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Original Research

Effects of some Activators and Inhibitors on the Activity of Palm Kernel Lipoxygenase

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Palm kernel lipoxygenase was partially purified and the effects of some chemical compounds as inhibitors and activators to the enzyme's activity were studied. The enzyme was purified **7** fold in ammonium sulphate precipitation, dialysis and gel filtration on Sephadex G-120 with the addition of 0.5mM $CaCl_2$ while it was purified 35 fold in the absence of $CaCl_2$. $AgNO_3$ was a competitive inhibitor, p-chloromercuribenzoate was uncompetitive and KCN was non-competitive to the enzyme's oxidation of linoleic acid. $NiCl_2$ was the least metal inhibitor to the enzyme's activity with Ki of 2.46 mM while $ZnCl_2$ was the highest metal inhibitor with Ki of 0.58Mm. The divalent cations were more activating to the enzyme's activity than non-metal reagents studied. The results of the experiments showed that palm kernel lipoxygenase had Fe^{3+} as its prosthetic group and some chemical compounds were activators while some were inhibitors to the enzyme's activity.

Keywords: Palm kernel, lipoxygenase, activators, inhibitors, enzyme activity, linoleic acid.

INTRODUCTION

Chemical compounds that bind to enzyme and thereby reduce or stop enzyme activity are called inhibitors and they could be reversible or irreversible inhibitors Activators on the other hand, are those chemical compounds that increase the activity of an enzyme upon binding to it [1]. Some chemical compounds had been found to either activate or inhibit lipoxygenase activity, According to Stevens *et al.*[2], Lipoxygenase is not inhibited by pyrophosphate, fluoride, cyanide, azide, mercury ions and diethyldithiocarbamic acid. The results are contrary to those obtained by Al-Obaidy and Siddiqi [3] in which they reported that the compounds gave varying degrees of inhibition to the activity of broad bean lipoxygenase. Kermasha and Metche [4] reported that potassium cyanide doubled haricot lipoxygenase activity at the enzyme concentration of 40mM.

Lipoxygenase (Linoleate:Oxygen oxidoreductase EC 1.13.11.12) catalyzes the oxidation of 1 - cis - 4 cis unsaturated fatty acids (that is, fatty acids containing cis-cis methylene interrupted diene structures) in the presence of oxygen to produce cis, trans-conjugated hydroperoxide as primary products [5,6.7]. Examples of its substrates are linoleic, linolenic and arachidonic acids [8,9].

The enzyme initiates free radical chain reaction in lipids [10,11]. Its primary products, cis, trans-conjugated diene hydroperoxides and the breakdown of the primary products such as aldehydes, ketones, alcohols and short hydrocarbons are the causes of either desirable flavours like fresh-vegetable flavours and scents of flowers or undesirable off-flavours that occur in stored and processed foods [12,13,14].

The enzyme had been used to bleach carotene [15], yellow wheat flour to white one [16] and browned yam tubers to their original white or yellow colour [17].Since lipoxygenase had been used in biotechnology, it is important to know the inhibitors and activators of the enzyme. In this study, some other inhibitors and activators that affect the activities of palm kernel lipoxygenase were studied.

MATERIALS AND METHODS

The palm kernels from the palm tree (*Elaeis guineensis* Rees) were purchased at the local market at Nsukka in Enugu State of Nigeria. The palm kernels were dehulled mechanically in a grinding machine, sundried and stored at 25°C. All the experiments were average of triplicate samples.

Extraction of crude lipoxygenase

The palm kernels were blended in Hammer mill and mixed with 50mM phosphate buffer, pH 7.0.The mixture was filtered through a double layer of cheese-cloth. The filtrate was centrifuged at 2000 rpm, for 30 min at 5°c and the supernatant, which was the crude enzyme, was stored at 0°C.

Purification of crude enzyme

Solid ammonium sulphate was added slowly to the crude enzyme with stirring to 30% saturation. The precipitate was dissolved In 50mM phosphate buffer pH7.0 and dialyzed for 18 h against 200 ml of the same buffer with 3 changes. The dialyzed proteins were applied to Sephadex G-120 column (2.5 x 40 cm) previously equilibrated with the same buffer . The proteins were eluted with the 50 mM phosphate buffer pH 7.0 at a flow rate of 0.5 ml/min and at room temperature.

Fractions of 6ml were collected. Protein content and enzyme activity in each fraction were determined and those fractions that showed enzyme activity were collected. To these collected fractions were added 0.5 mM $CaCl_2$ and incubated at 30°C for 15 min. The mixture was then applied to the Sephadex G-120 column and eluted as previously described.

