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Purification and characterization of lipoxygenase enzyme from Iraqi patients with type 2 diabetes mellitus

M. T. Lateef[1*], H. Abd Alhassan Mahmood[1], Yu. S. Abdulsahib[2]

[1] *Department of Applied Chemistry, College of Applied Sciences, University of Fallujah, Iraq*
[2] *Department of Clinical Pharmacy, College of Pharmacy, University of Misan, Iraq*

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Lipoxygenase (LOX) enzyme was partially purified from the sera of type 2 diabetic mellitus patients (T2DM) in the current study. The results indicated that three peaks from ion exchange chromatography and one peak from gel filtration chromatography had high activity for the patients. The solution was obtained by ammonium sulfate precipitation (30–50%) following a dialysis process, and the sample was purified by gel filtration using Sephadex G-100 column with purification folds up to (2.65) (10.65), respectively. The optimum temperature for the LOX enzyme was 40°C, and its pH was 8.0. According to SDS-PAGE electrophoresis, the enzyme's approximate molecular weight was 70 kD.

Key words: Diabetes mellitus, lipoxygenase ([LOX), oxidative stress, purification of enzyme.

INTRODUCTION

Diabetes mellitus (DM) and its consequences are the most important diseases that affect human health and pose a serious threat to worldwide public health [1]. DM is a multifaceted metabolic disorder that is categorized through chronic hyperglycemia occasioning from insulin product deficiencies, action of insulin, or both. The many complications caused by this disorder severely impact the body's organs and total systems [2]. Oxidative stress particularly mediated by free radicals including ROS and RNS is the foremost source of DM complaints. There is a relationship between hyperglycemia and oxidative stress in the development of diabetic complications, for instance: nephropathy neuropathy, retinopathy, besides cardiovascular diseases [3]. Hyperglycemia-induced oxidative stress begins with the excessive production of ROS due to elevated glucose levels [4].

Moreover, high levels of glucose (hyperglycemia) rise the generation of advanced glycation end products (AGEs) from a non-enzymatic reaction of the reducing sugars with cellular components as proteins, lipids or nucleic acids. In the meantime, AGEs are responsible for ROS creation by different pathways, as well as for metal-catalyzed oxidations. This series of oxidative stress might result in cellular dysfunction, injury and significantly affect various tissues and organs [5, 6]. Unlike healthy people, people with DM2 who are not on any medication, are inevitably at risk of developing peripheral vascular disease, stroke, and cardiovascular disease [7].

Lipoxygenases (EC 1.13.11.12) (LOXs) are a group of dioxygenases that contain non-heme iron and catalyze the bi-oxidation of polyunsaturated fatty acids (PUFAS) containing 1-cis-4-cis-pentadiene to convert them into fatty acid hydroperoxides [8]. Lipoxygenases come in three different isoforms: 5-, 12-, and 15-lipoxygenase. The 5-lipoxygenase isoform contributes leukotrienes which are potent inflammatory mediators implicated in a number of chronic inflammatory illnesses, such as asthma and allergic reactions. 12-Hydroxyeicosatetraenoic acid is synthesized by the 12-lipoxygenase isoform. The 15-lipoxygenase isoform contributes to the synthesis of lipoxins which have anti-inflammatory properties and help regulate the resolution of inflammation [9]. In addition to their role in inflammation, lipoxygenase isoforms have been linked to a number of physiological functions. Adipogenesis and development of adipose tissue have been associated with particular lipoxygenase variations, suggesting a potential role in controlling body weight and metabolism [10].

The diabetes disorder is linked with chronic inflammation [11]. Lipoxygenase, a rate-limiting enzyme that facilitates the process of arachidonic acid metabolism into leukotriene (LT), promotes inflammation by activating the production of inflammatory cells (neutrophils) and cytokines. A significant way to preventing inflammation is by reducing the production of LT through inhibition of the LOX enzyme in the body [12].

* To whom all correspondence should be sent:
E-mail: *mohammedtalib@uofallujah.edu.iq*

MATERIALS AND METHODS

The blood samples were collected in the period from January 1st, 2022 until the end of September, 2023 from Falluja Hospital. The study included 50 T2DM patients from both sexes, ages ranged between 35-65 years. Venous blood samples of 5 mL were obtained from each patient in a plain laboratory and the following laboratory experiments were performed.

