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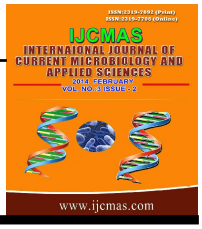


**Original Research Article**

# Effects of calcium on the purification of linoleic acid by lipooxygenase (E.C.1.13.1.13) From coconut (*Cocos nucifera)*

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**A B S T R A C T**

Peroxidation or auto-oxidation occurs in animal cells as well as in plant cells. It causes the off-flavors and odours observed in oils and supplies free radicals that initiate further peroxidation in animals which ultimately produces many pathological problems within the organisms. Peroxidation is catalysed *in vivo* by heme compounds and by lipoxygenase found in platelets and leukocytes. Lipoxygenase (EC.1:13.1.13) plays a key role in the oxidation of unsaturated fatty acids containing methylene interrupted system such as linoleic, linolenic and arachidonic acids to 1,4-cis-trans conjugated diene hydroperoxide. This enzyme was extracted from coconut *Cocos nucifera* and purified by ammonium sulphate precipitation, gel filteration on sephadex G-25 and by dialysis. The enzyme was purified about 70 folds and 30% of the enzyme activity was recovered. The enzyme was activated by addition of calcium chloride solution. Coconut is therefore a good source of lipoxygenase for biotechnological processes.

**K e y w o r d s**

Peroxidation, Lipoxygenase, purification, calcium and Coconut.

## Introduction

The coconut tree otherwise known as *Cocos nucifera* grows to a height of several meters, though dwarf species are known to occur and are only few. The stem of coconut is stout and may be 30 to 40cm in diameter. The life of coconut can extend to sixty years or more under favourable conditions. Coconut is essentially oil though every part of the palm is useful to mankind in some way. The oil is extracted by crushing the copra in rotary mills, expellers and hydraulic presses. Coconut oil has a high specification value, a low iodine number, and is used extensively for edible and industrial purposes (Thampan, 1981).

At present, the coconut palm is propagated only through seed (Menon and Pandaleu, 1958) though recent successful attempts to produce plantlets through tissue culture methods have been reported (Raju *et al*. 1984). Once the palm begins to bear fruit, it continues till year round throughout its life. Leaves are produced continuously at intervals of about one month. An

inflorescence is produced in each new leaf axil and since the coconut palm produces receptivity period of the female flowers to the male flowers giving rise to utilization varies with the season but usually lasts 4 to 7 days depending on the number of female flowers.

Both wind and insects are considered to be important pollinators in coconut (Harries, 1974). About 25 to 40% of the female flowers produced develop into mature nuts. After fertilization, the female flowers take about 11 to 12 months to mature into nuts. The fruit of the coconut is botanically known as a fibrous drupe and popularly as the nut. Thus, for propagation, open-pollinated, well-matured nuts are collected from high yielding selected mother palms. Selection is based on desirable phenotypic characters associated with high yield of nuts and copra output.

The seed nuts begin to germinate about 2 to 3 months after planting on sandy soil with high salinity and abundant sunlight and high humidity. When the seedlings are about 10 to 12 months old,a rigorous selection made based on characteristics such as early germination, good girth at collar and a large number of leaves.

The tall variety is late bearing, requiring 5 to 8 years for initial flowering whereas the dwarf variety of coconut bears fruit in the third and fourth years while green, yellow and red or orange and are planted mainly for ornamental purposes. Dwarf palms live up to 40 years.

*In vivo*, lipoxygenase has a serious deleterious effect. It has been implicated in several pathological conditions and such becomes imperative to be studied. Lipoxygenase has also shown to have economic effects in many industrial processes like in the manufacturing of liquid substituent like margarines. In the whole experiment, lipoxygenase activity was tested in order to ascertain the fate of the enzyme if it were to be treated with calcium solution.

## Materials and Methods

### Sources of coconut and identification

The consumable layer of coconut together with its rocky ectocarp was bought from a local market in Nsukka, Enugu state, Nigeria. The coconut was broken with the aid of a hammer. After which, the consumable layer usually white in colour but has a brown outer covering was removed and ground using a mortar and pestle.

### Apparatus

Test tubes Pyrex, Magnetic Stirrer Pyrex, *Co*nical flask Pyrex, Pipettes Pyrex, PH meter Gallenkamp, Glass rod Pyrex, Mortar / Pestle Nsukka, Aluminum foil Jazo , Refrigerated centrifuge Gallemkamp, Column Pyrex , Weighing balance Methey, Beakers Pyrex, Spectrophotometer and Measuring cylinder Pyrex .