Determination of protein content and enzyme activity

The methods used to determine protein content and enzyme activity were as described by Anokwulu [18].

Inhibition and activation studies

The methods of Al-Obaidy and Siddiqi [3] were used to determine the effect of inhibitors and activators on palm kernel lipoxygenase. Exceptions to the methods were that 50mM phosphate buffer pH 7.0 was used instead of deionized water in dissolving the chemical compounds. Moreover, the enzyme was incubated at 25°C for 10 min in the various compounds. Then I ml of the mixture (enzyme and compound solutions) was added to 2.9 ml of the substrate and the enzyme activities were assayed.

Fe^{3+} in palm kernel lipoxygenase

The varying concentrations of $FeCl_3$ in Table 4 were dissolved in 50 mM phosphate buffer. The enzyme solution (1 ml) was added to 1 ml of each of the dissolved $FeCl_3$ concentration. After the incubation of the mixture of the enzyme and $FeCl_3$ solutions for 10 min at 25°C,1 ml of each of the mixture was added to the substrate, the absorbance was read at 234 nm and percentage activity of the enzyme found.

In further studies to determine the presence of iron molecule in the enzyme, modified method of Axelrod *et al.* [20] was used. The enzyme solution was dialyzed in a mixture of 0.5 mM sodium arsenite (to remove Fe^{3+} from the enzyme) and 300 ml of 50 mM phosphate buffer. For control, the enzyme solution was dialyzed in only 300 ml of 50 mM phosphate buffer. The dialyses were done for 18 h with 3 changes.

RESULTS AND DISCUSSION

Table I shows the purification profile for palm kernel lipoxygenase. As indicated in the table, there was increase in specific activity and purification fold as the purification of the enzyme proceeded from crude extract to gel filtration on G-120. the increases were due to more purification of enzyme at each stage of the purification. On the other hand, there was decrease in total protein and percentage yield from crude extract to gel filtration which indicated that there was increase in the lost of proteins (including the enzyme) as the enzyme was purified from crude to gel filterations steps. However, upon addition of $CaCl_2$, there was increase in the purification of the enzyme when compared with gel filtration on Sephadex G-120 except in percentage yield. The results therefore suggest that calcium increases total protein, specific activity and purification fold in the purification of palm kernel lipoxygenase.

Table 1. Purification profile of palm kernel lipoxygenase

Purification step	Total protein (mg/ml)	Specific activity (unit/mg protein)	Yield (%)	Purification (fold)
Crude lipoxygenase extract	61.16	0.56	100	1.00
30% Ammonium sulphate precipitation	47.92	2.01	81.12	3.59
Gel filtration on Sephadex G-120	30.18	3.92	72.16	7.00
Gel filtration on Sephadex G-120 + $CaCl_2$	32.46	6.18	70.85	11.05

$AgNO_3$ was a competitive inhibitor of palm kernel lipoxygenase (Fig.1). In the competitive inhibition, the inhibitor binds to the free enzyme and thereby prevents the binding of the substrate; the V_{max} remains unchanged while the K_m is altered and increased.

