. The second, the peroxidases which were used in this study contains Ferriprotoporphyrin III (hematin). The heat treatment caused denaturation of the protein and resulted in the separation of the hematin, and in the presence of antioxidant, the oxidation of the heme group could be prevented and no peroxidase –Fe Free radical complex would be formed.

2.2.10. Effects of peroxidase on the quality of raw and processed fruits and vegetables:

The study of the peroxidase in food has attracted interest because of its capacity to modify food in both desirable and undesirable ways. Deterrents of flavor, color, texture and nutritional value (Fils et al, 1985), can be contrasted with the development of desirable texture through the formation of protein cross-linking (Matheis and Whitaker, 1984). The physiological role of POD in post-harvest of fruits and vegetables have been reviewed by several authors (Gorin and Heidema., 1976; Haard and Tobin, 1977; Rhotan and Nicolas, 1989 ; Miesle et al., 1991). POD had been involved in determinative changes in flavor, texture, and color of raw fruits and vegetables (Haard, 1973; Burnette, 1977; Vámos Vigyázo, 1981; Clemente and Pastor., 1998). Color, is the first quality attribute that attracts the consumer, since POD is involved in color changes or deterioration, so it will affect the quality and the acceptability of fruits and vegetables. Enzymatic browning is one of the most devastating reactions for many exotic fruits and
vegetables, in particular in tropical and subtropical varieties and it is estimated that over than 50% losses in fruits occur as a result of enzymatic browning (Whitaker and Lee, 1995). Osman ,1993, reported , from the work of other authors, that the anthocyanin pigments could be discolorized by POD. Ferrer et al. (1990) clearly demonstrated that, cell wall PODs catalyses co-oxidation of IAA and xanthene dyes.

Cano et al., (1990) declared that, banana fruits undergo rapid browning as a result of tissue disruption and exposure to oxygen during peeling and slicing operations prior to further processing. They stated that, POD catalyses the oxidation of phenols, and produce changes in raw fruits color, and during processing and storage can also produce significant quality loss of frozen fruits . Cano et al. (1990) found that, the activity of banana POD increases throughout the ripening and senescence stage, and the most anionic isoenzymes are liberated from the cellular membrane, therefore, deterioration of this membrane progress significantly. POD and the polyphenoloxidase (PPO), during senescence, were found to play a primordial role in fruit metabolism which leads to the darkening of peel and fruit (Palmer's, 1971). Oxidation, especially following tissue injury by freezing, apparently is responsible, at least partially for discoloration, browning, loss in flavor and production of certain objectionable unnatural flavor, which occurs during freezing, storage and thawing of fruits and vegetables were reported by Elshafie,1993 from the work of other authors, who also found that, some fruits such as pineapple and oranges are also subjected to deterioration during storage.

Darkening of freshly cut surfaces of apples is a reaction that is catalyzed by peroxidase in the presence of the air . Elshafie (1993),
from the work of other authors reported that, for the prevention of both fruit tissue and juice darkening, the inhibition of peroxidase is, therefore, of great importance in delaying the discoloration of the cut fruits.

Peroxidase may also contribute to color loss indirectly, the fall of the pH in the High Temperature Short Time, (HTST) processed green pea puree during storage as reported by Buckle and Edward (1970), since the acidity can result in the hydrolysis of the chlorophyll pigments to pheophytin leading to discoloration.

Many fruits and vegetables which are not properly blanched develop a very noticeable off-odor and off-flavor. With accordance to its high resistance to heat, and the ability to regenerate, peroxidase is able to destroy products quality in the long term storage, and there is a general agreement that peroxidase enzyme should be suppressed in order to maintain good quality of the products.

Peroxidases are involved in the metabolism of phenolics, which are plants secondary metabolites and has been characterized as potential causes of instability (George et al., 1992), as they are involved in the formation of undesirable sediments in juice products (Heatherbell, 1984), and formation of yellow and brown pigments (Montgomery, 1983).

The association of the peroxidase enzyme with off-flavor development in the products during the storage of the frozen, canned and dehydrated foods have been reviewed by Osman (1993). According to him, that, in the same sample after storage following blanching, although there was regeneration of peroxidase, undetectable off-flavor was found. Mitchell and Rutledge (1973) observed that, it is possible to reduce peroxidase activity to a level at which no noticeable quality change during
frozen storage of the blanched potatoes. Burnette (1977), stated that a small amount of residual activity of peroxidase, 1-5% for specific product may or may not cause deterioration in the canned products but not in the frozen vegetables.

