**TITLE PAGE**

**THE EFFECT OF TEMPERATURE ON GLUTATHIONE PEROXIDASE FROM FISH**

**BY**

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**GOU/12/2256**

**IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF BACHELOR OF SCIENCES (B.Sc) DEGREE IN BIOCHEMISTRY**

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**JULY 2016**

**CERTIFICATION**

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**DEDICATION**

I dedicate this to the God almighty from whom all good things come from, for all his unfathomable grace, mercy and abundant love one me through these years.

**ACKNOWLEDGEMENT**

I may find it difficult to express a heartfelt appreciation to all who have one way or the other contributed to my success in this research work. I say a big thank you the God for all that he has done and is yet to do for me in my life and in the course of this research.

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**ABSTRACT**

Glutathione Peroxidase (GPx) family of enzymes plays important roles in the protection of organisms from oxidative damage. GPx converts reduced glutathione (GSH) to oxidized glutathione (GSSG) while reducing lipid hydroperoxides to their corresponding alcohols or free hydrogen peroxide to water. This study was conducted to investigate the possible effects of temperature in vivo on Glutathione peroxidase (GPX) oxidative activity in fish tissues.An adult fully grown cat fish, (the African flathead catfish*, Clariasgariepinus*) about 0.7m in length and 0.15 in width, weighing 15Kg was obtained from the school pond. The fish was humanely killed and its liver was cut off from the rest of the body. Glutathione peroxidase was extracted and purified from the liver of the fish using the purification method of Eze*et al*[(2010)](#_bookmark9).The extracted and purified GPX was then divided into 23 test tubes and labeled. Each test tube was heated at different temperatures ranging from -30°C to 50°C, after which Glutathione and Hydrogen Peroxide substrates were introduced to each test tube.Results showed that measurements were higher in test tubes were heating temperature were either exactly at or close to the acclimation temperature of the African Flathead Catfish.

**CHAPTER ONE**

**INTRODUCTION**

* 1. **INTRODUCTION**

**1.1.1 FISH: SOURCE OF FOOD FOR MAN.**

Food is one of the basic needs of man (Morey, 1940; Pierce, 2010). Since agriculture produces the food that provides the calories and micronutrients essential for a healthy and productive life, it is interlinked in many important ways to human nutrition and health (Michael, 2011). These nutrients include carbohydrates, proteins, fats and oil, minerals, vitamins and water. Of these nutrients, it is the proteins that supply the body with amino acids necessary for growth and repair of damaged tissues. The sources of protein include plants and animals. Animal sources are preferred because of the presence of essential amino acids and higher digestibility. However, the major disadvantage is higher cost. The animal sources include fish, poultry, dairy, pork, snail, and rabbit.

Fish accounts for one fifth of world total supply of animal protein (FAO, 1991; Olagunju*et al.*, 2007). It is an important source of protein to a large number of Nigerians. It provides 40% of the dietary intake of animal protein of the average Nigerian (FDF, 1997; Sogbesan*et al*., 2006). According to Adekoya and Miller (2004), fish and fish products constitute more than 60% of the total protein intake in adults especially in rural areas.

Amiengheme (2005) enumerated the importance of fish in Human Nutrition as follows:

* Fish food has a nutrient profile superior to all terrestrial meats (beef, pork and chicken) being an excellent source of high quality animal protein and highly digestible energy;
* Fish is a good source of sulphur and essential amino acids such as lysine, leucine, valine and arginine. It is therefore suitable for supplementing diets of high carbohydrate contents;
* It is an extremely rich source of polysaturated (Omega III) fatty acids, which are important in lowering blood cholesterol level and high blood pressure, reducing the risk of sudden death from heart attacks and reduces rheumatoid arthritis, lowering the risk of age- related muscular degeneration and vision impairment; decreasing the risk of bowel cancer and reducing insulin resistance in skeletal muscles.
* Fish is also a good source of thiamine fat soluble vitamins (A, D and E), water soluble vitamins (B complex) and minerals (calcium, phosphorus, iron, iodine and selenium);

In Nigeria, fisheries occupy a unique position in the agricultural sector of the economy (Kudi *et al*., 2008). Its contribution to Gross Domestic Product (GDP) rose from 76.76 billion in 2001 to N162.61 billion in 2005 (CBN Report, 2005). Nigerians are large consumers of fish, with an annual average demand estimate at 1.4 million metric tonnes, (Kudi*et al*., 2008). Domestic fish production of about 0.5 million metric tonnes is supplied by artisan fishermen (85%), and fish farmers (15%) (Adekoya and Miller, 2004; Emokaro, 2010; BusinessDay, 2011). However a demand and supply gap of at least 0.7 million metric tonnes exists nationally with import making up the short fall at a cost of 400 billion United States dollars per year. According to FAO (2007), this figure (0.7 million metric tonnes) makes Nigeria the largest importer of fish in the developing world.

To take advantage of the large market created by this deficit, the immediate past and current Governments has promoted a backward integration plan for increased fish farming and production in Nigeria, Nigerians are also complementing Government efforts by increasing their participation in aquaculture, with many fish farmers focusing on African flathead catfish, *Clariasgariepinus* as they have been shown to have a potential market value of two to three times that of other cultivable species like Tilapia and Heterobranchus (FAO, 2000; Fafioye and Oluajo, 2005; Emokaro*et al*., 2010; Businessday, 2011).

