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Original Article

The Inhibitory Effect of KCN, NaN3 and some Bivalent Ions on Lipoxygenase Activity of the Purified Human Placental

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Abstract

Lipoxygenase (LOX) catalyzes irreversible transfer of oxygen molecule to Arachidonic and Linoleic acid to produce 13 Hydroproxy Octadecadienoic acid. Recent studies showed that the involvement of Lipoxygenase products, leukotrienes, in inflammations and Lipoxygenase pathways acts as mediators of early inflammatory events in atherosclerosis. The aim of the present study is purification and characterization of Lipoxigenase from Human placental.For this aim, the human placental Lipoxigenase was extracted and purified by normal butanol, acetone, ammonium sulphate (30-80%), and gel permeation chromatography on Sephadex G-150. After purification and characterization of LOX, the *in vitro* inhibitory effect of KCN, NaN3 and some selected bivalent ions such as Co^{2+}, Ni^{2+}, Cu^{2+}, and Zn^{2+} were checked on the activity of purified LOX. Results showed that specific activity was 123.16 u/mg proteins and the yield of purification was 21.84 percent. Also, it was found that Co^{2+}, Ni^{2+}, KCN, and NaN_3 at concentration of 20 mM had inhibitory effect on LOX activity and their inhibitory was 72.4, 58.2, 56.5 and 42.3% respectively. However, Cu^{2+} stimulated the lipoxygenase activity at the same concentration whereas Zn^{2+} has no significant effect on LOX activity. With respect to increase of LOX activity in the patient with cardiovascular diseases, Alzheimer disease, cancer, chronic obstructive pulmonary disease (COPD), artherogenesis, and also airway inflammation diseases, suggesting that LOX inhibition may have beneficial effects as a potential target to limit the severity of related symptoms of these diseases and therefore these inhibitors could be considered as an agent for decreasing the enzyme activity in association with the disease.

Keywords : Bivalent ions, Chemical Inhibitors, Chromatography, Humane placenta, LOX activity, LOX isolation.

1. Introduction

Lipoxygenases (LOXs) are dioxygenase enzymes that incorporate molecular oxygen into unsaturated fatty acids such as arachidonic acid , linoleic acid and produce 13-hydroperoxy octadecadienoic acid; in simple terms it catalyzes the oxidation of polyunsaturated fatty acids to form a peroxide of the acid [1]. Lipoxygenase is considered to be a key enzyme in the oxidative degradation of lipids (Hugo T. purified LOX for the first time in 1945) [2]. This enzyme is widely distributed throughout the plant and animal kingdoms, and is named according to their substrate. Depending on the source, lipoxygenase may be found in the form of

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various isoenzymes which differ from each other with respect to pH optimum, substrate specificity and degree of inhibition by various agents. The type of substrate used in the reaction also alters the activity of LOXs isozyme due to the preferences for different substrates [3]. The Lipoxygenases are a class of non-heme, iron-containing dioxygenases that use molecular oxygen for the oxygenation of polyunsaturated fatty acids with a 1, 4-cis, cis pentadiene moiety [4].

Originally it was thought that LOX occurred only in seeds of leguminous plants and some cereals [5] but now it is known to be distributed amongst higher plants too rich sources being soybean [6]and avocado peels [7]. In 1993, researchers isolated two isoenzymes of LOX from uterus of non smoking females by chromatographic techniques [8]. Various research conducted have led to the isolation of LOX from germinating barley [9], red Korean potato and Indian corn which produce the fresh smell of corn [10]. Lipoxygenase oxidizes the essential PUFA into stereo and regio- specific hydroper oxides which can in turn be converted to ketons as well as carbonyl compounds which contribute to the fresh flavor of vegetables or the off flavor due to degradation reactions as a result of storage [11]. Also Erika *et al*. (2003) isolated LOX from human platelets [12].

In animals, the LOX pathway is responsible for the production of the physiologically-active leukotrienes and lipoxins [13]. In mammals a number of lipoxygenases isozymes are involved in the metabolism of prostaglandins and leukotrienes. Several reports have indicated LOXs to be invoked in many diseases [1, 13, 14, 16]. As suggested by researchers, lipoxygenase seems to be implicated in the progression of certain cancers [16, 17], chronic obstructive pulmonary disease (COPD) [18], cardiovascular complicating and atherosclerosis [19], artherogenesis [20, 21] and airway inflammation [22]. Generation of oxidized LDL, modified LDL, and expression of some key proinflammatory proatherosclerotic such as Th1 cytokine and interleukin (IL)-12 are also increased by LOX [23].