Precipitation by concentrated ammonium sulfate (30%-55%) with dialysis

Solid ammonium sulfate was progressively added to the serum (5 mL) in order to precipitate the enzyme while constantly stirring at 5°C approximately till the solution became turbid. Then the mixture was centrifuged for 20 min at 4000 rpm to separate the precipitate from the supernatant. The precipitate was dissolved in Tris-HCl buffer (10 mM, pH 7.4) [13]. To perform dialysis, the protein solution made in the previous stage was put on the bottom of a tightly sealed cellophane bag into a Tris-HCl [10 mM, pH 7.4] buffer solution at 4°C while being continuously stirred. After the process of dialysis was completed, enzyme activity and protein concentration were determined and the final volume of the solution was calculated.

Ion exchange chromatography

Following column balancing and Tris-HCl (10 mM, pH 7.4) washing, the samples from the aforementioned procedures were run through a 15 cm DEAE-Sepharose anion column. In each pipeline, a 3 mL fraction aliquot was collected at a flow rate of 0.5 mL/min. An UV-VIS spectrophotometer was used to measure the absorbance at 280 nm. The fractions that provided the highest absorption were collected. Following measurements of the protein volume and enzyme activity, bound proteins were eluted using 100 mL of Tris-HCl (10 mM, pH 7.4) which included a linear gradient of NaCl (0.1–1M). A 3 mL fraction was collected at a flow rate of 0.5 mL/min in all pipelines. For all fractions, the amount of protein and enzyme activity was determined.

Gel filtration chromatography

After equilibrating and eluting with Tris-HCl buffer (10 mM, pH 7.4), the sample was loaded to a Sephadex G-200 column (20 × 1.5 cm). In each pipeline, an aliquot of 3 mL fraction was collected at a flow rate of 1 mL/min. Each fraction's absorbance was measured at 280 nm using a UV-VIS spectrophotometer, and the fractions with the highest absorption were used to gauge the activity of proteins and enzymes.

Lipoxygenase (LOX) activity assay

The activity of lipoxygenase to convert linoleate to its hydroperoxide was assayed by the Holman method using the LOX activity assay kit of Abcam company (China) [14].

Estimation of protein concentration [15, 16].

The total concentration of proteins in the serum was estimated using the kit biuret method of SPINREACT Company (Spain).

Estimation of molecular weight by electrophoresis using SDS – PAGE [17, 18].

Enzymes from diabetic patients were purified by SDS-PAGE gel electrophoresis at the University of Baghdad, Ibn Al-Haitham College of Education for Pure Science.

Kinetics properties

- Effect of pH on enzyme activity: The activity was determined using the eluted fractions from the Sephadex G- 100 column at optimum reaction conditions with several buffers in the pH range from 3 to 9 (glycine, Na-acetate and Tris-HCl). The rate of reaction was plotted *versus* pH to determine the optimum pH for LOX reaction.
- Effect of temperature: The activity of LOX was determined at temperatures of 25, 30, 35, 40, 45, 50, and 55°C in 10 mM Tris-HCl buffer of pH 8.

RESULTS AND DISCUSSION

Ion exchange chromatography

Patients with type 2 diabetes mellitus were used as a source of lipoxygenase which was isolated from their serum. $(NH_4)_2\ SO_4$ and dialysis process were used to precipitate 5 ml of serum for each patient. Proteins are usually concentrated in the first steps of enzyme removal by using a suitable amount of salt and water in order to reach a high level of purity. Ammonium sulfate is commonly used to concentrate proteins which dissolve easily in water, where salts are produced as a result of neutralizing the charge of protein in order to make salt. This decreases protein solubility and sedimentation, known as salting out effect [19]. Owing to its high efficiency, fast production and ability to separate bioactive compounds using simple reactions, it has a wide range of uses [20]. After the dialysis process, the samples were eluted using 50 mM Tris-HCl buffer with graded NaCl concentration at a pH of 7.0 using a DEAE-Sepharose column.