A survey by Addo (2005) revealed that Nigerian children below the age of 18 years, who make up about 47% of our total population are still victims of stunting, wasting and under-weight, so with the increased establishment of more aquaculture in Nigeria, it is possible to reverse this trend of malnourishment among Nigerians in this age bracket.

* 1. **THE AFRICAN CATFISH**

The African catfish – *Clariasgariepinus*, an omnivorous freshwater fish, is a popular delicacy in Nigeria. It is a prominent culture species because of its fast growth rate and resistance to diseases and stress factors like over-stocking and poor water quality (Olojo*et al*., 2005). It is distributed mainly in fresh waters of Africa hence the name African catfish, although it is also seen in Asia. It is named ‘catfish’ because they possess prominent barbels which resemble cat’s whiskers. It has a slender body, flat bony head and broad terminal mouth with four barbels. The pectoral fins have spines. Its dendritic organ is an accessory breathing organ and it is a modification of the gill arches (Ahmed *et al*., 2008). The adult of about 1.5m weighs up to 29kg (Teugel, 1986).

Habits: They are nocturnal omnivorous animals feeding on living as well as dead organic matter. They are capable of swallowing large prey because of large mouth (Teugel, 1986).They can crawl on the ground to escape drying pools but can also survive in shallow mud for long periods of time between rainy and dry seasons. They can produce croaking sound. They spawn mostly at night in the shallow areas of rivers, lakes and streams. Development of the larva is rapid as they are able to hatch 24 hours and swim within 48 – 72 hours after fertilization.

**1.3 ENZYMES AND THE GENERAL EFFECTS OF TEMPERATURE**

The living cell is the site of tremendous biochemical activity. Catalysis, through enzymes, makes possible biochemical reactions, which are necessary for all life processes. Enzymes are responsible for bringing about almost all of the chemical reactions in living organisms. Without enzymes, these reactions take place at a rate far too slow for the pace of metabolism.

Like most chemical reactions, the rate of an enzyme-catalyzed reaction is affected by temperature. Variations in reaction temperature as small as 1 or 2 degrees may introduce changes of 10 to 20% in the results. In the case of enzymatic reactions, this is complicated by the fact that many enzymes are adversely affected by high temperatures.

The enzyme Glutathione Peroxidase which catalyses the reduction of hydrogen peroxides is the main focus of this study. Understanding the effects of temperature on the activity of this enzyme in the catfish, could help in determining the best storage temperature for this fish such that harvested catfish can be kept fresh for a longwhile for consumption after

**1.4. STATEMENT OF PROBLEM**

Despite the clear increase in interest for aquaculture and fish consumption in Nigeria, a major problem of storage exists. This is clearly observed in the lack of fresh iced or well dried catfish options in the market. It is also manifest in the ‘point and kill’ marketing phenomenon, which has invariably restricted catfish farming to retail/subsistence levels intended for consumption by members of the immediate locale were the farm is sited, and discourages large scale catfish farming intended for consumers across state and even national borders.

**1.5. RESARCH OBJECTIVES**

The aim of this study is to isolate the enzyme Glutathione Peroxidase (GPx) from the liver of the African catfish (*Clariasgariepinus)* and carryout investigations in vitro to determine

1. The optimum temperature range wherein the enzyme Glutathione peroxidase best catalyses the reduction of hydroperoxides and other free radicals, thereby protecting the tissues of the Flat Head Catfish from oxidative stress and damage.
2. The temperature range wherein rate of the catalytic reduction activity of the enzyme Glutathione peroxidase is lowest.

**1.6. SCOPE OF STUDY**

This study will be carried on only the African Catfish (*Clariasgariepinus)*, as survey has shown that it is the by far the catfish variety most consumed by inahbitants of Eastern Nigeria.

**1.7. SIGNIFICANCE OF THE STUDY**

The importances of this study are as follows:

1. Results obtained at the end will accurately advise the best storage temperature for the Flat Head Catfish that will ensure optimum protection of its muscles and other edible tissues from oxidative stress and damage,
2. Thereby increasing its shelf life,
3. Encourage farmers to engage in larger cultivation of this popular species of fish for wider consumption,
4. This in turn reduces production and processing costs; and wastage and loss due to deterioration and decay,
5. Ensure that fish which is an important but currently expensive source of protein is made cheaper and consumed more frequently by citizens of all income and age brackets;
6. And finally help reduce the malnutrition problem earlier mentioned in the last.

**CHAPTER TWO**

**LITERATURE REVIEW**

Fish live in a constantly changing environment where temperature, oxygen availability, light and compounds in the water can fluctuate on an hourly, daily, and seasonal basis. To maintain internal homeostasis in the face of such fluctuations, fish are equipped with adaptable, integrated physiological control systems. One such system is the antioxidant enzyme glutathione peroxidase (GPx), which acts to maintain the redox balance of many cellular systems, including those of fish. Better put, it has been clearly shown that the GPx enzyme system in animals provide protection against oxidative damage and accumulation of free-radical products.

**2.1. CELLULAR REDOX ENVIRONMENT**

The cellular redox environment is a balance between oxidative and reductive reactions that involve the transfer of electrons between or within molecules (Ziegler, 1991). Depending on the degree, loss of this balance can immediately or overtime lead to oxidative damage of the cells, muscles, liver, kidney etc of fishes, which may in turn result to their death and reduced shelf life or freshness of the fish before consumption.

