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ISOLATION AND PARTIAL PURIFICATION OF CYCLOOXYGENASE-2 FROM BLADDER CANCER PATIENTS AND ESTIMATION OF ITS MOLECULAR WEIGHT

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Abstract

The study was carried out in the city of Mosul during the period 3/1/2022 until 6/1/2023 and included 75 people with bladder cancer, 68 males and 7 females, whose ages ranged between 50-80 years. The study included the isolation and purification of the cyclooxygenase-2 enzyme from blood serum. Patients with bladder cancer and studying its characteristics using techniques of salt displacement, membrane sorting, and ion exchange chromatography, and estimating its approximate molecular weight by electromigration technique. The study was carried out in the laboratories of the College of Science/University of Mosul. The results showed the presence of one distinct peak for the protein solution, and the purification process was relatively effective, with recovery of more than 50.82% of the enzyme activity. The approximate molecular weight was estimated using the electrophoretic migration technique, as a distinctive protein band was found that was used in estimating the approximate molecular weight of the enzyme, which is equal to (72).) kilodaltons, and the optimal conditions for measuring enzyme activity were 5 minutes for reaction time, 7.5 for pH, temperature of 40°C, and 0.6 mM concentration of the substrate.

Keywords: bladder cancer, cyclooxygenase-2, salt displacement precipitation, membrane sorting, ion exchange chromatography, electromigration technique

Introduction

Bladder cancer is among the leading causes of cancer-related deaths worldwide with an estimated 430,000 new cases and 165,000 deaths annually, and the prevalence of bladder cancer varies

widely in different regions, ranging from 5.9 in Asia to 23.9 in Europe (Rafiemanesh et al ., 2018). Bladder cancer is the second most common type of genitourinary cancer, accounting for 4.5% of all cancer cases worldwide. Although the cause is not clear, it is more common in men than in women, at 8.9% and 2.2% on average. respectively (Karavana et al., 2018). Transurethral resection of the tumor, intravesical chemotherapy, and immunotherapy form the basis of treatment for bladder cancer, and despite all these treatments, approximately 14% of these patients die, and the 5-year survival rate is 50% (Chen et al ., 2018). Therefore, it is necessary to develop different treatment methods and new agents that can be used to treat bladder tumor (ÖKSÜZ and BUĞDAY, 2019).

Cyclooxygenase (COX), also known as prostaglandin endoperoxide synthetase (PTGS), plays an important role in the inflammatory process by converting arachidonic acid to prostaglandins (PG) (Xu, 2002). There are two isoforms of the COX enzyme: COX-1, which is responsible for physiological activities and is therefore called constitutive, and COX-2 is expressed after cytokine, growth factor, and mitogen stimulation (Cossiolo et al., 2017). COX-1 is found in many tissues and is involved in the synthesis of prostaglandin PG. In contrast, COX-2 is not detected in most normal tissues but is often overexpressed in many tumor types (Wang et al., 2014). Since COX-2 appears to be involved in various aspects of carcinogenesis, inhibition of COX-2 activity results in antitumor and antiangiogenic effects in various human cancers (Xu, 2002).

COX-2 is one of the key proteins responsible for promoting angiogenesis, cell proliferation, and inhibiting apoptosis (structure of cyclooxygenase-2 is shown in Figure 1) (Gakis, 2014). COX-2 is overexpressed in many cancers, including bladder cancer, and is often an indicator of poor patient prognosis, so COX-2 overexpressed in cancer could be used as a target for the treatment and detection of bladder cancer to improve tumor detection during cystoscopy procedures. (Chen et al., 2018). Considering that COX-2 is involved in many biological processes, such as carcinogenesis, cell proliferation, angiogenesis, and mediating immune suppression, much evidence indicates that increased expression of COX-2 is closely associated with malignant progression (Trifan and Hla, 2003). In addition, it is also shown that carcinogenesis can be prevented using selective COX-2 inhibitors (Gasparini et al., 2003).

Figure (1): Structure of the enzyme cyclooxygenase-2 (Gakis, 2014).