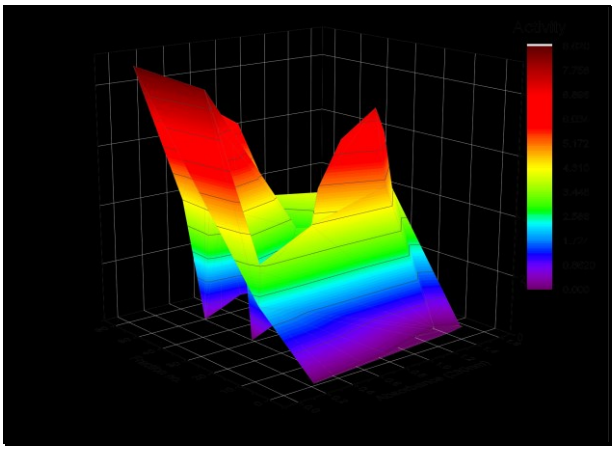

Fig. 1. Purification of LOX from sera of T2DM patients by ion exchange chromatography.

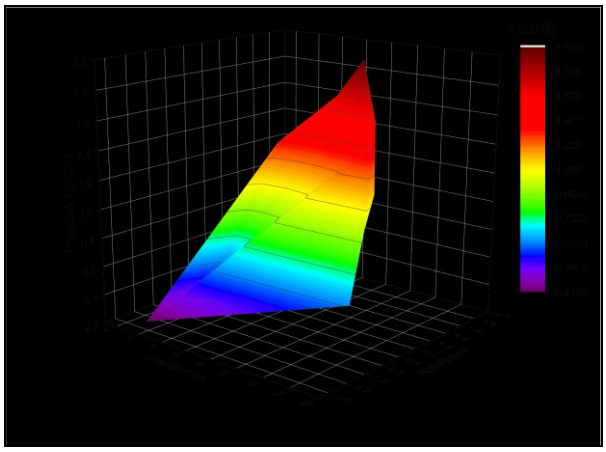

Fig. 2. Purification of LOX from sera of T2DM by gel filtration chromatography.

Three protein peaks (protein concentration and LOX activity) were identified, LOX data found in the beginning, wash fractions falling within 7–15 fractions. Three protein peaks are shown in Fig. 1, one in fractional washings, and two in rinsing fractions.

Gel filtration chromatography

Size-exclusive chromatography, which relies on the size of the particles separated, was employed as a crucial purification step. The concentrated active portion from the earlier stage made it through. After being cleaned and eluted with washing buffer, a 1.5 × 20 cm Sepharose 6B column was employed in this phase. The one active protein peak, shown by the fraction number [4-9] in Fig. 2, shows the lipoxygenase activity. Fig. 2 also shows the LOX activity in the fraction number [3-13] which indicates a single peak of protein.

Ion exchange produced three isomers, the specific activity of which was 8.92 $mg.ng^{-1}$ for the first, 11.14 $mg.ng^{-1}$ for the second, and 13.32 $mg.ng^{-1}$ for the third isomer. The purification folds were 1.2, 1.77, 2.22, 2.65, 10.65 and the yield percentages were 93.2, 77.12, 52.41, 32.41, 32.94, 27.48, respectively. The final step was gel filtration, which gave 53.4 $mg.ng^{-1}$ specific activity (see Table 1).

SDS-PAGE electrophoresis

SDS-PAGE electrophoresis was used to determine the approximate molecular weight of LOX. The first two peaks in the ion exchange chromatography patterns represented LOX activity whereas a sharp peak was obtained in gel filtration chromatography. The sample insert in SDS-PAGE electrophoresis (Fig. 3) shows an approximate molecular weight of LOX of ~ 70 000.

Table 1. Purification steps of LOX enzyme

	Volume, mL	Protein conc., mg/mL	Activity of LOX, ng/mL	Specific activity, mg/ng	Purification fold	Yield %
Crude	5	3.14	15.74	5.012	1	100
Participation $(NH_4)_2SO_4$	5	2.34	14.67	6.037	1.2	93.2
Ion exchange isoenzyme I	5	1.36	12.14	8.92	1.77	77.12
Ion exchange isoenzyme II	5	0.74	8.25	11.14	2.22	52.41
Ion exchange isoenzyme III	5	0.46	6.13	13.32	2.65	32.94
Gel filtration Sephadex G- 100	3	0.135	7.21	53.4	10.65	27.48