Peroxidase undergoes undesirable losses of quality other than discoloration and off flavor. Stevensson (1978) reported that, the heme group in the peroxidase structure may catalyze unspecific reactions leading to quality loss. The drop of the pH and the rising acidity in the processed food are reported to contribute to the discoloration and the product may be susceptible to mould growth. Haard (1977) reported that, the role of peroxidase in post-harvest may deteriorate the texture of certain fruits and vegetables lignification for example, may be controlled by POD can lead to loss of the quality of fruits and vegetables following harvesting.

Enzymes promote and catalyze a large number of different chemical processes take place in cellular metabolism, the enzymes allow these processes to occur rapidly at the physiological temperature and concentration. Peroxidases are involved in a series of biosynthetic and degradative functions in particular, phenolics (Berlin and Braz, 1975) and alkaloids metabolism (Perrey et al., 1989; Bloom et al., 1991).

**2.2.11. Applications of peroxidase:**

It has been well established that, POD has very distinct characteristics-being the most heat tolerant enzyme together with the ability to regenerate, the wide pH range under which the enzyme can operate, the various substrates and chemicals which can be modified by the catalytic activity of the enzyme via different types of reactions, these characteristics has qualified this enzyme to be widely used in various clinical and analytical applications as well as in some other industries.

Peroxidases, has been extensively used as important reagents in the clinical diagnoses and enzyme immuno-assay. Various enzymatic assays and several novel applications have been
suggested including the treatment of waste water containing phenols and aromatic amines (Klibanov et al., 1980; Lobarzewski and Ginalask., 1995; Wu et al., 1998) and their use as catalysts for phenolic resin synthesis (Dordick et al., 1987). This enzyme also can be used in bio-bleaching processes and lignin degradation in fuel and chemical production from wood pulp, or in the production of dimeric alkaloids, oxidations, bio-transformations of organic compounds (Macek et al., 1993). Mc Eldon and Dordick (1993) had related this great diversity of applications in large part to the wide substrate specificity of peroxidase catalysis.

Horseradish roots are the traditional source of commercial production of peroxidase (Krell., 1991). As new applications of peroxidase are being explored, their grow the need for other sources for the enzyme. Agostini et al. (1997) has introduced another source for the enzyme, the peroxidase anionic isoenzyme (A2) from turnip roots. This isoenzyme exhibited a great thermal stability and proved to retain its full activity for at least six months of storage at 0-4 °C. The author stated this isoenzyme (A2) could be used as a reagent for clinical diagnosis or in the enzyme immunoassay technique (EIA technique) and another application of this isoenzyme could be the enzymatic determination of serum metabolites such as uric acid. Chatterjee at al., (1999) introduced the cucumis melo hairy root as a source of the enzyme.
CHAPTER THREE
MATERIALS AND METHODS

3.1. Materials:
3.1.1. The horticultural crops:
Four fruits of unspecified varieties, namely apple, guava, orange and banana were obtained from the local market at commercial maturity. Undamaged fruits, which were free from infection were selected, washed and kept in the fridge (at about 4°C) till the time of the experiment. Banana and orange were peeled just before the time of the experiment while apple and guava were not peeled. The fruits were cut into small pieces before being weighed and used as an enzyme source.

3.1.2. Chemicals:
All chemicals used in this study were of analytical grade.

3.1.3. Preparation of substrates:

3.1.3.1. The hydrogen peroxide solution:
Solutions of hydrogen peroxide were freshly prepared from 35% (W/V) hydrogen peroxide (analar grade), which had previously been stored under refrigeration, in an appropriate buffer.

3.1.3.2. The guaiacol solutions:
Guaiacol solutions were prepared in 0.01M phosphate buffer at the required pH values i.e. at 0.5, 6.0, 7.0 and 8.0.

3.1.4. Preparation of buffer solutions:
Sodium phosphate buffers were prepared from 0.1M solution of monobasic sodium phosphate (13.8g NaH₂PO₄·2H₂O in 1 L), and 0.1M
solution of dibasic sodium phosphate (26.8g Na$_2$HPO$_4$ in 1 L). These solutions were mixed and adjusted to the required pH values.

**3.2. Methods:**

**3.2.1. Extraction of peroxidase:**

Extraction of crude soluble peroxidase from each of the four fruits (apple, guava, orange and banana) was performed according to the method described by Civélllo et al., (1995) with minor modifications.

