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Isolation and Partial Purification of Procollagen-Lysine 5-Dioxygenase from a Patient with Coronary Artery Disease

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Abstract
This pilot case study describes the isolation and partial purification of the Procollagen-lysine 5-dioxygenase (PLOD) enzyme from the blood serum of a single 42-year-old male patient diagnosed with coronary artery disease using different techniques, starting with precipitation with ammonium sulfate, dialysis and ion exchange technique. The results showed the presence of two peaks for protein solution; the first peak I showed the highest activity of the enzyme in (18.33 U/ml) band (A), with the specific activity (4.47 U/mg), and the highest activity for peak II was in (6.7 U/ml) band (B). A single band was obtained when applying electrophoresis to the enzyme purified by ion exchange that was used for estimating the molecular weight of the PLOD enzyme, which is approximately equal to (85) kilodaltons.

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Keywords: Coronary Artery Disease, Heart Attack, Ion Exchange Technique, Modified –Lowry Method, Electrophoresis Technique

1. Introduction

Procollagen-lysine, 2-oxoglutarate 5-dioxygenase (PLOD), classified as (EC. 1.14.11.4), also known as lysyl hydroxylase (LH), is an essential enzyme for collagen biosynthesis found in all living organisms [1]. It is found in the lumen of the endoplasmic reticulum, functionally this enzyme catalyzes the hydroxylation of the lysine residue in the peptide chains that form the collagen protein in the walls of blood vessels in humans [2,3]. Hydroxylysine resulting from the latter process is essential for the stability of collagen cross-links [4]. Disease conditions have been found to be associated with the occurrence of genetic mutations in this gene (*PLOD*) that encodes the construction of collagen protein, such as Thoracic Aortic Aneurysm disease (TAA), as genetic factors "genes," play a major role in atherosclerosis and coronary artery disease. Accordingly, studies have identified the association between genetic variations (Extracellular matrix genes) and coronary artery disease and have confirmed the importance of cell attachment to the extracellular matrix (ECM) in vascular diseases. Therefore, changes in proteins within the ECM may affect the cells and cause the development of coronary artery disease. Further studies are needed to understand the major role played by the ECM and the mechanisms by which ECM-associated proteins influence the development and progression of atherosclerosis [5].

Procollagen-lysine 5-dioxygenase (PLOD) or lysyl hydroxylase (LH) uses 2-oxoglutarate, iron ions Fe^{2+}, ascorbic acid, and an oxygen molecule as cofactors, to form hydroxylysine, which acts as the carbohydrate-binding sites in collagen and is essential for the formation and stability of the collagen triple helix structure [6,7]. Collagen is one of the most abundant proteins in the skin, tendons, ligaments, blood vessels, organs, and bones [8]. Lysyl hydroxylase (LH) contains three homologous enzymes in humans, which are (LH1, LH2, and LH3) and the genes encoding these enzymes are (*PLOD1*, *PLOD2*, *PLOD3*) respectively. These enzymes catalyze the hydroxylation of lysine residues in collagen, which is vital for the formation of covalent cross-links that stabilize collagen fibers, enhancing the structural integrity of collagen [9,10]. LH isoenzymes are characterized by an amino acid sequence identity of approximately 70% [5].

PLOD enzymes have distinct expression patterns and properties, and any dysfunction in their function may lead to varying degrees of structural abnormalities in bones and cartilage, hearing loss, vascular abnormalities, kidney and lung diseases, and cancer [8,11,12].

Genetic mutations in the PLOD gene that lead to reduced enzyme activity are associated with connective tissue diseases, including Ehlers-Danlos syndrome (EDS). Conversely, overexpression of PLOD and increased enzyme activity lead to malignant tumors [13]. Previously, it was believed that the LH enzyme is the same as the prolyl hydroxylase enzyme that catalyzes the process of adding hydroxyl to proline in collagen and they work on the same substrate and with the same enzyme cofactors. Later, it was shown that the two enzymes work at different sites. Figure (1) shows the work of the prolyl hydroxylase and lysyl hydroxylase enzymes [8,14].