The precise function of different lipoxygenase isoforms is still a matter of discussion. In addition, they have been reported to be involved in lipid reserve mobilization from lipid bodies [24, 25]. Major roles for products of the lipoxygenase pathways are in defense against pathogen attack, wounding and senescence [3, 26, 27, 28, 29]. A widespread of compounds can inhibit lipoxygenase activity in various sources, but effects of KCN, NaN_3 and bivalent ions of Co^{2+}, Ni^{2+}, Cu^{2+}, and Zn^{2+} on placental lipoxygenase activity has not yet been investigated. Thus, in the present work, the effects of aforementioned substances on placental lipoxygenase activity were studied.

2. Materials and Methods

2.1. Homogenization and Defatting

The placenta was washed in cold saline (4°C) 3 times and after removal of the outer layer, it was cut into pieces and homogenized in a homogenizer with 4 volumes of saline. After homogenizing, it was then centrifuged (1208 g, 10 min, 4ºC). The precipitate was discarded and supernatant was subjected to butanol treatment (4ºC, v/v) for removal of pigments .The mixture was centrifuged (1208 g, 10 min, 4ºC) the supernatant was discarded and lower layers were subjected to defatting. To eliminate the interference of lipids, sample defatting was done by using cold acetone (4ºC) followed by mechanical stirring for 24h after suspension of defatted enzyme in saline (1:10 w/v) [30] and then the resulting suspension was centrifuged (1208 g, I5 min) and the supernatant was discarded whereas the pellet was subjected to further purification.

2.2. Partial Purification and Protein Determination of LOX

All the purification steps were performed at a temperature of 4ºC unless stated otherwise. The partial purification of LOX was first initiated by the addition of solid ammonium Sulfate at 30% of saturation. The suspension was allowed to stand for 5 h and then centrifuged at 1208 g for I5 min to obtain the precipitate (0-30%). The resulting supernatant was saturated with 80% solid ammonium sulfate and the precipitate (30-80%) was obtained after centrifugation

(1208 g, I5 min). The subsequent supernatant was discarded and precipitate was collected and resuspended in 20 ml saline (4ºC). This solution was dialyzed against saline solution for a period of 24 h. The desalted enzymatic fractions were then subjected to permeation chromatography, kinetic studies and electrophoretic analyses after determination of protein concentration according to Lowry method [31]. Bovine serum albumin (Sigma Chemical Co., St-Louis, MO) was used as a standard for calibration.

2.3. Gel Permeation Chromatography

The partial purification was carried out by size exclusion chromatography using a Sephadex G-15ºcolumn. The column was equilibrated with sodium phosphate buffer solution (0.01 M, pH 7.0) and the sample (3 mg protein/ml) was solubilized in 200 µl of the buffer solution. Elution was performed at a flow rate of 0.5 ml/min and 3ml fractions were collected. The collected fractions were subjected to enzyme kinetics and protein determination.

2.4. Substrate Preparation

Substrate standard used for this study, included linolenic acid (cis-9,cis-12,cis-IS octadecatrienoic acid (purchased from Sigma Chemica1 Co). The preparation of stock solutions of substrate (5m M) was performed according to the procedure described previously by Kermasha *et al*. with slight modification [32].

2.5. Enzyme Assay

LOX activity was spectrophotometrically measured (Perkin Elmer, lambda 2) in accordance with the procedure described by Kermasha and Metche [32]. The reaction medium consisted of enzyme extract (0.5 ml), substrate solution (0.2 ml) to adjust the final volume with phosphate buffer to 5 ml for determination of enzyme activity in the partially purified extract (butanol, acetone and ammonium sulfate) and the purified fractions (chromatography). The specific activity of LOX was expressed as the increase in A (mg of protein)$^{-1}$ min^{-1} in 1964 by Surrey [33], where A is equal to 0.001 absorbance al 234 nm as reported by Ali Asbi *et al*. [34]. The purified extract was subjected to enzyme assay for optimum pH studies using sodium phosphate buffer (0.1 M, pH 3.0 - 8.0).

2.6. Effect of Inhibition and Activation on Enzyme Activity

The effect of KCN, NaN_3, Cu^{2+}, Zn^{2+}, Co^{2+} and Ni^{2+} on the LOX activity of the purified extract was determined using the enzyme assay procedure described above. The only difference was the adding of 0.2ml inhibitors (5mM) into each test tube before adding and incubating the substrate at room temperature for 16 h. The percentage of inhibition was spectrophotometrically determined at 234nm for control and samples.