Cox-2 has recently been shown to be overexpressed in various human tumors such as colon, breast, lung, and gastric cancer, and preclinical and clinical evidence suggests that this is associated with a more aggressive tumor type with unfavorable outcome (Xu, 2002). Furthermore, COX-2 expression and its relationship to clinical outcomes of chemotherapy or radiotherapy have been evaluated by several independent researchers who found higher treatment failure rates in cervical and ovarian cancers that express COX-2 (Kim et al., 2002).

COX-2 can be rapidly induced by a variety of mitotic and inflammatory stimuli and increase prostaglandin production, contributing to tumor pathogenesis and progression by modulating cell proliferation, cell death, and angiogenesis (Greenhough et al., 2009). In breast cancer, several studies have suggested that moderate to high expression of COX-2 is associated with breast tumorigenesis and that the expression level is associated with breast cancer aggressiveness, including large tumor size, positive axillary lymph node metastases, and positive tumor status (Spizzo et al., 2007). , 2003). Targeted inhibition of COX-2 inhibited the proliferation of breast cancer cell lines in vitro and prevented the development of murine breast cancer chemically induced by DMBA (Dai et al., 2012). Upregulated expression of cyclooxygenase (COX-2) is associated with human cancer and cancer progression, and COX-2 inhibitors show antitumor

activity in various human cancers. Thus, the combination of chemotherapeutic agents with COX-2 inhibitors has been shown to improve therapeutic effects on human cancers (Li et al., 2020).

Increasing evidence supports a role for COX-2 in promoting cancer cell proliferation (Raj et al., 2019). COX-2 can promote cancer cell proliferation by regulating aromatase gene expression, activating neutrophils and stimulating stromal cancer-associated fibroblasts (Krishnamachary et al., 2017). Moreover, upregulated COX-2/PGE2 expression stimulates levels of aromatase-stimulated estrogen and aromatase cytochrome P450 in a paracrine manner, leading to uncontrolled epithelial cell proliferation (Esbona et al., 2018). COX-2 also recruits macrophages and neutrophils to sustain proliferative signals respectively in cancer cells (Esbona et al., 2016). Cancer-associated fibroblasts also impart a chronic proliferative signal in cancer cells (Hull et al., 2017). In addition, changes in cell adhesion molecules are essential for cancer cell proliferation, and down-regulated E-cadherin expression is associated with down-regulated COX-2 expression (Watanabe et al., 2020). Notably, COX-2 can promote the malignancy of cancer cells in the liver (Sorski et al., 2016) and brain (Soto et al., 2016). Epithelial-to-mesenchymal transition (EMT) is a driver of cancer invasion (Watanabe et al., 2020), and COX-2 promotes EMT by regulating the expression of miR-526b (Majumder et al., 2015). In addition, COX-2 induces β1-integrin and membrane proteases-like matriptas e, which are involved in cancer cell invasion (Pan et al., 2016). Figure (2) shows the effects of overexpression of the COX-2 enzyme.