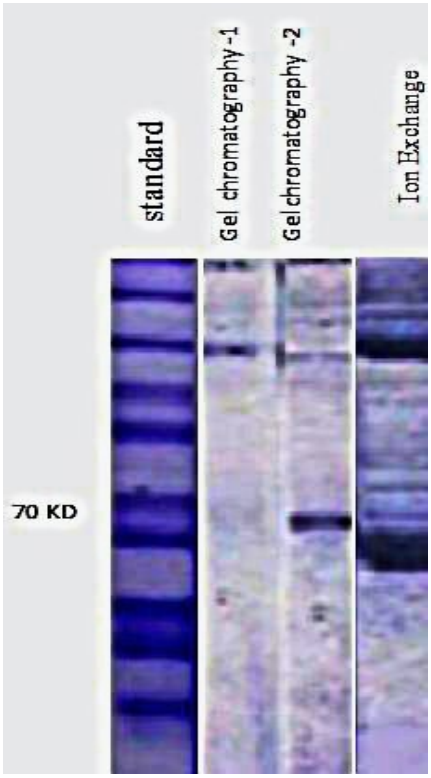

Fig. 3. Polyacrylamide gel electrophoresis of partially purified LOX enzyme from sera of T2DM patients.

Optimum temperature

The optimum temperature for enzyme activity was established, and the LOX assay was performed at 25–55 °C in 50 mM Tris-HCl buffer of pH 7.4, at 5-min intervals. The enzyme solution was placed in a water bath for 30 min and then allowed to stay at room temperature for 10 min in order to access thermal stability. Fig. 4 shows the measurement of LOX activity. Three repetitions of the experiments were made. The optimum temperature was 40ºC.

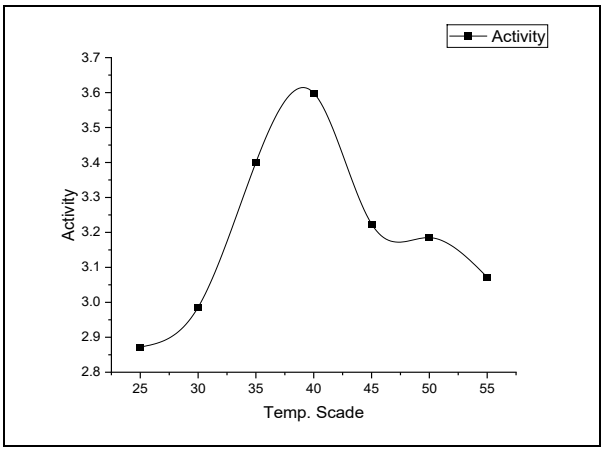

Fig. 4. Effect of temperature on the velocity of the LOX enzyme purified from the serum of T2DM patients.

The current study's findings indicated that the crude patients' specific activity was 5.012 mg.ng-1, followed by 6.037 mg.ng^{-1} in the precipitation step with ammonium sulfate.

Optimum pH

Citrate-phosphate buffer (50 mM, pH 3-5), sodium phosphate buffer (50 mM, pH 6-8), and Tris-HCl (50 mM, pH 8-10) were the buffer systems used. The optimum pH for enzyme activity was found to be between 3 and 10. After 30 min at 40°C with the enzyme solution in each buffer (at the optimum pH 8), LOX activity was measured with linoleic acid present. The plot of LOX activity *vs* pH is shown in Fig. 5.

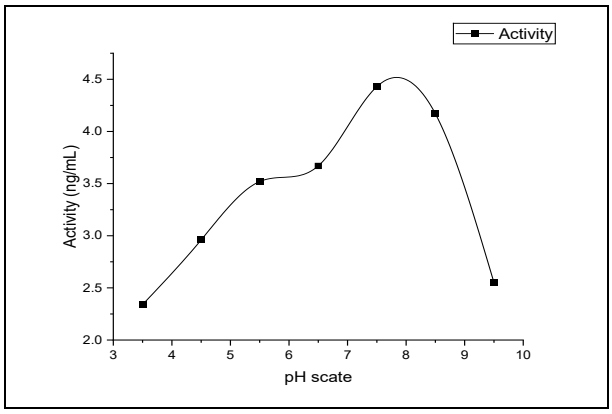

Fig. 5. Effect of pH on LOX activity.

CONCLUSION

The present study aimed to utilize sera of type 2 diabetic patients to purify lipoxygenase enzyme (LOX) by using protocol purification methods. The approximate molecular weight of LOX was estimated to be 70000 Dalton by using SDS-PAGE electrophoresis. The optimum pH was 8.0 and the optimum temperature - 40ºC.

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