The main threat to the redox balance within a cell is the presence or production of molecules with one or more unpaired electrons, termed free radicals (FR). Reactive oxygen species (ROS) are FR forms of molecular oxygen and represent a large biological threat to the redox balance of cells. There are many sources of ROS and other FR within an organism. One of the most significant sources of ROS is from mitochondrial respiration, where approximately 0.1 % of oxygen entering the electron transport chain is released as ROS — mainly as superoxide (O/” ) and hydrogen peroxide (Fridovich, 2004).

Moderate levels of ROS are important in cell function as they can act as signals for DNA synthesis, enzyme activation, gene expression and the cell cycle (Deplancke and Gaskins, 2002). However, high levels of ROS can result in damage to key biological molecules such as lipids, proteins, and DNA (reviewed by Kidd, 1997; Hayes and McLeIIan, 1999).

The cellular redox balance is maintained by reduction of FR, ROS and their reactive oxygen products by a suite of coordinated, integrated antioxidants (AO) that include the AO enzymes (glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase (CAT)), as well as small water soluble (such as GSH, Vit C, and uric acid) and lipid soluble (such as Vit E, Vit K, and §- carotene) molecules (see Ternay and Sorokin, 1997; Hayes and McLeIIan, 1999). If ROS production increases beyond the capacity of the AO system, this can result in membrane damage, enzyme malfunction, DNA strand breaks and cell death (reviewed by Acworth *et al*., 1997; Kidd, 1997).

This study is focused on Glutathione Peroxidase (GPx) antioxidant system and how temperature affects its levels and activity particularly in maintaining the redox balance in the cell. GPx is present in high intracellular concentrations relative to other antioxidants (up to 10mM), and is consequently considered part of the first line of defence against exogenous or metabolically produced ROS and electrophiles (Ziegler, 1991; Wilhelm- Filhoet al., 2000; Martinez-Alvarez *et al*., 2005).

**2.2. THE GLUTATHIONE PEROXIDASE (GPX) ANTIOXIDANT SYSTEM**

Figure 1



Antioxidant enzyme schematic

The antioxidant enzyme Glutathione Peroxidase (GPx) constitutes one of the main systems involved in the redox balance of cells. GPx is a tripeptide, composed of y-glutamic acid (y-glu), cysteine (cys) and glycine (gly). In the 59years since the discovery of GPx by Mills GCJ in 1957 (Obi, 1988), there have been numerous articles examining GPx metabolism and cellular roles.

The main function of GPx is to catalyze the reduction of HOOD and lipid hydroperoxide.

H2O2 + 2GSH -----> 2H2O + GSSG

In the process, GSH is oxidized (to oxidized glutathione — GSSG). The AO power of GPx relies on the ability of GSSG to be readily reduced back to GSH. This helps to maintain low levels of GSSG. A high GSSG level (10-50% of total GSH) is indicative of oxidative stress (Pastore*et al*., 2003).

**2.3. GLUTATHIONE PEROXIDASE (GPX) AND FISH**

Fish differ from mammals in several key aspects that may result in unique characteristics of GPx function and regulation. Fish can experience daily and seasonal fluctuations in body temperature and oxygen availability, both of which could potentially affect the rate of ROS production (Wilhelm-Filho*et al*., 2000; Davidson and Schiestl, 2001), and consequently the necessity of GPx. In addition, extensive gill and water contact make fish particularly susceptible to uptake of water-borne toxicants, suggestingfish may have a relatively large demand on their GPx system.

However, GPx levels turnover and activity are noted as being relatively low in fish compared to mammals (Wallace, 1989; Gallagher *et al*., 1992; Wilhelm-Filhoet al., 2000). There may be a number of reasons for this. Most notably, fish are able to excrete Hydrogen Peroxide in substantial quantities across their gills (approximately 0.8nmoI/min/g fish), and consequently the AO activity of GPx may be in lower demand (Wilhelm-Filho*et al*., 1994). As well, fish generally have lower mass-specific metabolic rates than mammals, even when corrected for temperature (Else and Hulbert, 1981), and consequently may produce less ROS from the mitochondria.

Work by Janssens*et al*. (2000) suggests AO levels in fish arerelated to metabolic rate, as they found out for GPx. As well, Wilhelm-Filho (1996) found active fish had higher AO levels than sluggish fish in the marine environment, but not in the freshwater environment. Wilhelm-Filho*et al*. (2000) proposed AO status would be proportional to water temperature in thermoconformers such as fish, as oxygen consumption is generally proportional to temperature, and consequently ROS production by the mitochondria. However, whether temperature or other influences of metabolism directly affect GPx activity in fish, or can explain the difference in GPx dynamics between fish and mammals have not previously been investigated.(Rosalind A, 1997)

The liver has the highest concentration of GSH upto10mM,(Kretzschmar and MoIler,1993;Kidd,1997). GPx is important in this organ, as the liver is the main site of exogenous and endogenous metabolite detoxification for excretion into the bile. As well, the liver is the main source of plasma GSH (Anderson *et al*., 1980; Lauterburg*et al*., 1984). In the plasma,GPx functions as an extracellular antioxidant (Hayes and McLeIIan, 1999),

GPx levels in extra-hepatic tissues such as the kidney and gill are closer to, equal to, or in some cases greater than levels in the liver (Gallagher and Di Giulio,1992;Otto *et al*., 1997a, 1997b; Lushchak*et al*., 2001). This suggests the kidney and gill of fish may have similar functions in antioxidant defence and detoxification as the liver (as hypothesized by Gallagher and Di Giulio, 1992).

