Chapter

Lipoxygenase and Colon Cancer

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Abstract

The enzymes involved in the oxidative metabolism of n-6 polyunsaturated fatty acids, such as lipoxygenase (LOX) and cyclooxygenase (COX), are significant in the pathogenesis of colorectal cancer. The aim of this study is to estimate the effectiveness and partial purification of LOX and measure gamma-glutamyl transferase (GGT) activity in the serum of patients with colon cancer in Baghdad. The study included samples from 80 male patients with colon cancer and 50 samples of apparently healthy males (control) as the comparison group. The result displayed a noteworthy increase in lipoxygenase effectiveness (805.0 ± 517.23 IU/L) in the serum of patients with colon cancer (stage pT3) compared with control (114.6 ± 49.77 IU/L). The enzyme was purified by the precipitation of the serum protein using 40% $(NH4)_2SO4$ and then removing the remaining salts by dialysis. The column of gel (Sephadex G.100) was used to separate the enzyme from another protein, in this step a single peak was obtained. The effective part of lipoxygenase is at yield 71.42% and folds 11.033. The ion exchange chromatography (DEAE–CeA50) was used to isolate LOX isoenzyme, and two bands (LOX1 and LOX2) were acquired with different degree of purity 16.372 and 12.16 folds, respectively. The result displayed a noteworthy increase in the GGT activity in patients (58.69 ± 16.94 IU/L) compared with control (12.79 ± 5.68 IU/L) $p \leq 0.0001$. The increase in the activity of LOX can be potentially used as a tumor marker for colorectal cancer.

Keywords: colon cancer, lipoxygenase (LOX), gamma-glutamyl transferase (GGT), LOX isoenzyme, linoleic acid

1. Introduction

Tumors usually arise as a result of mutations in the cellular DNA [1–7]. The mutations occur in two types of genes, oncogenes and tumor suppressor genes. Oncogenes stimulate cell division, and increasing the activity of these genes encourages cancer cells to grow abnormally and work on Protect cells from apoptosis. Tumor suppressor genes or apoptosis genes work to stop cell division and help the immune system protect tissue [8]. In the case of a tumor, these genes stop, because they oppose its formation by correcting errors in DNA transcription.

It should be noted that cancer occurs in all cases due to mutation, but not all mutations cause cancer. Cancer results from the abnormal activation of cellular genes that regulate cell growth and divisions. Determining the stage of the tumor expresses the extent of the tumor's progress and exacerbation and is necessary before starting the treatment. Thus we conclude that cancer is a disorder that results from the failure of cells to die, rather than the process of cell proliferation, as the proliferation is not matched by a sufficient number of cells that die, which leads to their accumulation [9–12].

The metabolism of fats in the human body, especially the arachidonic acid metabolism pathway, plays a major role in chronic inflammation and colon carcinogenesis [13], as phospholipase A2 (PLA2) enzymes stimulate the formation of free fatty acids such as arachidonic acid from phospholipids associated with the cell membrane, which have been shown to participate in the formation of cancer in laboratory mouse models [14].

LOX has an important role to stimulate inflammatory reactions. Reactive oxygen free radicals can cause inflammation that activates the release of cytokines and the activation of LOX. Inflammation is associated with many diseases, such as cancer, cardiovascular and neurodegenerative diseases. LOX contributes to the synthesis of leukotrienes and prostaglandins. These are associated with disease development [15]. The most important enzymes in the pathway of arachidonic acid metabolism [16] are LOX and COX, which are found in high concentrations in many tumors, including lung cancer [17], prostate cancer [18], brain cancer [19], rectal cancer [20], skin cancer [21], and breast cancer [22] where the GGT enzyme enters in the metabolic pathway of leukotrienes C4 [23].

2. Materials and methods

2.1 Collection of samples

Blood samples of 80 colon cancer patients (aged 40–80 years) who attend the Teaching Oncology Hospital at the City of Medicine and the National Center for Oncology, Baghdad for the period (18-2-2018 to 28-2-2019), were obtained.

A total of 50 blood samples were collected from apparently healthy individuals as a control group (aged 40–80 years). The samples were collected by drawing blood from the vein (5 mL) using a syringe and placing the blood in a gel tube.

The tubes were placed in the centrifuge at 1252 g for 10 minutes to obtain serum. The serum was kept by Eppendorf tube in deep-freeze at −20°C until testing.