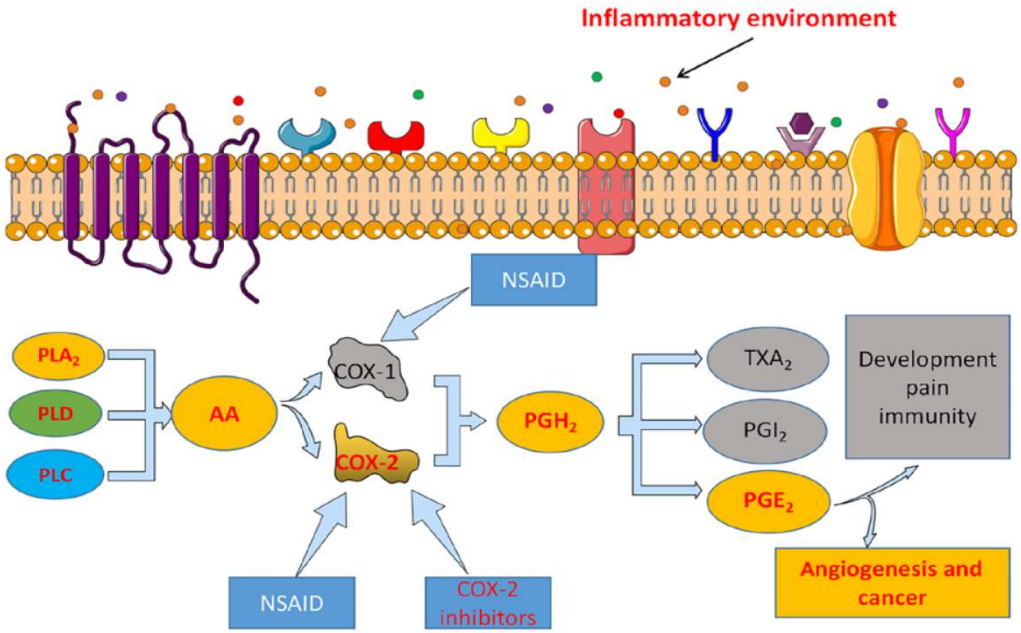


Figure 2: Cyclooxygenase-2 interactions within the tumor microenvironment. The extracellular environment is exposed to inflammation, and COX 2 is overexpressed in the cytoplasm of various cell types, converting arachidonic acid into prostaglandins and thromboxanes. PL, phospholipase;

AA, arachidonic acid. COX, cyclooxygenase; NSAID, nonsteroidal anti-inflammatory drug; PG, prostaglandin. TXA, thromboxane (GONG et al., 2021).

The current study aims to isolate and purify the cyclooxygenase-2 enzyme from bladder cancer patients and study the optimal conditions for its effectiveness.

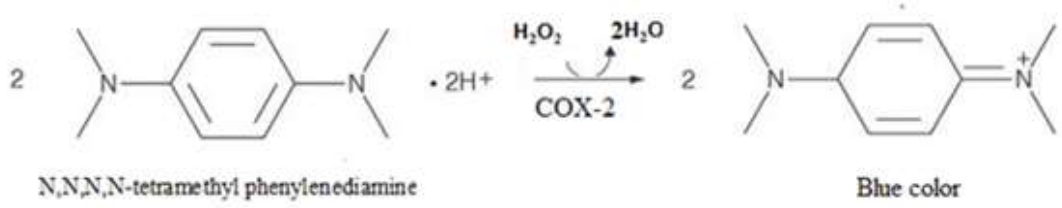
2-Materials and work methods

2-1- Materials used in the study: Materials: All materials were prepared from international companies such as Biolabo, Fluka, Sigma, and Aldrich.

2-2- Models used: Specimens used: (75) blood samples were collected from people suffering from bladder cancer, in cooperation with the clinic of Dr. Saud Hussein Mousa, a specialist in kidney and urinary tract surgery. Their ages ranged between 50-80 years for both sexes and included The sample was (68) males and (7) females, and the patients' information was recorded according to a questionnaire form prepared for this purpose.

2-3-Serum collection and preservation: Five blood samples of 10 ml were drawn from patients with bladder cancer from each patient in the clinic of Dr. Saud Hussein Musa Al-Jumaili, a specialist in kidney and urinary tract surgery in Mosul, after taking the ethical license from the patients, as the serum was isolated. From each sample, the amount required for purification was collected, which is equivalent to 50 ml of patients' serum, according to the instructions of the supervising doctor, so that a series of separation and purification operations were performed necessary to study the properties of the target enzyme, as explained later.

2-4-Determination of cyclooxygenase-2 activity: The activity of the cyclooxygenase-2 enzyme was estimated by the colorimetric method based on measuring the activity of the enzyme in oxidizing the compound N,N,N,N-tetramethyl phenylenediamine (TMPD) through the reduction of hydrogen peroxide. To give a blue-colored compound, its absorbance intensity was measured at a wavelength of 610 nm as a function of the activity of the enzyme cyclooxygenase-2, which depends on estimating the activity of the enzyme that catalyzes the peroxide reduction reaction, which is called peroxidase activity (Locklear, 2008), and as shown in the following chemical equation:



N,N,N,N-tetramethyl phenylenediamine Blue color

2-5-Isolation and purification of Cyclooxygenase-2 (COX-2) from seurm of bladder cancer patient.