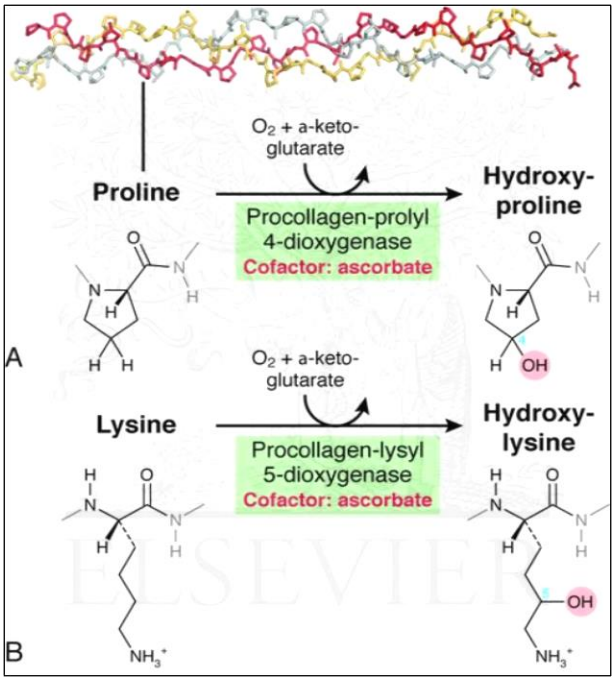

Fig 1*:* shows the work of the prolyl hydroxylase and lysyl hydroxylase enzymes [15]

1.1. Mechanism of Action of Procollagen-Lysine 5-Dioxygenase Enzyme

Procollagen-lysine 5-dioxygenase enzyme belongs to the group of 2-oxoglutarate dioxygenase (2-OG) as it works to add a hydroxyl group to the amino acid lysine and the reaction requires 2-oxoglutarate and oxygen in addition to iron (II) Fe^{2+} and ascorbic acid, which are cofactors for the enzyme [8,1]. These enzymes combine one atom of the two oxygen molecules O_2 with the hydroxyl group of the substrate and use the other oxygen atom to oxidize 2-oxoglutarate to succinate and release carbon dioxide. Lysyl hydroxylase and prolyl hydroxylase, which are involved in cross-linking and collagen formation, require ascorbic acid. Ascorbic acid is not directly involved in the reactions they catalyze, as these enzymes contain Fe^{2+} as a cofactor, which tends to oxidize to Fe^{3+} [16,17,18].

Vascular fragility leading to life-threatening aneurysms, aortic anomalies, and rupture of medium and large arteries is commonly observed in patients with PLOD1 deficiency [5] because its deficiency causes a molecular defect in type III collagen, an important component of blood vessel walls and organs [19,20]. The PLOD1 enzyme is also related to cardiovascular diseases, and the absence of LH1 leads to increased inflammation and, consequently the death of smooth muscle cells of blood vessels in the abdominal aorta [21].

2. Aim of the study

This pilot case study aims to isolate and purify the procollagen-lysine 5-dioxygenase enzyme from the blood serum of a specific patient with coronary artery disease, alongside estimating its molecular weight.

3. Materials and Methods

3.1. Materials Used

Materials were provided by global companies like (Whatman) for the ion exchanger (DEAE-Cellulose 52), (Shanghai Ideal Medical Technology, China) for ELISA measurement of PLOD enzyme, and the rest of the materials used in the research were provided by the Chemistry Department store in the College of Science and the central store of the University of Mosul from international companies: Sigma, Fluka, and Aldrich.

3.2. Specimen Used

Due to the specific clinical presentation and to serve as a baseline evaluation, this investigation was designed as a single-case study. A blood sample of (125 ml) was drawn from a 42-year-old male donor suffering from coronary artery disease and a heart attack for about 3 years. The patient was selected as the primary study case under the direct supervision of the field specialist at the Mosul Center for Cardiac Medicine and Surgery in Mosul City, after obtaining

the ethical license from the patient in compliance with the regulations of the Nineveh Health Department. As the serum was separated from the blood, the sample was subjected to a series of processes necessary to separate and study the properties of the PLOD enzyme.

3.3. Determination of Enzyme Concentration

The concentration of the procollagen-lysine 5-dioxygenase was assessed based on the sandwich ELISA method, using the standard curve, as in Figure (2), which was prepared by taking different concentrations of standard solution ranging from (0-40) IU/ml. The absorbance was read at a wavelength of 450 nm.