Sixty grams of each fruit which had been prepared as was described in section 3.1.1. The weighed fruits were homogenized in a blender with 100 ml ice-cold 0.01M phosphate buffer at pH 5.0 for three minutes. The homogenate was filtered through a double layer of cheesecloth. The filtrate was centrifuged at 15,000 r.p.m for 20 minutes at 4 °C using Heraeus Sepatech Suprafuge 22 centrifuge.

The supernatant was collected and retained for further analysis. The same extraction procedure was repeated at the other investigated pH values i.e. at 6.0, 7.0 and 8.0.

**3.2.2. Enzyme assay:**

Peroxidase activity was determined by a method based on monitoring the decomposition of hydrogen peroxide by peroxidase with a suitable hydrogen donor by measuring the rate of the color development at a suitable wave length.

Guaiacol method of assay is one of the most favorable methods for assaying peroxidase, which involves the oxidation of guaiacol with peroxidase and H$_2$O$_2$ and a tetruguaiacol compound is formed. This compound, tetruguaiacol, has a red brown color which can be easily determined spectrophotometrically.
In this study, guaiacol method was used for assaying peroxidase activity. The enzyme assay was carried out at room temperature, and the measurements were performed using a (JENWAY 6305 UV/VIS) spectrophotometer at 470 nm. The substrate for this enzyme, \( \text{H}_2\text{O}_2 \) as a hydrogen acceptor and guaiacol as hydrogen donor, were prepared in sodium phosphate buffer solutions as described in section 3.1.4. at the desired pH values.

The reaction mixture for this method of assay, consists of 0.1 ml guaiacol solution, 0.1 ml hydrogen peroxide solution (35% W/V), and 99.8 ml of 0.01 M phosphate buffer at each of the investigated pH values.

The spectrophotometer was calibrated using a reference cuvette,
into which, 2.9 ml of the reaction mixture mentioned above were pipetted, and 0.1 ml of 0.01M sodium phosphate buffer was added to make total volume 3 ml in the cuvette, and then the instrument was set to the zero at 470 nm. Into sample cuvette, 2.9 ml of the reaction mixture were pipetted.

The reaction was initiated by the addition of 0.1 ml enzyme solution (to make a total volume of 3ml), to measure the activity of the enzyme in each sample. The activity of the non heat-treated enzyme, was considered to be the enzyme maximum activity i.e. the 100%activity of the enzyme. The residual activity of the heat treated samples, was determined with the same procedure, and was expressed as a ratio to the maximum activity of the enzyme and was presented as percentage.

**3.2.3. Extract stability:**

In order to investigate the ability of the enzyme to retain its activity at different temperatures, the crude enzyme extracts (all at pH 5.0), from the four tested fruits, were kept at different conditions of temperature including freezing temperature (-18°C), fridge temperature (4°C) and room temperature (30°C). The activities of the extracted enzymes were determined every day for a period of eight days.

**3.2.4. Thermal inactivation:**

Heat inactivation experiment at pH 5.0 were carried out in the same manner described by Elshafie.,(1993), at four temperatures (60°C, 70°C, 80°C and 90°C) for various time intervals 2, 4, 6, 8 and 10 minutes. Enzyme extracts were diluted 1 in 10 (V/V) with 0.01 M sodium phosphate buffer. The same procedure was repeated at the other investigated pH values (5.0, 6.0, 7.0 and 8.0).
Aliquots (0.2ml) of the enzyme solution were pipetted into glass test tubes of the same diameters and covered with cotton. The test tubes containing enzyme solution were transferred to a thermostated waterbath, which was previously set at the desired temperatures (60°, 70°, 80° and 90° C). At different time intervals (2, 4, 6, 8 and 10 min) the test tubes were removed immediately and cooled in an ice -cold water and stored at -18°C till time for assay. The procedure was repeated at the other investigated pH values (6.0, 7.0 and 8.0).
Peroxidase is an ubiquitous enzyme in living cells, and its presence has been described in almost every cell of the different plant parts, moreover, it can be produced as a response to different stimuli. The high heat tolerance and the wide range of pHs which POD can work at, the ability to regenerate, all these factors led to the fact that, peroxidase is the most important enzyme in raw foods as well as in processed products. Furthermore, its relation to food quality particularly flavor and color (Burnette, 1977), is evident.

For enzyme characterization, in the present study, some variables (level of the enzyme from the different sources, temperature, heating time, pH, and the thermal stability) were analyzed on the crude soluble peroxidase extracts obtained from unspecified varieties of apple, orange, guava and banana fruits.