**2.4. THE EFFECT OF TEMPERATURE ON GLUTATHIONE PEROXIDASE (GPX) ACTIVITY IN FISH**

Fish live in a variable environment where factors such as temperature can fluctuate on a daily and seasonal basis. There are many physiological systems in place to maintain homeostasis in such environments. The antioxidant enzyme Glutathione Peroxide, GPX represents one such system that maintains the redox balance of cells. Generally, GPX levels, turnover and activity in some tissues were altered proportionally to acclimation temperature changes in fish. Increasing temperature may result in an increase in metabolically produced reactive oxygen species, and hence increase the demand for the reducing power of GPX. Whether short term changes may also challenge the redox status of cells and hence challenge the GPX system has not been well examined in fish.

The effect of temperature on GPx dynamics has been examined to some extent in fish and mammals. In mammals, hyperthermia results in a decrease in temperature followed by an increase in GPx levels in the blood, an increase in excretion of hepatic GSH, and an increase in lipid peroxidation (Skibba*et al*., 1991; Ohtsuka*et al*., 1994). Short-term heat stress has been found to decrease GPx levels and increase lipid peroxidation in catfish (Heteropneustesfossilis, Parihar*et al*., 1996; Parihar*et al*., 1997), and snakeheads (Channapunctata, Kaur *et al*., 2005).

Although no studies have directly addressed the effects of long-term temperature changes on GPx dynamics, seasonal changes in GPx levels have been associated in part with changes in temperature in various ectothermic animals (Dziubek, 1987; Pérez- Pinzén and Rice, 1995; Wilhelm-Filho*et al*., 2001; Gorbi*et al*., 2005). In addition, GPx levels were influenced by precursor supply, and not necessarily synthesis rate. Consequently, GPx levels may be rapidly increased in fish tissues during times of high demand, such as altered temperature.(Rosalind,1997).

GPx turnover was proportional to incubation temperature in a rainbow trout hepatomacell line in vitro. In killifish in vivo, tissue GPx levels enzymes were proportional to acclimation temperature. Although these showed that temperature influences GPx dynamics in fish, the effects of temperature could only partially explain the low GPx levels in fish compared with mammals.

Long-term changes in temperature influenced GPx dynamics in killifish, while acute temperature changes had little effect on GPx dynamics in rainbow trout in vivo or in vitro. However, altered GPx levels inconsistently altered the cellular response to acute heat stress in rainbow trout in vitro, and influenced the generalized and cellular responses to acute heat stress in vivo.

Acute temperature changes beyond the normal range a fish is exposed to results in the fish entering a stressed state. This may affect GPX dynamics in several ways. Stress can increase oxygen consumption (see WendelaarBonga, 1997) which can increase production of reactive oxygen species by the mitochondria (Muradian*et al*., 2002). This would increase demand for antioxidants such as GPX.

In fish, GSH levels are altered in response to stressors that directly threaten the redox balance of a cell, such as oxidative or toxicant stressors (Bell and Cowey, 1990; Otto and Moon, 1995; Almar et al.,1998; Pena-Llopis*et al*., 2001; Stephensen*et al*., 2002; Hughes and Gallagher, 2004). However, few studies have examined the effect of acute heat stress on GPX dynamics in fish. Acute heat stress in catfish (Heteropneus/esfossilis) decreased GPxactivity in gills (Parihar*et al*., 1997), and increased lipid peroxidation in liver (Parihar*et al*., 1996) during large temperature increases.

In addition, heat stress increases lipid peroxidation levels and hydrogen peroxide production from the mitochondria (Ohtsuka et at., 1994; Aréchiga et at., 1995;Davidson and Schiestl, 2001), indicating heat stress-induced changes in GPX dynamics are the result of oxidative stress.

**CHAPTER THREE**

**MATERIALS AND METHODS**

**3.1 MATERIALS**

**3.1.1 REAGENTS / REAGENT PREPARATIONS**

**3.1.1a REAGENTS**

Most of the reagents used in this analysis were of analytical grade, only few were of reagent grade. Reagents were obtained from Godfrey Okoye University chemical stock store and some from the chemical store at Ogbete Main market, Enugu State. The list of the chemicals used are summarized Table 3.1

Table 1

Summary of the reagents used for the analysis

|  |  |
| --- | --- |
| Reagents  | Formula  |
| Disodium hydrogen phosphate | Na2HPO4 |
| Phosphoric acid | H3PO4 |
| Phenol red | C19H14O5S |
| Hydrogen peroxide | H2O2 |
| Distilled water | H2O |
| Sodium hydroxide | NaOH |
| Sodium chloride salt | NaCl |
| Sephadex G-75 gel |  |
| DEAE cellusose |  |
| Sodium carbonate | Na2CO3 |
| Copper tetraoxosulphate (VI) |  |
| Sodium potassium tartrate |  |
| Folin-Ciocalteau phenol reagent |  |
| Hydrochloric acid | HCL |
| O-dianisidine |  |

**3.1.1b REAGENTS PREPARATION**

**0.1 M phosphate buffer:**

14.3 g of disodium hydrogen phosphate salt was weighed and dissolved in appropriate quantity of water. 2.3 ml of phosphoric acid was added to make up the pH to 7.0 with the solution made up to 1000ml

**0.5 M NaOH**

Exactly 20 g of NaOH was dissolved in distilled water and volume made up to 1000 ml.