Specimen used: Five blood samples of 10 ml were drawn from patients with bladder cancer from each patient in the clinic of Dr. Saud Hussein Musa Al-Jumaili, a specialist in kidney and urinary tract surgery in Mosul, after obtaining ethical clearance from the patients. The serum was isolated from each sample and the required amount was collected. For purification, which is equivalent to 50 ml of serum for patients according to the instructions of the supervising doctor, so that a series of separation and purification operations are performed necessary to study the characteristics of the target enzyme, as explained later.

2-6-Precipitation and separation of protein by using ammonium sulphate: The processes used for protein precipitation began with the external salting out process (Moscoso et al., 2013). This process is summarized by adding inorganic salts such as ammonium sulphate, which forces large amounts of water molecules to leave the Protein and binding to salt molecules because they are more soluble than slow-soluble protein molecules, so the protein molecules aggregate together and precipitate due to the strong interactions between protein - protein that overcome protein - solvent interactions (Robyt and White, 1987). The protein present in the serum is precipitated depending on the The degree of saturation of the solution, as blood serum was used, and ammonium sulfate was added to it in its solid state at a saturation level of 50%, gradually while stirring the mixture with a magnetic stirrer at 4°C for 60 minutes, then leaving the mixture in the refrigerator for 24 hours, after which the precipitate was separated from the filtrate. Using a refrigerated centrifuge for 20 minutes at a speed of 6000 rpm, after that the precipitate was obtained and dissolved with the smallest possible amount of distilled water. Then the volume of the resulting protein precipitate solution was calculated and the amount of protein was estimated by the Biuret method using ready-made solutions from the French company Biolabo, and it was measured. The activity of the cyclooxygenase-2 enzyme, as previously described, was applied to the protein precipitate solution before performing the subsequent purification steps, then the protein precipitate solution was stored at a temperature of -20°C until it was used again.

2-7-Dialysis: This technique was used to get rid of the ammonium sulfate added in the protein precipitation process and to also get rid of substances with small molecular weights by using a cellophane bag with a semi-permeable membrane that allows the passage of small molecules and

does not allow the passage of large molecules, as each membrane has A fixed value called the molecular weight cutoff, Molecualr weight cut off, allows the passage of compounds whose molecular weights are less than this value (Ahmed and Al-Hilali, 2010).

The membrane sorting process was carried out by placing the previously prepared protein solution in a cellophane tube that was previously tied tightly at the bottom, then tied tightly at the top with a thread, then placing the tube in a volumetric container containing (2.5) liters of 0.1 M ammonium bicarbonate solution NH4HCO3, and the sorting process was completed. membrane at 4°C with continuous stirring with a magnetic motor. The process continued for 24 hours, taking into account the replacement of the membrane sorting solution from time to time (Miltko et al., 2010). After completing the membrane sorting process, the final volume of the resulting solution was calculated, and the amount of protein was estimated using a method. Biuret, as well as measuring the activity of the cyclooxygenase-2 enzyme in the solution resulting from membrane sorting, then storing the solution at a temperature of -20°C until it is used again (Al-Sadoun and AL-Sabaawy, 2016).

2-8-Ion exchange chromatography:

Filling the column: Prepare the ion exchanger diethyl amino ethyl cellulose (DEAE-Cellulose 52), according to the instructions of the supplied company (Whatman). Use a glass separation column with dimensions of 2.5 x 40 cm, and fill the column with the activated ion exchanger resin by pouring it gently over walls of the column to prevent the formation of air bubbles and leave it until the resin particles settle. After that, most of the excess buffer solution was withdrawn from the column and then another amount of resin was added. This process was repeated until the resin level reached a height of 35 cm. Then the resin was allowed to settle by allowing the buffer solution to settle. (Tris-buffer pH=8, 0.1M) by descending from the lower opening of the column, taking care to keep the resin level constant to the required height by adding a new amount of resin whenever its level decreases due to its compaction. Leave the column for 12 hours at a temperature of 4°C to stabilize.