4.1. Peroxidase levels:

During the course of this research, extraction and the subsequent examination of the properties of peroxidase enzyme obtained from unspecified apple, orange, guava and banana varieties was performed, and among the three distinct POD fractions (Gkinis and Fennema, 1978), soluble and bound (ionically and covalently), only the soluble fraction was considered in this study, as it was reported to be the dominant form in many plant tissue (Mohamed., 1983; Elamin., 1987; Elshafie., 1993; Osman., 1993; Neves., 2002).

Extraction of the soluble peroxidase from the four fruits at pH 5.0 was done as was described in section 3.2.1. using low ionic strength buffer (0.01M phosphate buffer). The activity was determined spectrophotometrically. The levels of the soluble peroxidases were
reported in Table 4-1 as units of the activity per ml enzyme extract. The level of POD for each sample was an average of three determinations.

**Table 4 - 1: Peroxidase activity of the investigated fruits:**

<table>
<thead>
<tr>
<th>PH values</th>
<th>POD activity (U ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Apple</td>
</tr>
<tr>
<td>5.0</td>
<td>5.45</td>
</tr>
<tr>
<td>6.0</td>
<td>5.92</td>
</tr>
<tr>
<td>7.0</td>
<td>5.64</td>
</tr>
<tr>
<td>8.0</td>
<td>5.55</td>
</tr>
</tbody>
</table>

- Each value is an average of three measurements.
- Enzyme activity was determined by guaiacol method of assay.
- One unit of peroxidase activity (U) is defined as a change of one absorbance unit (ml) per minute.

The Results show that, POD enzyme was found in all investigated fruits though with different levels. The presence of this enzyme in many fruits and vegetables was observed by many workers (Gorin and Hiedema ,1976; Haard.,1977; Müftügil.,1985; McLellan and Robinson.,1987; Rhotan and Nicolas ., 1989; Miesle et al.,1991; Neves.,2002; Llano et al.,2003). From the results here obtained, it can be clearly seen that, the level of the enzyme varies considerably between the investigated four fruits, and the lowest level achieved was at PH 5.0 for the four fruits. Banana, apple and guava were found to contain high POD levels at all investigated pH values, higher levels were found at pH 6.0 and 7.0 with the highest activity being at pH 6.0. Relatively low levels were observed for orange POD at the four investigated pH values. The variation of POD level and activity was reported to be affected by several factors, the source of the enzyme
(Müftügil., 1985; and Civello et al., 1995), the ripening stage of the fruits, POD activity has been shown to increase with the ripening of the fruits and gradually falls as the fruits turn from ripe to senescence stage, and that, the maximum specific activities were found at "small green" and "large green" ripening stage (Palmers., 1971; Haard., 1977; Flurkey and Jen., 1978; Kokkinakis and Brooks., 1979; Silva et al., 1990; Cano et al., 1990; Civello et al., 1995; Neves., 2002). The pH value also found to affect the enzyme activity (Mohamed., 1983; Halpin et al., 1989; Silva et al., 1990; Civello et al., 1995; Bestwick et al., 1998; Neves., 2002).

4.2. Extract stability:

In this work, the stability of the crude soluble peroxidase extracted from the unspecified varieties of apple, orange, guava and banana under different storage conditions was tested. The extraction of the soluble fraction performed as described in section 3.2.1. with low ionic strength sodium phosphate buffer (0.01 M) at pH 5.0 from the four fruits, the activity was determined spectrophotometrically at 470 nm and the results were expressed as percentage of the original activity (activity of the enzyme at the first day of the extraction at room temperature). Results are shown in table (4 - 2) and figures (4 - 1), (4 - 2), (4 - 3) and (4 - 4) for apple, orange, guava and banana, respectively.

Figure (4 - 1) represents the results of keeping the crude soluble peroxidase extracted from apple fruit at pH 5.0. (Appendix-1). From the graph it can be clearly seen that, the enzyme has kept 100% of its activity after 8 days of storage at -18°C. The enzyme has lost about 16% of its original activity when stored at 4°C, as the residual activity detected at the 8th day was 84%. The enzyme activity was 54% in the 8th day of leaving at the room temperature (about 30°C) i.e. the
enzyme has lost 46% of its original activity.

Figure (4 - 2) represents the results of the stability of the soluble peroxidase extracted from orange fruit at pH 5.0. (Appendix-2). The enzyme has retained its full activity at -18°C, but it lost about 19% of its activity when kept at 4°C after 8 days as the activity detected was 81%. A 50% activity was detected after 8 days when kept at the room temperature and the loss of activity was therefore 50%.