**0.5 M HCl**

Hydrochloric acid; HCl (37%) 41.5 ml was added to distilled water and volume made up to 1000ml.

**PREPARATION OF COMPONENT REAGENTS FOR PROTEIN DETERMINATION**

**Solution A:** an alkaline sodium carbonate (Na2CO3) was prepared by dissolving 2 g of Na2CO3 in 100 ml of 0.1 M NaOH (0.4 g of sodium hydroxide pellets were dissolved in 100 ml of distilled water).

**Solution B:** a copper tetraoxosulphate (VI)- sodium potassium tartarate solution was prepared by dissolving 0.5 g of CuSO4 and 1 g of sodium potassium tartarate in 100 ml of distilled water (as the stock solution). It was freshly prepared by mixing the stock solution.

**Solution C:**Folin-Ciocalteau phenol reagent was made by diluting the commercial reagent with water in a ratio of 1:1 on the day of use.

**Solution D:** standard protein (Bovine Serum Albumin) , the crude extract, finally, the purified protein (sample).

**Solution E:** freshly prepared alkaline solution was made by mixing 50ml of solution A and 1 ml of solution B.

**Preparation of 2 mg Bovine Serum Albumin (BSA); standard protein**

0.2 mg of BSA was dissolved in 100 ml of distilled water and then used as protein stock solution.

**3.1.2 APPARATUS AND EQUIPMENTS**

pH meter

Model: PHS-3C

Serial No: 600410089018

Manufacturer: Lifecare Medical, India

Electronic Weighng Balance

Model: YP-502N

Serial No: CS09B0372

Manufacturer: BilanciaUsoScientifico, Italy

Spectrophotometer

Model:

Voltage: 220V

Frequency: 50Hz

Power: 40W

Serial No: 23A09254

Manufacturer: Midfield Equipment & Scientific LTD, England

Centrifuge

Model: 80-2

Manufacturer: Health Medical Equipment, England

Volumetric flask

Conical flask

Round bottom flask

Beakers

Reagent bottles

Test tubes

Test tube racks

Glass rod (stearer)

Pipettes

Spatula

Whatman Filter paper

Laboratory electric blender

Refrigerator

Foil

Masking tape

EDTA container

Water bath

**3.2 METHODS**

**3.2.1 PROTEIN DETERMINATION**

According to the method of lowry*et al* (1951), using BSA as standard protein

For Protein Standard Curve, the reaction mixture contained 0.1-1.0ml of the protein stock (0.2g BSA in 100 ml distilled of water) in test tubes arranged in triplicates. The volume was made up to 1 ml by adding distilled water. But for the test mixture, 0.1 ml of sample enzyme was mixed with 0.9 ml of distilled water. In either case, 2 ml of solution E was added and allowed to stand for 10 minutes at room temperature. Then 0.5 ml of solution C (dilute Folin-Ciocalteu phenol reagent) was added with rapid mixture and allowed to standard for 30 minutes (this was to ensure that the protein reacted with Folin-Ciocalteu reagent) . Then the absorbance was read at 750nm using UV-spectrophotometer. The absorbance values were converted to protein concentration by extrapolation from the standard curve.

**3.2.2 SAMPLE COLLECTION**

The fish was obtained from the Godfrey Okoye University Biological Science Fish pond and the fish was humanely killed and the liver was cut off from the rest of the body. It was stored in the freezer after which it was homogenated using a blender and 600ml of phosphate buffer.

**3.2.3 SAMPLE PREPARATION/ ENZYME EXTRACTION**

45.66g of the liver Clariasgariepinus (fish) was homogenated using electric blender in 600ml of phosphate buffer of pH 7.0. The homogenate was centrifuged was using an electric centrifuge with speed of 10,000 × g for 15mins after which the supernatant (crude) was stored in the refrigerator as the source of the protein (crude glutathione peroxidase).

**3.2.4 ENZYME ASSAY**

The activity of peroxidase in the crude was determined according to the method ofEze*et al*., (2010). The reaction contained 2 ml phosphate buffer, 0.4 ml crude enzyme (sample), 0.2 glutathione (substrate), 0.2 ml hydrogen peroxide (oxidizing substance). O-dianisidine was subsequently used in place of glutathione for further experiments. The absorbance was read at 470nm at 0 second and 30 seconds in a UV-Spectrophotometer at 25°C. The activity was measured by finding the difference between the absorbance at 30 seconds and that of 0 seconds.

**NOTE:** The absorbance was measured following addition of hydrogen peroxide.

**3.2.5 ENZYME PURIFICATION STEPS**

**3.2.5a FIRST PURIFICATION STEP**

**GEL FILTRATION, SIZE EXCLUSION, OR GEL PERMEATION CHROMATOGRAPHY**

**PRINCIPLE:** proteins are separated based on size. The solid phase consists of cross-linked polymer beads with an engineered pores or cavities of a particular size. Example of such polymer is Sephadex (G-10 to 200). Large proteins cannot enter the pores and so emerge (elute) from the column sooner than the small ones. On the other hand, small ones get trapped in the pores and their movement retarded, so they elute later from the column. This method can also be used to approximate the size of a purified protein.

**PROCEDURE:**

**Gel preparation:**

A column of sephadex G- 75 (Pharmacia) was prepared. A 5 g of the dry sephadex G- 75, was suspended in 65ml of phosphate buffer (pH7.0). It was heated in a water bath for 30 minutes at 40⁰C. The column was placed vertically on a flat surface. Distilled water was added to the column as to fill the empty outlet tube. The slurry was poured in order to completely fill the column of 1.3 cm diameter and 40 cm length specifications. It was left undisturbed for 24 hours, as distinct layers of gel and water would appear.