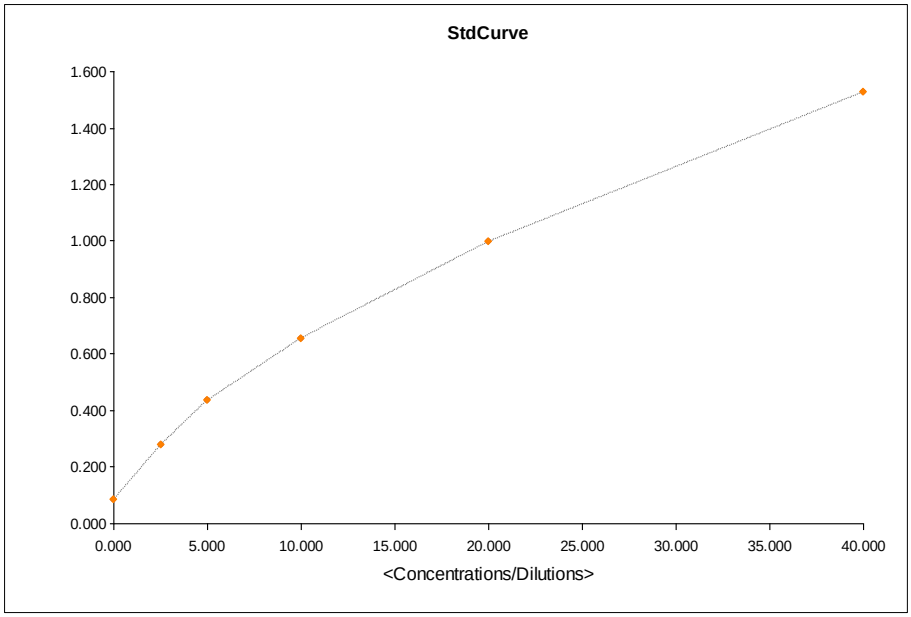

Fig 2: Standard curve for procollagen-lysine 5-dioxygenase enzyme

3.4. Isolation and Purification of Procollagen-Lysine 5-Dioxygenase (Plod) From the Patient's Blood Serum

The processes used to precipitate the protein in blood serum began with the salting out process, and the protein in blood serum was precipitated using ammonium sulfate at a saturation of (70%), at a temperature of (4ºC), the processes described in [22], then the amount of protein and enzyme activity in protein precipitate solution were estimated before carrying out the subsequent purification steps, then protein precipitate solution was stored at (- 20 ºC) until it was used in the subsequent step (Dialysis) which described in [2]3,2[4].

The ion exchange technique was performed using diethylaminoethylcellulose (DEAE-Cellulose 52) resin [23]. Then lyophilization (the freeze-drying process) was applied [25].

Lowry method [26], modified by Schacterle and Pollack (1973) [27] was used for quantitative estimation of protein level (estimate minute amounts of proteins). Finally, the electrophoresis technique described by Roy and Kumar (2014) [28] was adopted to estimate the molecular weight of the PLOD enzyme.

4. Results and Discussion

4.1. Isolating of Procollagen-Lysine 5-Dioxygenase from Blood Serum of a Patient with Coronary Artery Disease

Different separation and purification methods were used to isolate procollagen-lysine 5-dioxygenase enzyme (PLOD) from the blood serum of a patient with coronary artery disease. It was precipitated by salt displacement, then purified by membrane sorting and ion exchange chromatography as will be mentioned:

4.1.1. Protein Separation by Salting Out

The protein was precipitated by salting out by adding an excess of ammonium sulfate salt to the serum [24]. The highest precipitation was obtained when using the saturation ratio (70%) Then, the precipitate obtained was re-dissolved in the smallest possible amount of buffer solution at (pH 8). The results shown in Table (1) indicated that the specific activity of the PLOD enzyme obtained after the precipitation process increased to (2.86) U/mg protein; that is, it doubled by (1.72) times what it was before purification. The total enzyme activity recovery amounted to (105.16%) compared to the total activity of the raw enzyme, meaning that there is a tangible purification of the enzyme by this process.

4.1.2. Membrane Sorting (Dialysis)

The next process followed in the separation and purification of the PLOD enzyme was the membrane sorting process. Through this process, the specific activity of the enzyme increased to 3.56 U/mg protein, meaning that it doubled by (2.14) times what it was before the purification, and the total recovery rate of the enzyme activity reached (139.42%) compared to the total activity of raw enzyme as shown in Table (1), and this indicates that the membrane sorting process plays an important role in partially purifying the enzyme and thus increasing its effectiveness.