Adding the model to the ion exchange column: After preparing the separation column containing the DEAE – Celluose 52 resin, pass the sample resulting from the dialysis process by quietly injecting it at the top of the column, then pass the Tris-buffer solution with a concentration gradient from 0.05 M to 0.3 M and pH = 8. An alternating pump was used to collect the filtrated parts from the end of the column into test tubes, at a rate of 5 ml in each tube. The speed of filtering the solution from the column was (5 min/5 ml) manually, and the leached protein parts were tracked by measuring the absorbance at a wavelength of 280 nm using a spectrometer. The activity of the enzyme cyclooxygenase-2 was monitored in each of the separated parts for the purpose of monitoring the activity during the separation process. The protein parts containing the enzymatic activity were then collected.

: Lyophilization is the freeze-drying process that involves freezing water and then removing it from the form by sublimation (primary drying) and adsorption (secondary drying) (Nireesha et al., 2013). Lyophilization was done using a Lyophilizer. The process was done by freezing the solution to be lyophilized in a plastic beaker by placing it in the freezer at -20°C and then placing it in the lyophilizer.

2-9- Estimating the approximate molecular weight using the electromigration technique

Basic principle: The method described in Kamal and Hasan (2019) was adopted to estimate the approximate molecular weight using the SDS-PAGE electrophoresis device, which includes the addition of sodium dodecyl sulfate (SDS), which has the ability to separate protein units into secondary units and give them a large number of negative charges and thus remove The original charge on the molecule makes the separation depend on its molecular weight. The following is a description of the method:

Basic solutions Stock solution:

A. Dissolve 30 g of Acrylamide with 0.8 g of N,N-methylene-Bis-Acrylamide in 100 ml of distilled water.
B. Dissolve 6 g of Tris-Hydroxy methyl amino methane with 48 ml of 1 M HCl in 100 ml of distilled water to give pH = 6.8.
C. Dissolve 36.3 g of Tris-Hydroxy methyl amino methane with 48 ml of 1 M HCl in 100 ml of distilled water to give pH = 8.8.
D. Dissolve 10 g of SDS in 100 ml of distilled water to give a (10%) solution.
E. Dissolve 1.5 g of ammonium persulfate in 100 ml of distilled water.

And - N,N,N,N-tetramethylethylenediamine (TEMED).

G- Dissolve 30.3 g of Tris-Hydroxy methyl amino methane with 144 g of Glycine and 10 g of SDS in a liter of distilled water to give pH = 8.3.

Working solutions:

A- Separating polyacrylamide gel. To prepare 30 ml of separation gel at a concentration of 7.5%, I mixed the following ingredients: 7.5 ml of solution (A) with 3.75 ml of solution (C) with 0.3 ml of solution (D) with 1.5 ml of solution (e) with 16.93 ml of distilled water and with 1.5 ml of solution (f).

B- Stacking polyacrylamide gel. To prepare 20 ml of the initial gel at a concentration of 3.75%, I mixed the following ingredients: 2.5 ml of solution (A) with 5 ml of solution (B) with 0.2 ml of solution (E) with 1 ml of solution (d) With 0.015 ml of solution (f).

All solutions prepared using the previous methods were stored in dark bottles at a temperature of 4°C.

To prepare a column of gelatin, take 3 ml of the solution prepared in paragraph (2) and place it in a plastic tube with a length of 12 cm and a diameter of 0.5 cm. Carefully place a layer of distilled water on top of this solution to obtain a horizontal or flat surface of gelatin, and leave it for a period of time until the gelatin process occurs. Polymerization Then the initial polyacrylamide gel was placed on top of the first gel in the plastic tube after pouring the previously placed layer of water. 0.5 ml of this solution was added on top of the separation gel layer and the tubes were then placed close to a fluorescein light source until the polymerization process was completed, which takes between 20-25 minutes usually.