Figure (4 - 3) represents the results obtained from guava peroxidase (Appendix-3). The results declare that, the enzyme activity was 100% at the 8th day at -18°C, and a percentage of 83% residual activity was detected when kept at 4°C for 8 days as the loss of activity was 17%. The loss of activity was greater when stored at room temperature. The enzyme has lost 51% of its original activity which is more than half its activity at the 8th day.

Figure (4-4) represents the results obtained from the banana peroxidase (Appendix-4). As in the previous cases, at -18°C the enzyme activity was 100% after 8 days. The activity was lightly affected when kept at 4°C as the detected activity of the crude enzyme in the 8th day was 86%, only 14% of the activity was lost. The results of the keeping under room temperature were incomplete because the crude extract of the enzyme was turbid in the 3rd day of the storage.

From these results, it is obvious that the enzyme is quite stable under freezing conditions for the four fruits, as the enzyme has retained its full activity at the freezing condition for at least 8 days of keeping at 18°C. The enzyme has shown to be reasonably stable under refrigeration as the loss of activity was less than 20% for the four fruits after 8 days of storage (see Appendix)
Fig 4-1: Apple Extract Stability at pH 5.0

- Each value is a mean of three determinations
- Each value is a mean of three determinations.
Fig 4-3: Guava Extract Stability at pH 5.0

![Graph showing remaining activity (%) over time at different temperatures]

- Each value is a mean of three determinations.
Fig 4-4: Banana Extract Stability at pH 5.0

-Each value is a mean of three determinations.
but the loss of activity was higher, about 50% of the original activity when stored at room temperature.

These results are similar to the results obtained by Elshafie (1993), from the study of the soluble peroxidase extracted from potatoes and sweet potato. The author stated that, the enzyme shown to be stable when kept under refrigeration for at least 8 days of storage, loosing only 25% and 23% of its original activity for potato and sweet potato respectively. This finding support to an earlier work reported by Mohamed (1983) and Elamin (1987) for potato and sweet potato respectively. However, the results differ from those reported by Agostini et al (1997), who reported that the purified peroxidase anionic isoenzyme \( A_2 \) from turnip roots has exhibited a great thermo-stability and has retained full active for at least six months during storage at \( (4^\circ C) \). The enzyme had shown to be less stable when stored at room temperature but no supporting data found for storage of crude enzyme extract at room temperature.

4.3. Thermal inactivation of peroxidase:

It is well known that, peroxidases are the most heat stable enzymes when undergoing thermal treatments. This character is advantageous in reflecting the adequacy of the blanching processes (Halpin et al., 1989; Polyák-Fehér et al., 1992). However, the resistance to these treatments depends on the source of the enzyme, moreover, in a given source it varies from one isoenzyme to another (Civéllo et al., 1995). Halpin et al (1989) stated that, the basic isoenzymes were much more thermally resistant than the other neutral isoenzymes, also present in the system.

The present study has undertaken the effect of the three factors temperature, heating time and different pH values on the rate of the
heat inactivation of the soluble peroxidase enzyme extracted from apple, orange, guava and banana fruits. The similar trends were regarded in the extraction and the subsequent treatments for the four enzyme extracts.

Thermal inactivation of the POD from the four fruits under study was performed as described in section 3.2.4.

4.3.1. Thermal inactivation of apple peroxidase:

Heat inactivation of apple POD was done as was described in section 3.2.4. Results obtained are presented in Table (4 - 3), reflecting the residual activity after heat-treatments. The kinetics of heat inactivation of apple POD are plotted in figure (4 - 5), (4 - 6), (4 - 7) and (4 - 8) at the different investigated pH values (5.0, 6.0, 7.0, and 8.0). From the results obtained, it can be clearly seen that, the rate of loss of activity is temperature-time dependant. Initially, the inactivation rate was rapid followed by a much slower inactivation rate. For instance, heating at 60°C for 10 min resulted in residual activity of 57.0%, 61.9%, 60.2%, and 58.0% at pH values of 5.0, 6.0, 7.0 and 8.0 respectively. This pattern was more or less true for the other temperatures. It worth noting that, the maximum stability of the enzyme found at pH 6.0. The enzyme has lost approximately 80% after 2min exposure to 80°C, and 90% of its activity after 2 minutes exposure to 90°C both at pH 5.0, and was totally inactivated after 4 min. At the other investigated pH values, total inactivation of the enzyme was accomplished in 6 – 8 min of exposure to 90°C.
Table 4 - Thermal inactivation of apple peroxidase:

<table>
<thead>
<tr>
<th>a) pH 5.0</th>
<th>% Remaining peroxidase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
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</tr>
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</tr>
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<table>
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<th>b) pH 6.0</th>
<th>% Remaining peroxidase activity</th>
</tr>
</thead>
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</tr>
<tr>
<td>90°C</td>
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<table>
<thead>
<tr>
<th>c) pH 7.0</th>
<th>% Remaining peroxidase activity</th>
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<tr>
<td>90°C</td>
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<table>
<thead>
<tr>
<th>d) pH 8.0</th>
<th>% Remaining peroxidase activity</th>
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</thead>
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<tr>
<td>90°C</td>
<td>100</td>
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50
Fig 4-5: Thermal Inactivation of Apple Peroxidase at pH 5.0

- Each value is a mean of Three determinations.
Fig 4-6: Thermal Inactivation of Apple Peroxidase at pH 6.0

- Each value is a mean of Three determinations.
Fig 4-7: Thermal Inactivation of Apple Peroxidase at pH 7.0

- Each value is a mean of three determinations
Fig 4-8: Thermal Inactivation of Apple Peroxidase at pH 8.0

- Each value is a mean of three determinations
4.3.2. Thermal Inactivation of Orange Peroxidase:

Heat inactivation of orange POD was done as was described in section 3.2.4. Results obtained are presented in Table (4 - 4), reflecting the % residual activity after heat-treatments. The kinetics of heat inactivation of orange POD are plotted in figure (4 - 9), (4 - 10), (4 - 11) and (4 - 12) at the different investigated pH values. From the results obtained, it can be clearly seen that, the rate of loss of activity is temperature-time dependant. Initially, the inactivation rate was rapid followed by a much slower inactivation rate. For instance, heating at 60°C for 10 min resulted in residual activity of 54.1%, 62.3%, 61.9% and 56.1% at pH values of 5.0, 6.0, 7.0 and 8.0 respectively. This pattern was more or less true for the other temperatures. It worth noting that, the maximum stability of the enzyme was found at pH 6.0. Orange POD showed noticeable reduced thermal stability among the four fruits under study. The enzyme has lost activity after 4, 6, 6 and 5 min exposure to 90°C at pH values of 5.0, 6.0, 7.0 and 8.0 respectively.
Table 4 - Thermal inactivation of orange peroxidase:

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</table>
Fig 4-9: Thermal Inactivation of Orange Peroxidase at pH 5.0

- Each value is a mean of three determinations
Fig 4-10: Thermal Inactivation of Orange Peroxidase at pH 6.0

- Each value is a mean of three determinations
Fig 4-11: Thermal Inactivation of Orange Peroxidase at pH 7.0

- Each value is a mean of three determinations
Fig 4-12: Thermal Inactivation of Orange Peroxidase at pH 8.0

- Each value is a mean of three determinations
4.3.4. Thermal inactivation of guava peroxidase:

Heat inactivation of guava POD was done as was described in section 3.2.4. Results obtained are presented in Table (4 - 5), reflecting the % residual activity after heat- treatments. The kinetics of heat inactivation of guava POD are plotted in figure (4 - 13), (4 - 14), (4 - 15) and (4 - 16) at different investigated pH values. From the results obtained, it can be clearly seen that, the rate of loss of activity is temperature-time dependant. Initially, the inactivation rate was rapid followed by a much slower inactivation rate. For instance, heating at 60°C for 10 min resulted in residual activity of 62.5%, 74.1%, 68.7%, and 63.0% at pH values of 5.0, 6.0, 7.0 and 8.0 respectively. This pattern was more or less true for the other temperatures. It worth noting that, the maximum stability of the enzyme was at pH 6.0. Guava POD showed higher heat stability and was not completely inactivated under all tested conditions.
Table 4 - 5 Thermal inactivation of guava peroxidase:

<table>
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<th></th>
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<td>4 min</td>
<td>6 min</td>
<td>8 min</td>
</tr>
<tr>
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<td>15.4</td>
<td>10.2</td>
<td>7.3</td>
</tr>
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<td></td>
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<td>11.4</td>
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<td>5.1</td>
</tr>
<tr>
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<td>49.8</td>
<td>32.5</td>
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<td>22.9</td>
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<tr>
<td></td>
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<td>19.8</td>
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<td>15.0</td>
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<tr>
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<tr>
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<td>16.7</td>
<td>9.3</td>
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</tbody>
</table>
Fig 4-13: Thermal Inactivation of Guava Peroxidase at pH 5.0