**APPLICATION OF SAMPLE**

The outlet tube was opened and the distilled water present in column was removed until a small layer was on the top of column. The precipitated extract (50 ml) was then applied on the surface of the column and the outlet was opened. The sample was allowed to penetrate in packed column. The elution was carried out by 0.1 M phosphate buffer of pH 7.0 at a constant drop rate. The activity of the fractions was determined and their protein content estimated.

**3.2.5b SECOND PURIFICATION STEP**

**ION EXCHANGE CHROMATOGRAPHY**

**PRINCIPLE:**

This technique separates substances based on their charges in the presence of an ion exchanger as the stationary phase. It is used to separate ions and polar molecules based on their affinity to the ion exchanger. The column matrix is a synthetic polymer (resin) containing bound charged groups; anionic and cationic groups termed anion exchangers and cation exchangers respectively. In cation exchange chromatography, positively charged molecules are attracted to negatively charged solid support. Conversely, negatively charged molecules are attracted to positively charged solid support. The general ideology is that “like charges repel and unlike charges attract”.

The affinity of each protein for the charged groups on the column is affected by the pH (which determines the ionization state of the molecule) and the concentration of competing free salt ions in the surrounding solution. Separation is optimized by gradually changing the pH and/or the salt concentration of the mobile phase so as to create a pH or salt gradient. By increasing the salt concentration (generally by using linear salt gradient), the molecules with weakest ionic interactions start to elute from the column first while those with higher ionic interaction require higher salt concentration and elute later in the gradient. Furthermore, the pH of the mobile phase buffer must be between the pI (isoelectric point) or the pKa (acid dissociation constant) of the charged molecules and the pKa of the charged group on the solid support.

**PROCEDURE:**

**PREPARATION OF COLUMN**

A column of DEAE (Diethylaminoethyl) cellulose was prepared by the method of Kelley and Reddy (1986). The resin was gradually added to the 0.1M phosphate buffer (pH7.0) until slurry was prepared. It was heated in a water bath at 40⁰C for 5 hours, without drying the slurry. Buffer was passed through the column to fill the outlet tube and slurry was poured into the column of 1.3x40 cm specifications. It was left on a level surface for 24 hours. Then, the buffer was removed from the column after opening the outlet tube and closed when just small amount was present on top of the column.

**WASHING THE COLUMN WITH BASE**

The column was washed with 50 ml of 0.5M NaOH, which was allowed to flow through and out of the column. After the complete removal of base, distilled water was passed through the column until the pH of eluent was 7.

**WASHING THE COLUMN WITH ACID**

An amount of 50 ml of 0.5M HCl was poured on the surface of the column. It was allowed to flow through and out of the column and then distilled water was passed until the eluent pH was 7.0

**EQUILIBRATION OF COLUMN**

The column was equilibrated with phosphate buffer of pH 7. It was achieved by washing it continuously with buffer overnight so that the pH of eluent remains the same with that of buffer.

**APPLICATION OF SAMPLE**

The outlet tube was opened and the buffer already present at the surface of column was allowed to flow, until a small amount was left on the top. The fractions with the highest activity (5, 6, 7, 8, 9, & 10) up to 30 ml were poured on the surface of the column. The outlet was then opened for samples to penetrate into the column bed. The elution of the sample was carried out with 0.05-0.3 M NaCl gradients prepared in 0.1 M phosphate buffer of pH 7.0 the drop rate of the eluted sample was kept constant and fractions collected. Finally, the fractions were subjected to enzyme assay and protein estimation.

**3.3TEMPERATURE VARYING OF ENZYME ACTIVITIES**

**PRINCIPLE:**

Temperature denatures or influences the activity of enzymes. High temperature is known to reduce the activity of enzymes which are proteins, and proteins are known to denature in high temperature. The chance of having an enzyme with low activity is 90% possible as the temperatures increases. The experiment is done to determine the effects high temperature on this enzyme.

**PROCEDURE**

After the gel filtration and ion exchange chromatography purification was done the fractions was collected in twenty three fractions (23) and grouped into four with four temperature intervals ranging from 30°C to 50°C. the temperature interval was as follows

* 30-35°C
* 35-40°C
* 40-45°C
* 45-50°C

In twenty three test tubes 2ml of phosphate buffer was added into the test tubes, followed by 0.4ml of purified extract and then heated using the water bath. After each temperature interval was attained, the samples were collected and 0.2ml glutathione and hydrogen peroxide was added to each tube and measured using UV spectrophotometer with a wavelength of 470nm for 0secs and 30secs.

**NOTE:** The absorbance was measured following addition of hydrogen peroxide. (I.e. immediately the hydrogen peroxide was added to each test tube the absorbance was measured).

**CHAPTER FOUR**

**RESULTS AND DISCUSSION**

**4.1 ENZYME ACTIVITY OF CRUDE**

After extraction was done by hamogenating the fish liver with the working buffer, the homogenate was centrifuged at the speed of 10,000 x g for 15mins. This table shows the absorbance of the of the unpurified extract also know as the crude extract.