2.2 Measuring the LOX activity in blood serum

The method of measuring the activity of the LOX enzyme [24] is based on stimulating the oxygen reaction with the unsaturated fatty acids containing (cis, cis −1.4-pentadiene). It consists of a sequential system of double bonds that increase absorption at a wavelength of 234 nm where the absorption intensity is directly proportional to the concentration of the enzyme [25]. The unit of enzyme is defined as the amount of enzyme that changes in absorbance by 0.001 / sec at wavelength 234 nm under ideal conditions.

2.3 Estimation protein concentration

The biuret method was used to estimate the concentration of the protein in the samples [26].

2.4 Separation and purification of LOX from serum patients of colon cancer

LOX is purified using the following steps:

2.4.1 Precipitation by ammonium sulfate

The serum proteins were deposed by adding 0.9 gm of ammonium sulfate (0.40%) to 4 ml of serum for patients with colon cancer, which was gradually added in ice bath with magnetic stirrer (15 minutes) until all the ammonium sulfate has been dissolved. Then the solution was placed in the centrifuge for 15 minutes and at a speed of 17,608 g to separate the precipitation from the leachate, the precipitate was dissolved with the least amount of the buffer solution (Buffer phosphate pH 7(0.001 M)). Then, the enzyme activity and protein concentration were measured.

2.4.2 Dialysis

The process of dialysis for the dissolved protein was done to remove the ammonium sulfate residues that were used to precipitate the proteins, using a dialysis bag. The dissolved protein was added into the bag and immersed in the buffer solution (Buffer phosphate (0.001 M) pH 7). This process was carried out for 24 hours, with the solution being changed periodically. This step of purification was done at 4° C to maintain the activity of the enzyme. The activity and protein concentration of the enzyme were measured after the end of the process.

2.4.3 Gel filtration

The gel filtration technique is based on the difference in molecular weights. This step was used to purify the LOX enzyme from proteins and associated salts. The filter column of the Sephadex G.100 was used.

- A column separating diameter of 2 cm and length of 70 cm with a filter at the end of which prevents the granulation of the resin outside was used, the process of casting the column was performed by using resin solution and pouring the resin solution on the walls slowly and homogeneously so as not to form air bubbles that impede the separation process, the column was then washed with a quantity of buffer solution (Buffer phosphate (0.001 M) pH 7), and the flow velocity was set at 1 mL/min.
- 4 mL of product in dialysis step were added slowly and gradually over the resin surface and on the column walls and left for 5 minutes to soak into the resin.
- The gel filtration process was initiated using 250 mL of the buffer solution (Buffer phosphate (0.001 M) pH 7). The extracts were extracted from the gel filtration column at a size of 5 mL per part.
- The activity and the protein concentration of the LOX enzyme were evaluated.

2.4.4 Ion exchange chromatography

This technique was used to purify the isoenzyme of the LOX.

- DEAE-Cellulose (A50) was prepared by dissolving 20 gm of DAEA-Cellulose A50 in 250 mL of Buffer phosphate pH 7, leaving the solution suspended for 24 hours and at 4° C. The solution was switched several times from time to

time to remove the soft minutes from the suspended solution until the pH reaches 7.

- NaCl solution (1 M) was prepared by dissolving 5.85gm of sodium chloride in 100 mL of (Buffer phosphate (0.001 M) pH 7) solution. Other solution was obtained with graduated concentrations of NaCl (0.1, 0.25, 0.5, 0.75 M).

- A glass column diameter of 3 cm and length of 30 cm contains a filter at the end, which prevents the resin granules from leaking out of it was used, the process of casting the column was performed by using resin solution with pouring the resin solution on the walls slowly and homogeneously so as not to form air bubbles that impede the process of ion exchange, then the column was washed with 250 mL of the buffer solution (Buffer phosphate (0.001 M) pH 7) and the flow time and velocity were set at 1 mL/min.

- 3 ml of protein from the gel filtration step were added slowly on the column walls and left to soak into the column. The separation process was initiated using (500 mL) of the buffer solution containing NaCl (25, 50, 75, 100 mM) progressive concentrations and the elute parts (3 mL) were collected for each part. Then the activity of the LOX and the protein concentration was evaluated.

2.4.5 Measuring GGT activity in blood serum

The Szasz method [27] was used to measure the effectiveness of the GGT enzyme, and the reaction equation is shown in Eq. (1):

$$\begin{aligned} &L-\gamma-\text{Glutamul}-3-\text{carboxy}-4-\text{nitroanilid}+\text{Glycylglycin} \overset{\gamma-GT}{\Rightarrow} L-\gamma \\ &\quad -\text{Glutamul}-\text{Glycylglycin}+5-\text{Amino}-2-\text{nitro}-\text{benzoat} \end{aligned} \tag{1}$$

In Eq. (1) the reaction of measured the effectiveness of the GGT.