- Each value is a mean of three determinations
Fig 4-14: Thermal Inactivation of Guava Peroxidase at pH 6.0

Each value is a mean of three determinations.
Fig 4-15: Thermal Inactivation of Guava Peroxidase at pH 7.0

- Each value is a mean of three determinations
Fig 4-16: Thermal Inactivation of Guava Peroxidase at pH 8.0

- Each value is a mean of three determinations
4.3.4. Thermal inactivation of banana peroxidase:

Heat inactivation of banana POD was done as was described in section 3.2.4. Results obtained are presented in Table (4 - 6), reflecting the % residual activity after heat- treatments. The kinetics of heat inactivation of guava POD are plotted in figure (4 - 17), (4 - 18), (4 - 19) and (4 - 20) at the different investigated pH values. From the results obtained, it can be clearly seen that, the rate of loss of activity is temperature-time dependant. Initially, the inactivation rate was rapid followed by a much slower inactivation rate. For instance, heating at 60°C for 10 min resulted in residual activity of 62.2%, 74.0%, 72.1% and 63.0% at pH values of 5.0, 6.0, 7.0 and 8.0 respectively. This pattern was more or less true for the other temperatures. It worth noting that, the maximum stability of the enzyme was at pH 6.0. Banana POD showed higher heat stability and was not completely inactivated under the tested conditions.
Table 4-6 Thermal inactivation of banana peroxidase:

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<th>a) pH 5.0</th>
<th>% Remaining peroxidase activity</th>
</tr>
</thead>
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</tr>
<tr>
<td>90°C</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>b) pH 6.0</th>
<th>% Remaining peroxidase activity</th>
</tr>
</thead>
<tbody>
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<table>
<thead>
<tr>
<th>c) pH 7.0</th>
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<table>
<thead>
<tr>
<th>d) pH 8.0</th>
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</table>
Fig 4-17: Thermal Inactivation of Banana Peroxidase at pH 5.0

- Each value is a mean of three determinations
Fig 4-18: Thermal Inactivation of Banana Peroxidase at pH 6.0

- Each value is a mean of three determinations
Fig 4-19: Thermal Inactivation of Banana Peroxidase at pH 7.0

- Each value is a mean of three determinations
Fig 4-20: Thermal Inactivation of Banana Peroxidase at pH 8.0

- Each value is a mean of three determinations
From the results obtained after heat-treatment, it is obvious that the heat inactivation of the POD follows the same patterns of inactivation. The plots show that, the greater the temperature and treatment time, the smaller the residual enzymatic activity detected for the four fruits, which reflects lower stability.

The enzyme from the four fruits was reasonably stable at 60°C. A gradual decline of the activity was observed with the time at the same temperature, and as expected, an increase in the heating temperature inactivates the enzyme more quickly and the loss of activity was greater for the higher temperatures. Banana and guava peroxidases showed higher stability than that of orange and apple peroxidases. Neves and Lourenço., (1998) reported that, peach peroxidases, soluble and bound, showed distinct heat liability, and this fact was also observed for isolated enzymes from apple ( Moulding et al., 1989 ), papaya (Silva et al., 1991) and orange ( Clemente and Pastore ., 1998) . These results find support to the results obtained in this study. Several workers reported that, the heat inactivation process of POD is biphasic, among them are, Adams., (1978); Mohamed., (1983); Osman.,(1993); Yemenicloglu et al.,(1999); and Neves.,(2002). From the results presented in tables (4 - 3), (4 - 4), (4 - 5) and (4 -6), biphasic curves of inactivation were observed, for inactivation temperatures of (60°C - 90°C) for guava and banana POD ,while the biphasic curves were observed only at (60°C - 80°C) for apple and orange POD. Heating temperature of 90°C at pH 5.0 has inactivated the enzyme very quickly even in the first two minutes of the treatment. This phenomenon, the rapid inactivation at first followed by much slower inactivation, is generally accepted to be due to the presence of iso enzymes of different heat stabilities (Winter.,1971; Henley and Sadana.,1985 and change et al .,1988). On the other hand, the deviation of POD thermal inactivation from the first
order kinetics were due, as suggested by Winter (1971), to the formation of enzyme aggregates with different heat stabilities. Several workers impute this nonlinear character to the formation, during heating of denaturated protein forms and groups of peroxidase molecules that remain active (Lu and Whitaker, 1974; Khan and Robinson, 1993; Neves and Lourenço, 1998; and Clemente and Pastore, 1998), or to different conditions of the enzyme after heating (McEllan and Robinson, 1984; Hemeda and Klein, 1991; Adams, 1997; Clemente and Pastore, 1998; Neves and Lourenço, 1998).