Table 1: Stages of purification of the enzyme procollagen-lysine 5-dioxygenase in the blood serum of a patient with coronary artery disease

Purification steps	Volume (ml)	Protein concentration (mg/ml) × 10	Total Protein (mg) × 10	Total activity (unit)	Enzyme activity (U/ml)	Qualitative effectiveness (U/mg ×10⁻¹)	Purification times	Retrieval (%)
Raw	30	4.88	146.4	243.00	8.10	1.66	1.00	—
Precipitation with ammonium sulphate (70%)	19	4.71	89.49	255.55	13.45	2.86	1.72	105.16
Dialysis	22	4.33	95.26	338.80	15.40	3.56	2.14	139.42
Ion exchange chromatography peak (A)	55	4.10	225.50	1008.15	18.33	4.47	2.69	414.88
Ion exchange chromatography peak (B)	35	3.01	105.35	234.50	6.70	2.23	1.34	96.50

4.1.3. Purification by Ion Exchange Chromatography

The ion exchange technique was applied to the protein solution obtained from membrane sorting. By tracking the protein concentration in the model's elution solution, it was found that there were two distinct peaks (I, II) for the presence of the protein, as shown in Figure (3), where the elution volume of the two peaks was (95) ml and (220) ml respectively. By tracking the activity of the PLOD enzyme in each peak, it was found that the enzyme activity was concentrated in the elution solutions of the protein peak (I) and that the highest enzyme activity was at the elution volume of (95) ml (band A) Figure (3), where the enzyme activity reached about (18.33) U/ml. The specific activity reached (4.47) U/mg protein; that is, it doubled by (2.69) times what it was before purification process. The recovery rate of total enzyme activity reached (414.88%) compared to the total activity of the raw enzyme. PLOD enzyme also showed activity at the protein band B (II). The highest enzyme activity was at the volume of (215) ml as in Figure (3), where the enzyme activity reached about (6.7) U/ml and the specific activity reached (2.23) U/mg protein, meaning that it doubled by (1.34) times what it was before purification process. The total recovery rate of enzyme activity reached (96.50%) compared to the total activity of raw enzyme. Since PLOD enzyme showed activity at both bands A and B, the enzyme likely has more than one isoform, this result is consistent with previous studies [9].

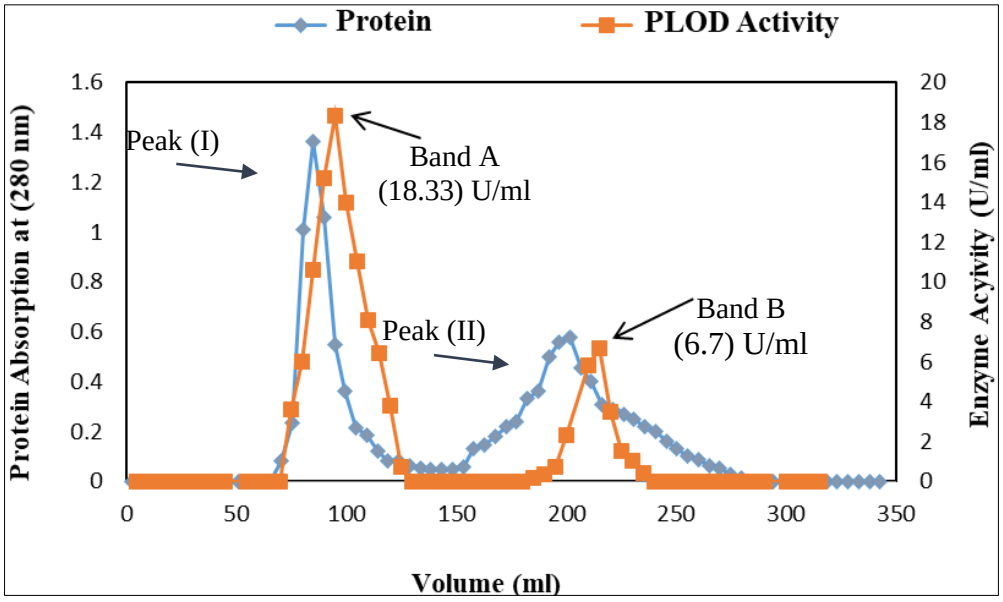

Fig 3: Purification of the procollagen-lysine 5-dioxygenase enzyme by the ion exchange technique