The activity of the enzyme is directly proportional to the formation of 5-amino-2-nitro-benzoate at a wavelength of 405 nm.

2.5 Statistical analysis

Statistical analysis was carried out using SPSS (version 16). Graphs were drawn using Excel (2010), where ANOVA, arithmetic mean and standard deviation were used. The minimum probability factor ($p \leq 0.05$) was statistically significant.

3. Results and discussion

The study included 80 males with colon cancer. The study also included 38 samples of healthy (control) males. The age range for both groups was between 40 and 80 years.

3.1 Measurement of LOX activity in blood serum

The activity of LOX was estimate in patients with stage pT3 colon cancer.

The results of the study included the statistical values of colon cancer patients and the biochemical variables measured in patients and control group.

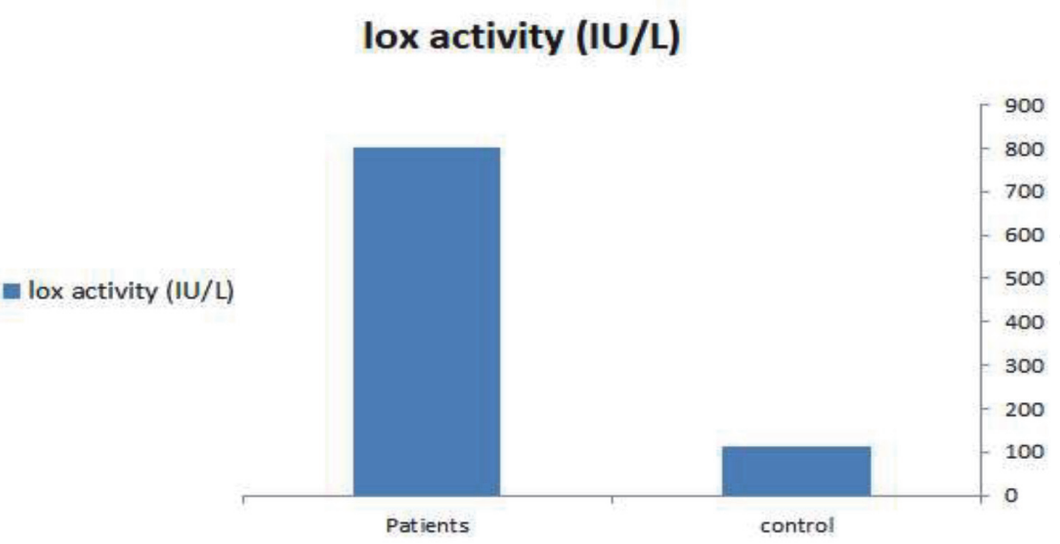
The results showed that there was an increase in the activity of LOX in the blood serum of patients with colon cancer. A statistical comparison between the effectiveness of LOX in patients' and control showed a significant excess in enzyme effectiveness in patients with probability $P \leq 0.0001$ compared with control, as shown in **Figure 1**.

Overall, the results indicated an increase in the activity of LOX in the serum of colon cancer patients, previous scientific literature did not indicate that the enzyme's activity was measured from the serum of colon cancer patients, but indicated an increase in the activity of the enzyme in human colon cancer cell lines [28–30], this high effectiveness was reported to be highly correlated with reproduction of cancer cells, angiogenesis and resistance to apoptosis [31, 32].

Also the increase in enzyme activity is due to the increase in the digestion of unsaturated fatty acids and the release of Eicosanoid compounds that promote the growth of cancerous tumors [33].

Separation and Purification of LOX from Serum Patients of Colon Cancer: LOX was separated and purified in several steps as shown in the **Table 1**.

The first step was precipitating and separating the enzyme from blood serum by using ammonium sulfate salt at a concentration 0.40%. In the second step, the dialysis was performed to obtain a degree of purity and desalting. In the third step size-exclusion chromatography technique was used to purify the LOX from the proteins and other salts associated with the enzyme. The filtration column of the Sephadex G-100 resin was used in this step, a single peak was obtained at yield 71.42% and 11.033 times of purification as shown in **Figure 2**.