TABLE 2

Result For crude Enzyme Activity

|  |  |  |  |
| --- | --- | --- | --- |
| SAMPLE | 0 SECONDS | 30 SECONDS | ACTIVITY |
| CRUDE  | 0.297 | 0.521 | 0.224 |

The protein activity is gotten from subtracting the absorbance at 0 seconds from the absorbance 30 seconds. The absorbance was measured at 470nm using the UV Spectrophotometer.

Also the absorbance of the protein content of the crude extract is 0.591, and was measured at 550nm.

**4.2 SIZE EXCULSION CHROMATOGRAGHY.**

After the extract has been purified using Sephadex G-75 gel, the results were obtained and represented thus

Sephadex G-75 of Gluthatione peroxidase Activity

**DISCUSSION**

The eluent was collected in 23 tubes and the protein content and enzyme activity as measured at 750nm and 470nm respectively. The enzyme activity was measured at 0seconds and 30seconds. The activity was then obtained by subtracting the value of 0secs from the value of 30secs. This graph shows the point at which enzyme activity and protein content are high at a temperature of 25°C (without heating) is from tubes 6-19. In tube 6 there is a rapid increase in activity and a slight increase in protein content. Protein content was most high in tube 19 and very low in tube 1. Enzyme activity was most high in tube 6 and 19 and low in tube 1.

**4.3 ION EXCHANGE CHROMATOGRAPHY**

After the size exclusion chromatography was done and the graph was plotted the tubes with highest activities and protein content (,7,8,9,10,12,13,14,16,17,18,19,) were collected and used for the ion exchange chromatography using the DEAE CELLULOSE gel. The eluent was collected in 50 test tubes and the result is thus.

DEAE - CELLULOSE ION EXCHANGE CHROMATOGRAPHY FOR GLUTHANIONE PEROXIDASE

**DISCUSSION**

From the graph, we notice that protein content were high from the eluent in tube 6. Further increase was seen in tube 8 and 17 for the protein content. And the enzyme activity was at its best in tube 7 and more 17. From the result, the eluent in tube 17 has the highest protein content and the highest enzyme activity. These results were obtained at a temperature of 25°C enzyme activity as lowest in tube 27 while the protein content was low at tube 44.

Below is the table showing results after the extract was purified.

**TABLE 3**

**Purification Table**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| sample | Volume (ml) | Total Protein (mg/ml) | Activity (IU/ml) | Total Activity (U/ml) | Specific Activity (IU/mg) | Purification fold | % Yield |
| Crude | 500 | 33.71 |  212 | 106000 | 6.28 | 1.0 | 100 |
| Sephadex G-75 | 50 | 27.11 | 128 | 6400 | 4.72 | 0.75 | 6.03 |
| DEAE CELLULOSE | 30 | 19.42 | 242 | 7260 | 12.46 | 1.98 | 6.84 |

**4.4 EFFECT OF TEMPRATURE ON THE PURIFIED EXTRACT**

After purification was done, the purified sample was collected in 23 test tubes and subjected to different temperatures starting from 30°C to 50°C, at the end of every temperature interval the absorbance of the enzyme activity was measured and recorded at 0secs and 30secs. I divided the tubes into four groups with four temperature intervals, 30-35°C, 35-40°C, 40-45°C and 45-50°C. the absorbance was measured 470nm and the result is thus

**DICUSSION**

From this chart, the enzyme activity most stable at the temperature interval of 30-35°C, these enzymes had the least activity when measured at 25°C (when it was not heated). As the temperature increased the enzyme activity is seen to be reduced. Enzyme activity was also seen to be lowest at the temperature interval of 45-50°C. From the result of the enzyme activity after purification (when it was not heated) the tubes with the highest activities were those with a notable decrease in the absorbance when measured after heating, this shows that enzyme activity was low or decreased as the temperature increased.

**CHAPTER FIVE**

**CONCULSION AND RECOMMENDATION**

**5.1 CONCUISION**

The result show that measurements were higher in test tubes were heating temperature were either exactly at or close to the acclimation temperature of the African Flathead Catfish which is temperature of the pond at which the fish was gotten which is between 25°C to 30°C. but these test tubes were the those which recorded the lowest protein content and activity. Based on the above, GPx turnover, content and activity in fish would decrease with increasing temperature, and that differing temperatures to some extent would explain the GPx dynamics in fishes.GPx dynamics in fish aids in the fish to be extremely beneficial to the consumer.The pioneering observations of Bang and Dyenberger on Greenland Eskimos regarding the association between high ﬁsh intake and reduced incidence of atherosclerotic heart disease has led to numerousepidemiologic studies highlighting a signiﬁcant effect also on a variety of diseases not necessarily related to atherosclerosis, such as diabetes, psoriasis, and multiple sclerosis. It was suggested that the potential health beneﬁt was due to ﬁsh oil, and mainly to its high content of n-3 long chain polyunsaturated fatty acids (PUFA ) which is because the fish has not been subjected to oxidative stress.

In live and freshly caught ﬁsh the antioxidant levels are generally high and able to counteract most oxidative attacks. Depletion of glutathione peroxidase and other related molecules like ubiquinol and vitamin C in post-mortem ﬁsh muscle and liver represents an important indicator of an ongoing process of oxidative stress, which is proportional to the storage temperature, and is followed by oxidative damage to PUFA (polyunsaturated fatty acid), proteins, and DNA, and by increased activity of lipases and phospholipase A2, with a consequent generation of free fatty acid. High levels and glutathione peroxidase and ubiquinol or vitamin C could be taken as the main indicators of freshness in ﬁsh.