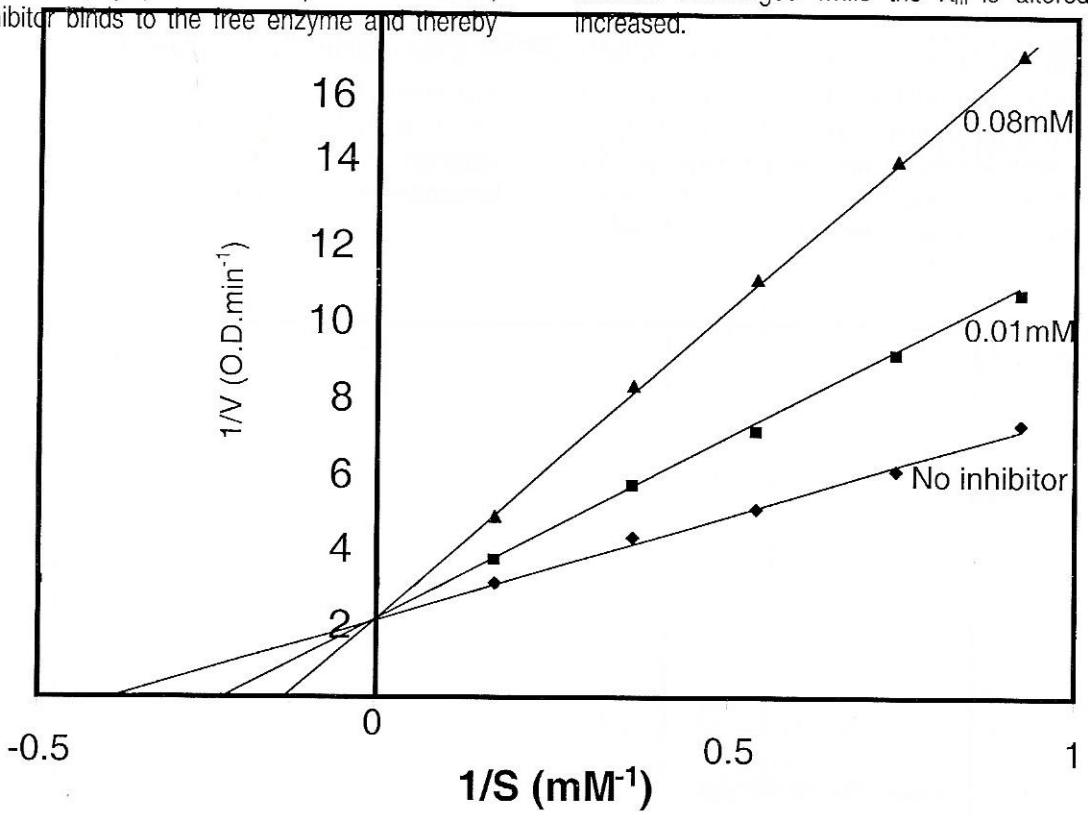

Fig. 1. Competitive inhibition of palm kernel lipoxygenase's oxidation of linoleic acid by $AgNO_3$

As indicated in Fig. 2, KCN was a non-competitive inhibitor of the enzyme's oxidation of linoleic acid. In the non-competitive inhibition, the inhibitor binds to either the enzyme or enzyme-substrate complex, resulting in reduced and altered V_{max} while K_m remains unchanged.