2.7. Electrophoresis

10% Polyacrylamide gel electrophoresis (PAGE) was performed on fractions exhibiting high specific activity. The electrophoresis run and the staining of the separated protein bands were performed in accordance to the procedure described by Reisfeld *et al*. [35]. In this study, one unit of LOX activity is that amount of enzyme required to produce 1 µM 13-hydroperoxy octadecadienoic acid per hour at 28˚C and pH 5.

3. Result and Discussion

3.1. Partial purification of lipoxygenase

Table 1 shows a scheme of the partial purification of the LOX extract from human placental cells. The results indicate that the fraction precipitated by acetone, ammonium sulfate at 30-80% of saturation, and chromatography have 1.67, 12.8, and 34.59 fold purity respectively, compared to that of the Butanol precipitation. The results also show that the fractions precipitated by 80% saturation of ammonium sulfate have the highest specific activity with 45.65 u/mg, however it did not exceed 26.9% of the total LOX activity.

In addition, our finding showed a 12.8 fold increased in LOX purified from Human placental by am monium sulfate precipitation at 30-80% of saturation. In the case of acetone precipitation a recovery of 29.9% and a 1.67 fold increase in purification was observed compared to the control.

Table 1. Purification of lipoxygenase enzyme from human placenta.

Purification step	Vol. ml	Total Protein	Total	Specific activity	Recovery	Fold
Butanol extract	340	9.5	33.83	3.56	100	1
Acetone extract	290	1.7	10.14	5.95	29.9	1.6
Ammonium sulfate (30-80%)	125	0.2	9.13	45.65	26.98	12.8
Chromatography on sephadex G150	45	0.06	7.39	123.16	21.84	34.59

3.2. Electrophoresis

PAGE electrophoresis was performed to check the sample purity and possible LOX isozymes. From the 50 fractions retrieved fromchromatography techniques, 3 fractions with the highest specific activity were combined to determine the enzyme assay and electrophoretic profile. From the electrophoresis conducted one major band was detected which is the result of extensive purification and indicate the presence of one isozyme of LOX, figure 1.

3.3. Optimum pH and Kinetics of the Purified Lipoxygenase

To obtain the optimum pH for purified LOX, its activity was determined in a wide pH range from 3.0-8.0. The results indicate that the maximum activity was seen at pH 5, which suggests for studying its kinetic factors by using this optimum pH (table 2) and also confirm electrophoresis data about presence of just one isozyme for our purified LOX. table 2 shows that the apparent Km values at pH 5.0, for the LOX extract from human placenta, which were calculated using Lineweaver-Burk plots [36]. The km value obtained was 4.761 X10-2 M and the Vmax was 34.59 U/ml.

3.4. Effect of KCN, NaN_3 , CO, Cu^{2+}, Ni^{2+}, ZN^{2+} on Enzyme Activity

Our data concerning the effects of Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, KCN, and NaN_3 on the activity of purified placental LOX are summarized in table 3. At a concentration of 20 mM of NaN_3, or KCN the enzyme activity was reduced to 57.6% and 43.4% compare to the control respectively figure 2, the inhibitory effect was 42.4% and 56.6%. Ni^{2+} seemed to decrease the enzyme activity at 20mM resulting in the same amount of decrease as KCN being about 58.2% inhibitory effect on LOX. Although CO^{2+} was functioned better than the rest and decreased the enzyme activity to 27.6% with having an outstanding inhibitory effect of 72.2% proving to be the best inhibitor amongst the other inhibitors used in this experiment. At a concentration of 20mM, Cu^{2+} increased the enzyme activity to about 40% compare to the control and Zn2+ has no significant effect on LOX activity.

4. Conclusion

In the present study, conducted n-butanol

Table 2. kinetic parameters of purified lipoxygenase extract.

Enzyme	pH optima	Kinetic studies	
		K_m (μM)	V_{max} units
Lox from placenta	5.0	4.761	34.59

Table 3. The profile effect of 20 μM concentration of Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, KCN, and NaN_3 on activity of purified human placental Lipoxigenase.