5.2 **RECOMMENDATION**

I recommend that fish should be stored at a temperature less than 25°C this is because it will help the enzyme glutathione peroxidase in fish. As seen, high temperature reduces the activity of the temperature which will cause oxidative stress to the fish. This will in turn cause a lot of harm to fish and make the fish unfavorable or bad for consumption.

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**APPENDIX I**

**TABLE 4**

RESULT FOR ENZYME ACTIVITY WITHOUT HEATING AFTER PURIFICATION

|  |  |  |  |
| --- | --- | --- | --- |
| SAMPLE | 0SECS | 30SECS | ACTIVITY |
| 1 | **0.210** | **0.216** | **0.006** |
| 2 | **0.191** | **0.198** | **0.007** |
| 3 | **0.151** | **0.184** | **0.033** |
| 4 | **0.106** | **0.163** | **0.057** |
| 5 | **0.492** | **0.447** | **0.085** |
| 6 | **0.634** | **1.554** | **0.92** |
| 7 | **0.809** | **1.638** | **0.829** |
| 8 | **0.945** | **1.765** | **0.82** |
| 9 | **0.317** | **1.926** | **0.609** |
| 10 | **1.123** | **2.0** | **0.877** |
| 11 | **1.514** | **2.0** | **0.486** |
| 12 | **1.064** | **1.921** | **0.857** |
| 13 | **1.122** | **2.0** | **0.878** |
| 14 | **1.196** | **1.966** | **0.664** |
| 15 | **1.509** | **2.0** | **0.491** |
| 16 | **1.046** | **2.0** | **0.945** |
| 17 | **1.037** | **1.856** | **0.819** |
| 18 | **1.096** | **1.958** | **0.862** |
| 19 | **0.843** | **1.629** | **0.786** |
| 20 | **1.605** | **1.470** | **0.365** |
| 21 | **0.839** | **1.165** | **0.326** |
| 22 | **0.078** | **0.086** | **0.008** |
| 23 | **0.827** | **1.167** | **0.34** |

The activity was gotten by subtracting the absorbance at 0secs from the absorbance at 30secs.

**APPENDIX II**

**TABLE 5**

PROTEIN CONTENT ABSORBANCE TABLEAFTER PURIFICATION

|  |  |
| --- | --- |
| SAMPLE | PROTEIN ABSORBANCE |
| 1 | **0.075** |
| 2 | **0.170** |
| 3 | **0.137** |
| 4 | **0.075** |
| 5 | **0.184** |
| 6 | **0.375** |
| 7 | **0.340** |
| 8 | **0.437** |
| 9 | **0.590** |
| 10 | **0.467** |
| 11 | **0.660** |
| 12 | **0.707** |
| 13 | **0.694** |
| 14 | **0.803** |
| 15 | **0.725** |
| 16 | **0.933** |
| 17 | **0.747** |
| 18 | **0.652** |
| 19 | **1.008** |
| 20 | **0.900** |
| 21 | **0.704** |
| 22 | **0.806** |
| 23 | **0.824** |

**APPENDIX III**

**TABLE 6**

ENZYME ACTIVITY ON VARYING TEMPRATURE AFTER PURIFICATION

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| SAMPLEZ | TEMPERATURE °C | OSECS | 30SECS | ACTIVITY |
| 1 | **30-35** | **0.575** | **0.589** | **0.014** |
| 2 | **30-35** | **0.473** | **0.475** | **0.002** |
| 3 | **30-35** | **0.480** | **0.483** | **0.003** |
| 4 | **30-35** | **0.475** | **0.479** | **0.004** |
| 5 | **30-35** | **0.493** | **0.495** | **0.002** |
| 6 | **30-35** | **0.523** | **0.553** | **0.03** |
| 7 | **35-40** | **0.557** | **0.532** | **-0.025** |
| 8 | **35-40** | **0.590** | **0.583** | **-0.007** |
| 9 | **35-40** | **0.564** | **0.583** | **0.019** |
| 10 | **35-40** | **0.553** | **0.554** | **0.002** |
| 11 | **35-40** | **0.504** | **0.509** | **0.005** |
| 12 | **35-40** | **0.473** | **0.477** | **0.004** |
| 13 | **40-45** | **0.247** | **0.255** | **0.008** |
| 14 | **40-45** | **0.235** | **0.239** | **0.004** |
| 15 | **40-45** | **0.235** | **0.236** | **0.001** |
| `16 | **40-45** | **0.223** | **0.224** | **0.001** |
| 17 | **40-45** | **0.205** | **0.211** | **0.006** |
| 18 | **40-45** | **0.199** | **0.212** | **0.013** |
| 19 | **45-50** | **0.835** | **0.840** | **0.005** |
| 20 | **45-50** | **0.760** | **0.760** | **0** |
| 21 | **45-50** | **0.828** | **0.831** | **0.003** |
| 22 | **45-50** | **0.763** | **0.765** | **0.002** |
| 23 | **45-50** | **0.657** | **0.664** | **0.007** |

The activity was gotten by subtracting the absorbance at 0secs from the absorbance at 30secs.

**APPENDIX IV**

For the purification table, the values were obtained through the following methods.

Total Protein (TP), A = ECL

Total Activity, T A = activity × volume

Specific Activity, S A = activity ÷ mg of protein

Purification Fold, P F = specific activity of each fraction ÷ specific activity of the crude.

Percentage(%) Yield = total activity of each fraction ÷ total activity of the crude × 100÷1



**APPENDIX V**