Sample preparation and injection Sample application: The sample was prepared by mixing 50 microlitres of protein dissolved in solution (B) at a concentration of 2 mg/ml with 10 microlitres of 10% SDS solution (d) with 3 microlitres of 2-mercaptoethanol, then heat the mixture in a boiling water bath. For 3 minutes, an equal volume of 20% sucrose and 10 microlitres of bromophenol blue dye at a concentration of 0.005% were added to the mixture. Then 25 microlitres of this solution was taken and placed on the surface of the gel after filling the tanks of the separator device with solution (g) diluted by (1 :10) With distilled water. Then he connected the positive electrode (Anode) to the lower tank and the negative electrode (Cathode) to the upper tank, and the electrical current was connected at 80 milliamperes and with a voltage estimated at 150 volts. The process continued until the bromophenol dye reached the bottom of the tube. At that point, the flow of electrical current was stopped and the columns were removed. The gel columns were removed from the tubes manually.

Fixation: The gel columns were immersed in a 20% TCA solution for 20 minutes, then washed with distilled water and immersed in the dye solution.

Staining: Coomasie brilliant blue R125 was used to show proteins separated by electrophoresis. The stain was prepared by dissolving 1.25 g of the dye with 227 ml of methanol and 46 ml of glacial acetic acid. The volume was completed to 500 ml with distilled water, and the gel columns were placed in an appropriate amount of Dye solution for 3 hours with slight stirring from time to time during.

Dye removal: Excess dye bound to the gel was removed only by immersing the gel columns for 48 hours in the dye removal solution, which was prepared by mixing 7 ml of methanol with the

same volume of glacial acetic acid. The volume was brought to 100 ml with distilled water, then washed several times with distilled water.

3- Results and discussion

3-1- Isolating the enzyme cyclooxygenase-2 from the blood serum of bladder cancer patients and studying its properties

Several methods were relied upon to partially separate and purify the cyclooxygenase-2 enzyme from the blood serum of bladder cancer patients. Salt displacement precipitation and membrane sorting methods were used to prepare the sample for use in the ion exchange chromatography technique, which detected the presence of two distinct peaks for the protein solution, and the highest activity of the enzyme appeared in the peak. The first (A). This summit was adopted to determine the optimal conditions and estimate the approximate molecular weight using electrophoresis technology. The following is a description of the purification stages used:

1- Separation of protein by salting out: According to this method, the protein was precipitated by increasing the concentration of the salt added to the protein solution. Due to the properties contained in ammonium sulfate salt, it was used to precipitate proteins in blood serum (Roby and White, 1987). Precipitation was done using a saturation rate of 50% (Van der Ouderaa et al., 1977). The resulting precipitate was redissolved in the smallest possible amount of buffer solution at pH = 8. The results shown in Table (1) indicated that the specific activity of enzyme 2 -COX after the sedimentation process became 69.11×10-3 U/ml, meaning that it increased by 1.02 times what it was before purification. The amount of recovery of the total activity of the enzyme amounted to 66.23% compared to the total effectiveness of the crude enzyme.

2- Dialysis: After performing the membrane sorting process, a volume of 19.3 ml of the protein solution resulting from the patients' blood serum was obtained. The results of Table (1) indicated that the specific activity of the cyclooxygenase-2 enzyme after the membrane sorting process amounted to 81.38 x 10 - 3 U/ml, meaning that it doubled by 1.20% from what it was before purification, and the amount of recovery of the total activity of the enzyme amounted to 74.63% compared to the total activity of the crude enzyme.

Table (1): Characteristics of the cyclooxygenase-2 enzyme isolated from blood serum.

Retrieval (%)	rification times	Qualitative effectiveness) U/ml×10^{-3}(	Activity total	Activity)U/ml×10^{-3}(	Total protein (mg)	Protein))mg/ml(	VOLUM (ml)	Purification steps

			)U/ml×10 $^{-3}$(					
-	1	67.26	12.417	477.6	184.6	7.1	26	Raw
66.23	1.02	69.11	8225	470.2	119	6.8	17.5	Precipitation with ammonium sulphate (before dialysis)
74.63	1.20	81.38	9267	480.2	113.87	5.9	19.3	Membrane sorting
50.82	2.93	197.17	6311	180.34	31.5	0.9	35	(after dialysis)

3- Ion exchange:

After the membrane sorting process, the protein solution was passed over the separation column containing the negative ion exchanger (DEAE-cellulose; A-50), and the cyclicity pattern obtained from the purification indicated the presence of one distinct peak (Figure 3). The graph shows a column of colorimetric resin. The resin consists of small particles coated with a substance bound to proteins. The protein solution is placed at the top of the column, and the proteins are then separated as they flow through the column. The volume of the drug at the top of the package was 20 ml, and when monitoring the activity of the cyclooxygenase-2 enzyme in the package, it was found that the highest activity of the enzyme at the top was at a volume of 35 ml, as can be seen in Figure (3), and the specific activity of the enzyme at this peak was 197.17 x 10 - 3 U/ml, meaning that it multiplied by 2.93 times what it was before purification, and the amount of recovery of the total activity of the enzyme amounted to 50.82% compared to the total effectiveness of the crude enzyme.