4.2. Determination of molecular weight

The approximate molecular weight of procollagen-lysine 5-dioxygenase enzyme partially purified from the blood serum of a patient with coronary artery disease was estimated by applying the electrophoresis technique of the type SDS-PAGE, where the sample of the protein solution obtained from the peak (I). The electrophoresis experiment was performed, and through this process, a protein band was distinguished, as shown in Figure (4). This band was adopted for estimating the approximate molecular weight of the PLOD enzyme, and it was found to be equal to (85) kilodaltons using the standard curve Figure (5).

Another study concluded that the molecular weight of the PLOD enzyme purified from humans is approximately (82-85) kilodaltons [2,9], This minor variance in molecular weight (85) kilodaltons may primarily explained by post-translational modifications, which occur within the lumen of the endoplasmic reticulum. Where another study also found that the molecular weight of the PLOD enzyme isolated from humans is about (81) kilodaltons, which is slightly less than the molecular weight of (82) kilodaltons for the chicken PLOD enzyme, and that the overall identity between human and chicken lysyl hydroxylase sequences was (76%) at the level of the encoded amino acids [30,31].

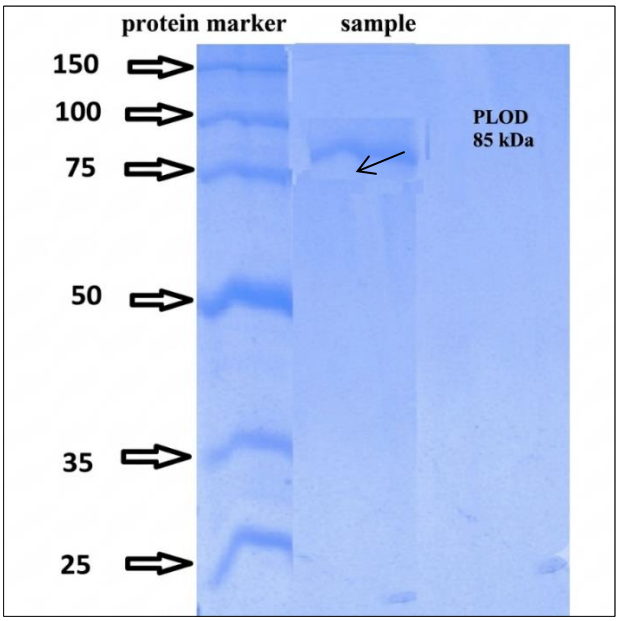

Fig 4: Protein bands separated on the gel after applying SDS-PAGE electrophoresis

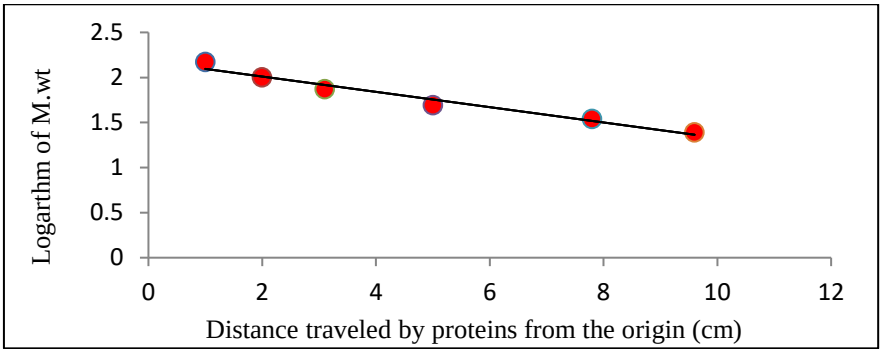

Fig 5: Molecular weight estimation curve of procollagen-lysine 5-dioxygenase enzyme using SDS-polyacrylamide gel electrophoresis

5. Conclusion

We conclude from the above that the PLOD enzyme has more than one isoform when using the Ion exchange technique. The highest activity was in the band (A) (18.33) U/ml, and the highest activity for band (B) was (6.7) U/ml. A single band was obtained when applying electrophoresis to the enzyme purified by ion exchange in coronary artery disease patient, and it had an approximate molecular weight of (85) kilodaltons.

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