Lipoxigenase activity without Metal Ions	enzyme with Co^{2+}	enzyme With Ni^{2+}	enzyme with Cu^{2+}	enzyme with Zn^{2+}	enzyme with KCN	enzyme with NaN_3
U/ml						
7.39	2.04	3.09	10.12	7.1	3.21	4.26

is used as a solvent for extracting the pigments and lipids present in the study material. Cold Butanol is used drop by drop so as to prevent the denaturing of the proteins. Acetone is used to precipitate the proteins as it breaks the hydrogen bonds between the amino acids of a protein and causes the proteins to lose their tertiary structure and break off their bonds with the water molecules and precipitate at the bottom. This solvent is also used in cold conditions to avoid denaturing of the proteins. Ammonium sulfate is used at 30-80% saturation to precipitate proteins. 30% saturation causes proteins with high molecular weight to precipitate while as the saturation increases the molecular weight of the proteins precipitated decreases accordingly. By removal of the ammonium sulfate, acetone and n-butanol proteins renature to their original tertiary structure.

Figure 1. Electropherogram of purified enzymatic extract from human placenta.

We have previously reported the effect of Mg^{2+}, Fe^{2+} and Mn^{+2} on Soya bean purified Lipoxigenase (37). Our previously reported data showed that the Soya been lipoxygenase activity was inhibited by Mg^{2+} and Mn^{2+}, however it was increased by Fe^{2+} in the reaction mixture sharply. In this study, the lipoxygenase was purified from human placental and we showed that the presence of some selected components or divalent cations such as KCN, NaN_3, Co^{2+}, and Ni^{2+} have inhibitory effect on the LOX activity in the reaction mixture. Cu^{2+} increased the LOX activity whereas Zn^{2+} has no significant effect on LOX activity. The possible mechanism for this inhibition could be through the competitive reduction of ferric ion, which is necessary for LOX activity. Our data demonstrate that KCN, as well as NaN_3, at concentration of 20 μM can readily reduce ferric ion, suggesting that they may have a similar reduction phenomenon with the ferric ion on the human placental LOX activity, leading to enzymatic inactivation. On the basis of our results Co^{2+} had the highest and NaN_3 had the lowest inhibitory effect on activity of human placental lipoxygenase.

Unlike the Co^{2+} and Ni^{2+}, we believe that Cu^{2+} cause the enzyme conformational change, causing higher affinity of the enzyme to the substrate and thereby increase the enzyme activity. Also, it is thought that these ions could be a part of the enzyme structure or they might be necessary for enzyme function as a co-factor for the enzyme activity, as the enzyme activity increases to a great extent in the presence of these ions.

The results gathered in this study indicated that the purification of human placen-

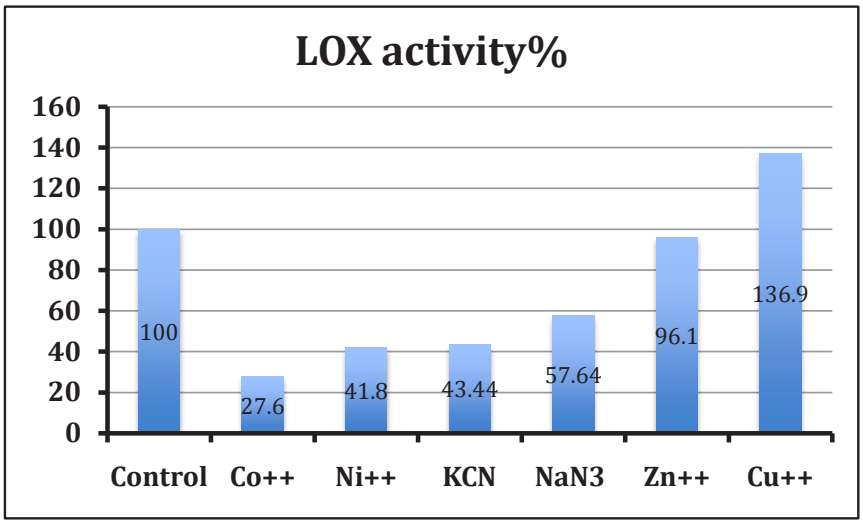

Figure 2. Effect of 20 µM concentration of Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, KCN, and NaN_3 on purified LOX activity of human placental.

tal cells led to the isolation of LOX enzyme, which showed optimal activity at pH 5.0. We have reported that some selected divalent ions, such as Co^{2+} and Ni^{2+}, partially inhibits activation of human placental LOX and activation of this enzyme is increased by some ions like Fe^{2+} [36] and Cu^{2+}. In addition, LOX activity is induced in various diseases including Alzheimer's disease [38], cardiovascular diseases [39], cancer [40], chronic obstructive pulmonary disease (COPD) [18], atherosclerosis [19], artherogenesis [20, 21] also airway inflammation [22], etc. therefore these inhibitors could be considered as an agent for decreasing the enzyme activity in association with the disease, however the nature and mechanism of this inhibition needs to be investigated.

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