The enzyme showed high enzymatic activity at pH 6.0 and 7.0, the maximum being at pH 6.0. This result is similar to that obtained by Civello et al (1995) for the strawberry fruit. Halpin et al. (1989) stated that, the optimum pH for peroxidase depends on the hydrogen donor, therefore, for a given enzyme, the optimum pH changed according to whether guaiacol or pyrogallol or any other hydrogen donor was used.

The plots on the graphs show that the enzyme preserves activities more than 50% of its maximum activity at 60°C for the four fruits at the investigated pH values. The enzyme showed to be more stable for heat treatments at pH between 6.0 and 7.0. Civello et al. (1995) has reported that the enzyme is stable in the range of pH 4-11, and the enzyme stability is totally lost at pH lower than 3.0. This effect, already reported for other systems, has been ascribed to the loss of the heme group at low pH (Burnette, 1977).
4.4. Conclusion and recommendations:

4.4.1: Conclusions:

From this work we can conclude that:

- Peroxidase enzyme was found in all investigated fruits.
- Different enzyme levels were found to occur with accordance to the source.
- High peroxidase levels were detected in banana, guava and apple fruits whereas the lower levels were detected in the orange peroxidases, at the same investigated pH values.
- Extracts of the four fruits investigated have retained 100% enzymic activity when kept at -18°C for 8 days.
- Approximately, 20% loss of the activity for the four fruits was detected after 8 days of storage at 4°C.
- More than 50% loss of activity was detected for the four fruits after 8 days of storage at 30°C.
- The rate of the thermal inactivation was found to be temperature-time dependant.
- The rate of loss of activity was found to increase with both increased time and temperature.
- Banana and guava peroxidases exhibited higher thermal stability than apple and orange peroxidases.
- The thermal inactivation of peroxidase was observed to be a biphasic process at all investigated temperatures and pH values, except for apple and orange peroxidases at 90°C at pH 5.0.
- The same patterns of heat inactivation were observed for the peroxidases extracted from the investigated four fruits.
- Banana and guava peroxidases were not completely inactivated at the tried conditions.
• Less heat severity was needed to inactivate apple and orange peroxidases.
• Apple and orange peroxidases were completely inactivated at 90°C at all pH values investigated.
• Peroxidase inactivation was found to be pH dependant.
• The highest levels, activities and resistances to thermal inactivation were achieved at pH of 6.0 and 7.0 with the higher being at 6.0.
• The lower enzymatic activities and thermal stabilities for the four fruits was found at pH 5.0 for all investigated fruits.

4. 4. 2. Recommendations:

Further researches are needed for better and deep understanding of this enzyme. Further suggested work include:
• Separation of the different isoenzymes and the individual determination of the heat stability.
• Search for ways and means of inactivation other than heat, or trying combined methods for the inactivation of the enzyme in order to reduce severity of heat needed for inactivation, to save energy and to maintain good quality of the products, since the heat can alter many organoleptic properties of foods and diminish the contents or decrease the bio-availability of some constituents.
REFERENCES


Civéllo, P. M.; Martínez, G. A.; Chaves, A. R. and Añón, M. C.


Schobien: (1947). In: Chemistry and Methods of Enzymes.


**Van Huystee, R. B. ((1987).** Some molecular aspects of plant


APPENDIX

Appendix-1: Numerical values for Fig. 4-1: Apple Extract Stability at pH 5.0:

<table>
<thead>
<tr>
<th>Temperature</th>
<th>1st-day</th>
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<th>4th-day</th>
<th>5th-day</th>
<th>6th-day</th>
<th>7th-day</th>
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<tbody>
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<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
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<td>Fridge 4°C</td>
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<tr>
<td>Room temp 30°C</td>
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Appendix-2: Numerical values for Fig 4-2: Orange Extract Stability at pH 5.0:

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<tr>
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### Appendix-4: Numerical values for Fig 4-4: Banana Extract Stability at pH 5.0:

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<td>Room temp30°C</td>
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