### Chemicals

Disodium hydrogen phosphate (hydrous) BDH, Sodium dihydrogen phosphate (hydrous) BDH,NaOH BDH, Phosphoric acid May and Barker, Tween 80, PFC (Pharmacia fine), Dilute HCI May and Barker, Ammonium sulphate BDH, Linoleic acid BDH, 10-15% CuSO4-nH2o Merck, Calcium chloride Sigma, Distilled water Nsukka, Oxygen gas Nsukka, Folin ciocateur May and Baker,

Sephadex G-25 PFC (Pharmacia fine) and Natrium carbonate M & B.

## Methods

### Buffer preparation

The quantity of solutions used in buffer preparation is as follows: 11.996g of sodium dihydrogen phosphate ($NaH_2P0_4$) and 35.814g of disodium hydrogen phosphate ($Na_2Hpo_4$) were each dissolved in l000ml of distilled water The buffer was prepared when needed from these constituents. Firstly, 300m1 of $NaH_2Po_4$ was mixed with 16m1 of ($Na_2Hpo_4$) to get a phosphate buffer of pH 7.0.

### Extraction of lipoxygenase

Coconut (272.6g) was ground using pestle and mortar until it was finely. After this, it was homogenized using 250ml of phosphate buffer and was subsequently strained using a double layer of cheese cloth. The filtrate was then collected in a beaker while the cake was discarded. The filtrate was later centrifuged at 10, 000Xg for 20 minutes. The resultant supernatant was recovered as the crude enzyme; the volume of the supernatant was then measured using cylinder and stored in the refrigerator at 2°C.

### Preparation of substrate solution

About 0.5m1 of linoleic acid was pipetted into a beaker. This was followed by introduction of 0.5m1 of tween 20 into the same beaker. Later 0.1M NaOH was introduced into this mixture drop by drop until the mixture became clear. The clear mixture was poured into a measuring cylinder and made up to 25ml with distilled water. Throughout this process, the mixture was shaken to get a homogenized solution. The eventual mixture was stored in a refrigerator.

### Preparation of calcium solution

0.11g of calcium chloride was dissolved in 1000ml of distilled water to get 1mM of $CaCl_2$ solution. This solution was stored in a reagent bottle and labeled accordingly.

### Protein content determination

The Lowry method for determination of protein was used. 2% NaCO3 was prepared by mixing 2g of disodium carbonate with l000ml of 0.1M NaOH. 49ml of this solution was poured into a conical flask with a subsequent introduction of 0.5ml 10:15% CuSO4. nH2O. Later, 2.7g of Sodium potassium - to was dissolved in lOOmIs of distilled water to get 0.1M Sodium potassium to. 0.5ml of sodium potassium tartarate was then mixed with the other substituents. After ten minutes, 2ml of distilled water was mixed with 2m1 Folin ciocateur and 0.5ml of this mixture was introduced into the mixture in the flask. The content was allowed to, incubate at room temperature After incubation, 5ml) of this mixture was added into three different test tubes with a corresponding addition of 0.5ml of the crude enzyme and read with visible spectrophotometer at the wave- length of 700nm before taking their absorbance. Some quantity of the prepared substrate was flushed with oxygen gas for two minutes and this provides the appropriate environment for the enzymes optical density determination. Later, 2.8ml of this flushed substrate was emptied into a test tube. The control had 2.9ml of the substrate in it. In those test tubes containing 2.8ml of the substrate was added 0.lml of calcium chloride solution. This was followed with inclusion of 0.lml

of the lipoxygenase to make up to 3m1 in each test tube. To the control, was added 0.lml of the enzyme and the whole test tubes were read in a spectrophotometer cell using a wave- length of 480nm. The change in optical density of the whole test tube content was used to determine the absorbance of the lipoxygenase in the absence of calcium

**Partial purification of the crude enzyme 20- 80% ammonium precipitation**

The remaining crude enzyme which was stored in the refrigerator was measured using graduated cylinder for ammonium sulphate precipitation. The crude extract was precipitated using 21.8g of solid ammonium sulphate to the sample with consistent stirring until the mixture was saturated. The saturated extract was stored in the refrigerator for one hour at later, the refrigerated solution was centrifuged at 10,000rpm for 20mins. It was later observed that after this time, some proteins settled at the base of the centrifuged enzyme. A middle layer of the column was collected with gradual removal of the upper-most layer which contained some lipids. More solid ammonium sulphate was added to the middle layer for more precipitation and centrifuged as before after cooling for one hour in the refrigerator. The proteins that were precipitated were dissolved in the initial buffer (1:2) and enzyme activity as well as protein content in the precipitated protein was determined. Following this was an eventual pouring of the remaining enzyme into measuring cylinder to ascertain it's volume and thereafter pouring it into analysis sac for dialysis.