Parameter	Control Mean±S.D N=50	Patients Mean±S.D N=80	T test	P value
LOX (IU/L)	114.6± 49.77	805± 517.23	5.94	0.000

Figure 1.
The effectiveness of LOX in sera of control and patient.

Step	Elute (ml)	Activity (IU/L)	Total activity (IU)	Protein con. (g/L)	Total protein (g)	Specific activity (IU/g)	Purification (fold)	Yield %
Crude	6	420	2.52	78.3	0.4698	5.363	1	100
Ammonium sulfate (0–40)	5	480	2.4	24	0.12	20	3.729	95.23
Dialysis	4	540	2.16	13.6	0.0544	39.705	7.403	85.71
Gel filtration sephadex G100	5	360	1.8	6.093	0.0365	59.17	11.033	71.42
Ion exchange DEAE-C A50 Isoenzyme-II	3	180	0.54	2.05	0.00615	87.804	16.372	28.57
Isoenzyme-I	3	120	0.36	1.84	0.00552	65.217	12.16	21.42

Table 1.
Separation and purification of the lox enzyme from serum patients of colon cancer yield.

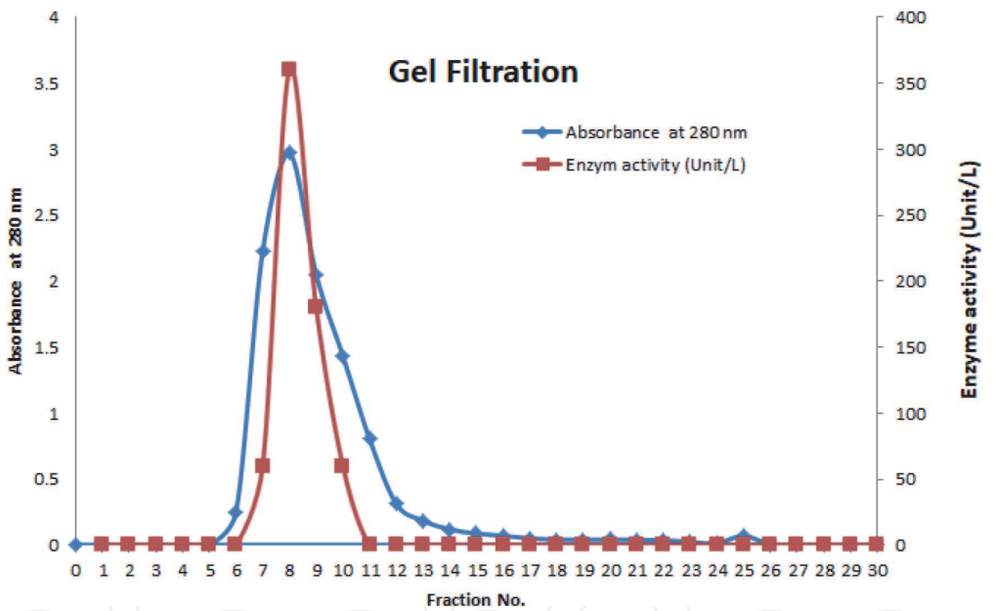


Figure 2.
Activity and absorbance at 280 nm for the fraction of gel filtration step of Sephadex G-100 resin.

In the final ion exchange chromatography technique step was used to separate the LOX isoenzyme that based on the difference in charge. DEAE-Cellulose A50 resin was used, two isoenzymes were obtained with varying degrees of purity at a yield 28.57%, 21.42%, respectively and times of purification 16.372, 12.16 as shown in **Figure 3**.

It has been noted in previous scientific literature that LOX was purified from various sources such as the serum of patients with cardiovascular disease [34], with asthma [35] and with breast cancer [36].

Previous scientific literature has also indicated that the enzyme was purified from the colon cancer cell line [37] but did not indicate that the enzyme was purified from the serum of colon cancer patients. Also the scientific literature indicated that the enzyme was purified from various other sources, including