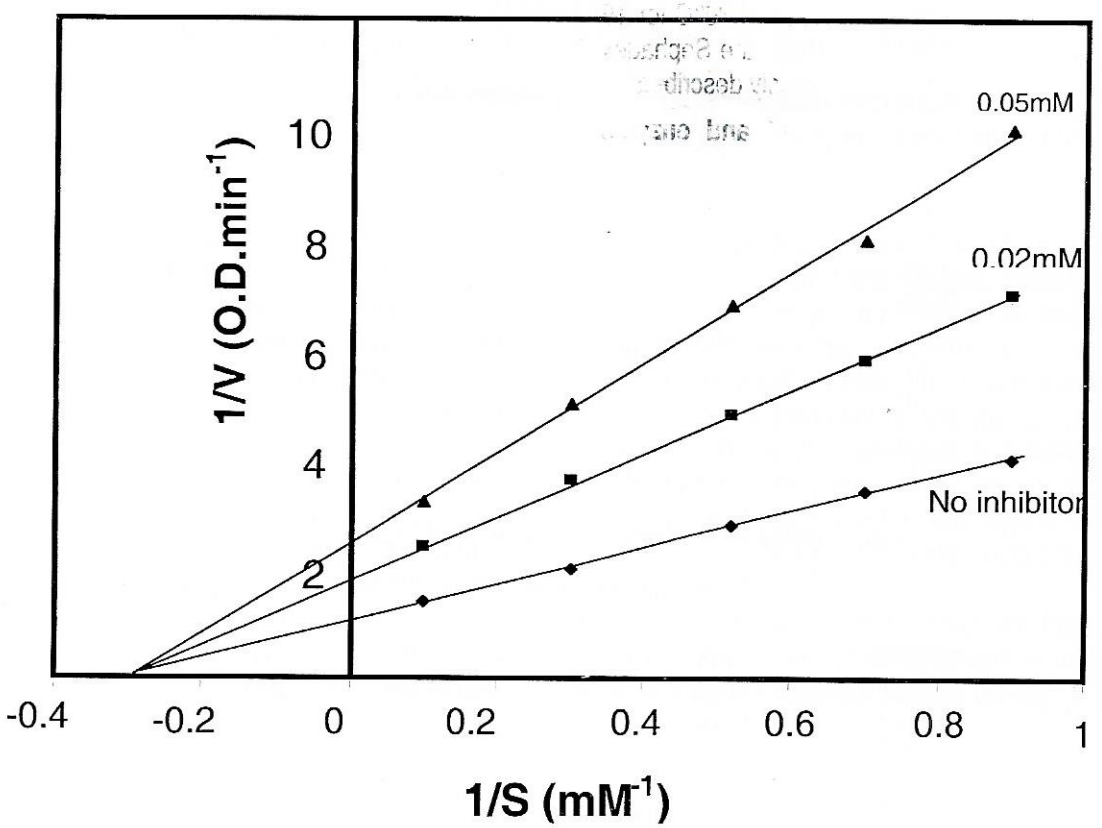

Fig. 2. Noncompetitive inhibition of palm kernel lipoxygenase's oxidation of linoleic acid by KCN

p-Chloromercuribenzoate acted as an uncompetitive inhibitor to the oxidation of linoleic acid by the enzyme (Fig. 3). In such uncompetitive inhibition, the inhibitor binds to only the enzyme-substrate complex and both the V_{max} and K_m are altered and reduced. These thiol reagents, namely $AgNO_3$, KCN and p-chloromercuribenzoate, might have acted as antioxidants by donating their hydrogen atoms to the free radicals formed at linoleic acid so that the oxidation of linoleic acid by palm kernel lipoxygenase is inhibited.

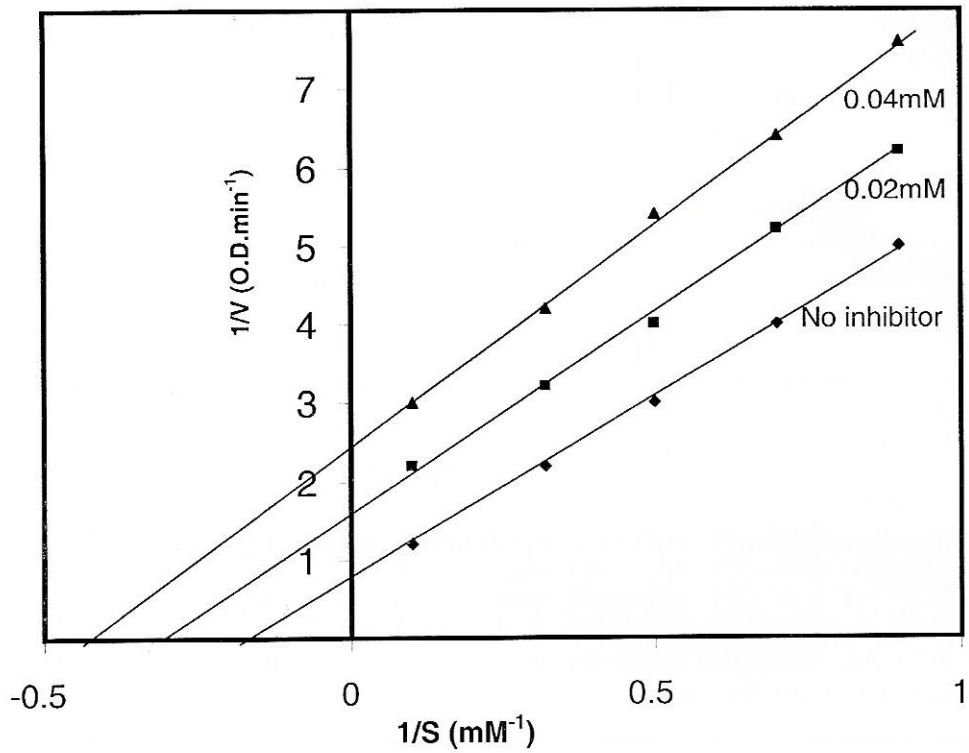

Fig. 3. Uncompetitive inhibition of palm kernel lipoxygenase's oxidation of linoleic acid by p-chloromecuribenzoate

As seen in Table 3, divalent cations (metal ions), namely $MnCl_2$, $MgCl_2$ and $CoCl_2$, seemed to have more activating effects on the oxidation of linoleic acid by the enzyme than the other three reagents with $CoCl_2$ having the most oxidating effect on the unsaturated fatty acid at the concentration of $2x10^{-3}$ M. According to Koshland [19], the binding of an activator on an enzyme alters the structure of the enzyme in such a way that the substrate can easily bind at the active site of the enzyme.