**Purification by dialysis**

With the enzyme in the dialysis sac, a beaker was two-third filled with the original buffer and the dialysis sac with the enzyme in it was kept in the refrigerator. After every four hours, the buffer in which the dialysis sac was submerged was changed with a new buffer making sure that the buffer covered the sac completely. This technique was done three times. After which, the content of the dialysis sac was removed into a beaker and protein content as well as enzyme activity was determined in the enzyme after measurement of the volume of the dialyzed enzyme. The remaining solution was stored in the refrigerator at -2°C for purification.

## Results and Discussion

From Table 1 above total protein increased from 0.27 in the crude enzyme to 1.90mg/ml in ammonium precipitate. This value is also greater than that obtained in dialyzed sample 0.86 and that of the ion exchange chromatography. In essence, ammonium precipitate had the greatest protein while ion exchange had the least protein. Specific activity was greatest in crude enzyme without calcium when compared to what happens in other purifying stages. It was least in desalting. In the presence of calcium appreciable increase in specific activity was equally seen in the crude enzyme while that of desalting was much bigger than it was in the absence of calcium. Percentage yield was greatest in the crude enzyme both in the presence and absence of calcium. This value decreased as the process of purification proceeds. Purification fold was least in the crude both in the presence and absence of calcium. The value was greatest in ammonium precipitate followed by ion exchange; all in the presence and absence of calcium. Henceforth, crude enzyme had the greatest percentage yield as shown in (table 1).

**Table.1** The result obtained from the purification steps both in the presence and absence of calcium

| Purification Step | Volume (ml) | Total protein (mg/ml) | Specific activity (unit/mg) without calcium | Specific activity (unit/mg) with calcium | Yield (%) without calcium | Yield(%)with calcium | Purification fold with calcium | Purification fold without calcium |
|---|---|---|---|---|---|---|---|---|
| Crude lipoxygenase extract | 250 | 0.27 | $8.88x10^{2}$ | 1.02x10-1 | 100 | 100 | 1 | 1 |
| Ammonium Sulphate precipitation | 100 | 1.90 | $3.157_{X}\ 10^{-2}$ | $4.0X10^{2}$ | 14.2 | 15.2 | 39.36 | 35.55 |
| Desalting using dialysis | 50 | 0.86 | $7.744x10^{-3}$ | $9.69x10^{3}$ | 1.744 | 1.898 | 4.745 | 4.360 |
| Iron exchange chromatography using sephadex G-25 | 1.5 | 0.23 | $3.04x10^{-2}$ | $4.35x10^{-1}$ | $2.05x10^{-1}$ | 2.56 | 6.40 | 5.135 |

Calcium ion has an activating effect in the activity of coconut lipoxygenase. In addition of calcium, a considerable decrease in the activation energy of the system was observed (Meyes, 2000).
Studies have been carried out on lipoxygenase in quantifying its characteristics in various biochemical conditions. Therefore, the essence of this research was to further throw more light on what happens to the enzyme if it was treated with small amounts of calcium ion. The enzyme was sourced from coconut (*Cocos nucifera*).

The protein content of coconut lipoxygenase (EC.1.13.1.13), value got from ammonium sulphate precipitation had the largest protein content while that from ion exchange chromatography had the least quantity of protein in it.

Specific activity was greatest in crude enzyme without calcium when compared to what happens in other purifying stages. In the presence of calcium appreciable increase in specific activity was equally seen in the crude enzyme while that in desalting was much bigger here than it was in the absence of calcium. Percentage yield was greatest in the crude enzyme both in presence and absence of calcium.

In the whole experiment, dual verification was carried out. Firstly, the enzymes activity with calcium and that in the absence of calcium. In conclusion, the enzyme activity of lipoxygenase could be activated in the presence of calcium.

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