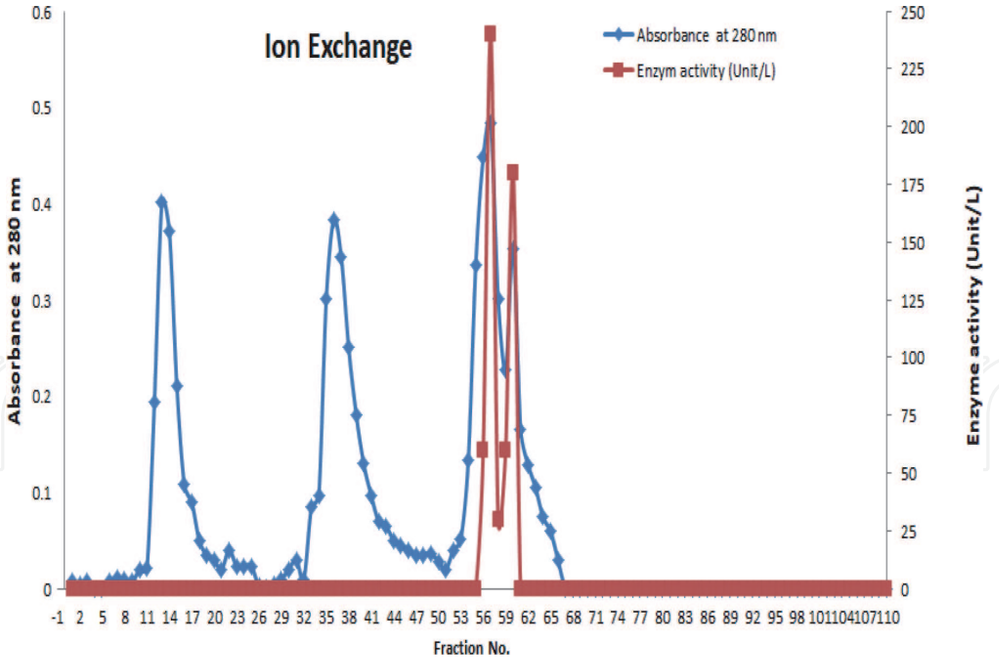


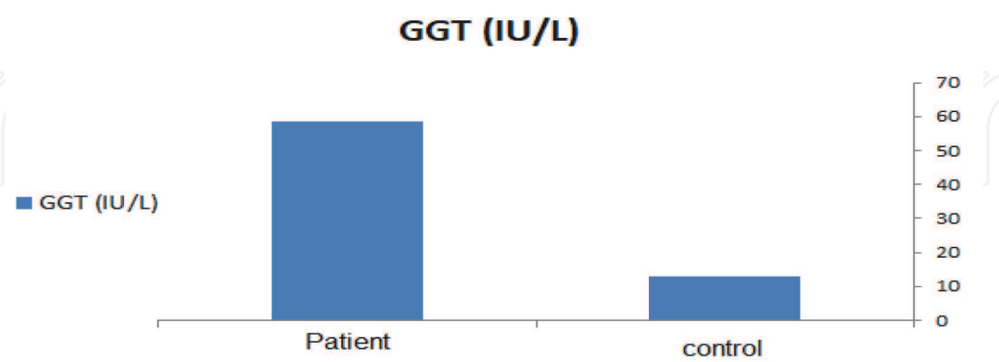
Figure 3.
Activity and absorbance at 280 nm for the fraction of ion exchange step by using DEAE cellulose A-50 resin.

soybeans, where the number of times of purification was 7.7 times at yield of 41% [38]. The enzyme was also purified from Human Placenta with a yield of 21.84% [39].

3.2 Measurement of GGT activity in blood serum

The results of the statistical analysis also showed a higher activity of GGT in colon cancer patients compared to control as shown in **Figure 4**.

Previous scientific literature has indicated a high GGT activity in the serum of colon cancer patients [40, 41]. The reason for the high activity of GGT is due to that the GGT is involved in generating free radicals and peroxidation



Parameter	Control Mean±S.D N=50	Patients Mean±S.D N=40	T test	P value
GGT (IU/L)	12.79± 5.68	58.69 ± 16.94	11.48	0.000

Figure 4.
The activity of GGT in sera of patients and control groups.

of unsaturated fatty acids, which are involved in various stages of tumorigenesis [42, 43].

4. Conclusion

1. This and other studies show an increase in the activity of LOX in patients with colon cancer compared to the healthy group. This increase in enzyme activity in patients can potentially be used as a tumor marker to detect the presence of colon cancer and also measure disease activity, before and after treatment, in conjunction with other tumor markers.

2. There was a significant increase in the activity of the enzyme GGT in patients with colon cancer compared to the healthy group. This may have clinical and prognostic significance. Further work is progressing in this field.

Authors' declaration

We hereby confirm that all the Figures and Tables in the manuscript are mine ours. Besides, the Figures and images, which are not mine ours, have been given the permission for re-publication attached with the manuscript. The author has signed an animal welfare statement.

Ethical clearance

The project was approved by the local ethical committee in Tikrit University.

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