$NiCl_2$ had the least effect in the inhibition of the oxidation of the substrate by the enzyme (Table 2) since it had the highest inhibition constant of 2.46 mM while $ZnCl_2$ had the highest effect in the inhibition with Ki of 0.58 mM. The inhibitors in Table 2 might have decreased the oxidation of linoleic acid by the enzyme by binding at the enzyme and altering its structure so that linoleic acid cannot bind to its active site easily [19].

Table 4 indicates that there was inhibitory effect of Fe^{3+} on the oxidation of linoleic acid by palm kernel lipoxygenase and the inhibition increases as the concentration of Fe^{3+} increases. The results in the table suggest therefore that the addition of Fe^{3+} to the enzyme's reaction causes inhibition of the oxidation of linoleic acid.

Table 2. Effect of some metal inhibitors to the oxidation of linoleic acid by palm kernel lipoxygenase

Inhibitor	Mode of Inhibition	Concentration (x 10^{-3} M)	Inhibitor Constant ki (mM)
$ZnCl_2$	Competitive	0.1 1.0 1.5	0.58
$CuCl_2$	Competitive	0.1 1.0 1.5	0.91
$AlCl_2$	Non-Competitive	0.1 1.0 10.0	1.22
$NiCl_2$	Competitive	0.01 0.1 1.5	2.46
$BaCl_2$	Competitive	1.0 1.5 5.0	1.82
$HgCl_2$	Uncompetitive	1.0 2.0 5.0	2.03

Table 3. Effect of some activators on palm kernel lipoxygenase-catalyzed linoleic acid oxidation

Activator	Concentration (X 10^{-3} M)	Relative activity (%)
Control	-	100
$MnCl_2$	1.0	106
	2.0	110
	3.0	128
$MgCl_2$	0.5	135
	1.0	112
	1.5	102
$CoCl_2$	0.5	120
	1.0	140
	2.0	184
Oxyquinoline	1.0	104
	1.5	119
	2.5	101
o-Phenanthroline	0.5	125
	1.5	110
	2.0	104
Mercaptoethanol	1.0	103
	1.5	106
	2.0	115

Table 4. Effect of Fe^{3+} on the oxidation of lonoleic acid by palm kernel lipoxygenase

$FeCl_3$ (x 10^{-3} M)	Percentage activity (%)
No $FeCl_3$ (Control)	100
1.0	90
2.0	70
5.0	60
7.0	52
10.0	6

The dialysis of the enzyme solution in the presence of sodium arsenite showed no enzyme activity while dialysis of the enzyme solution without sodium arsenite showed 100% enzyme activity. There was still no enzyme activity in the enzyme solution dialyzed in a mixture of 0.5 mM sodium arsenite, $1x10^{-3}$ M $CaCl_2$ and 300 ml of 50 mM phosphate buffer. On the other hand, there was 108% enzyme activity in the dialyzed enzyme solution with $1x10^{-3}$ M $CaCl_2$, 300 ml of 50 mM phosphate buffer and without sodium arsenite, indicating that Ca^{2+} activiated the activity of the dialyzed enzyme. It then shows that sodium arsenite removed the Fe^{3+} present in palm kernel lipoxygenase. Therefore, Fe^{3+} could be a prosthetic group of the enzyme. Moreover, since there was no enzyme activity when the prosthetic group was removed during dialysis and there was 100% of enzyme activity when $FeCl_3$ was not added, it follows therefore that Fe^{3+} could be an important part of the enzyme.

Axelrod [20] noted that each of the three isoenzymes of soybean lipoxygenase contained one atom of Fe^{3+} as prosthestic group, Sanjay and Rajendra [7] contended that lipoxygense has iron atom at its active site. According to Al- Obaidy and Siddiqi [3], studies to investigate the presence of metal ion in lipoxygenase showed that only urd beans, mung beans and chick peas had Fe^{3+} as prosthetic group. Rafelson and Binkley [21] argued that ion in lipoxygenase was in the form of Fe^{2+} and they proposed that Fe^{2+} is oxidized to Fe^{3+} when Fe^{2+} loses electron to cis, trans conjugated diene hydroperoxyl radical during the oxidation of linoleic acid by the enzyme to form cis, trans conjugated diene hydroperoxide.

On the contrary, Stevens *et al.* [2] did to detect any presence of a prosthetic group in soybean lipoxygenase, Moreover; Yamamoto *et al.* [22] contended that lipoxygenase had no prosthetic group. Furthermore, Mita *et al.* [23] reported that lippoxygenase had no iron atom.

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