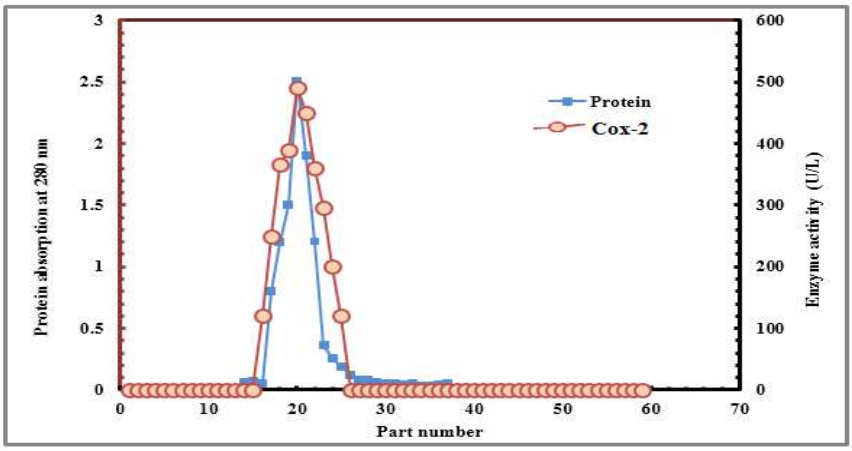


Figure (3): Purification of the cyclooxygenase-2 enzyme by the ion exchange method.

The results of the purification experiment showed that the protein in question was successfully purified, as the protein was concentrated and its activity increased, and the purification process was also relatively efficient, with more than 50.82% of the enzyme activity recovered. The purification process can be improved by optimizing the conditions for each step. For example, the concentration of ammonium sulfate can be adjusted to precipitate more protein, and the membrane can be changed to separate different sizes and charges of proteins. An ion exchange chromatography column can be changed to separate different charges of proteins. The purification process can also be made more efficient by reducing the number of purification times, using more efficient equipment and automating the process.

3-2- Estimation of the molecular weight of the enzyme cyclooxygenase-2

The approximate molecular weight of the cyclooxygenase-2 enzyme partially purified from the blood serum of bladder cancer patients was estimated using the electromigration technique. The sample protein solution (prepared from the lyophilized package isolated by the ion exchange technique) was injected onto the surface of the separation gel. The electromigration experiment was performed and the distance traveled by the proteins was calculated. Towards the positive electrode, a distinct protein band was found at a distance of (55) mm from the origin, as shown in Figure (4). This distance was adopted in estimating the approximate molecular weight of the enzyme, which is equal to (72) kilodaltons, by taking advantage of the standard curve shown in Figure (4). 5), which showed that the fifth package is the package for the enzyme, which is identical to Figure (4).

The molecular weight of the COX-2 enzyme is usually about 70 kDa, and the current results indicate that the COX-2 enzyme from bladder cancer patients is slightly larger, with a molecular weight of about 72 kDa. These results are consistent with previous studies that showed that the

molecular weight of the COX-2 enzyme cyclooxygenase-2 from bladder cancer patients is slightly larger than the molecular weight of cyclooxygenase-2 from healthy individuals, and this difference in molecular weight may be due to overexpression of cyclooxygenase-2 in bladder cancer cells.

The results are based on the following calculation:

Molecular weight (kDa) = crude volume (µl) * relative fluorescence RF /6033*10-3

For example, for scope 5, the calculation is as follows:

Molecular weight (kDa) = 55 * 0.301 / (6033 * 10-3) = 72.03

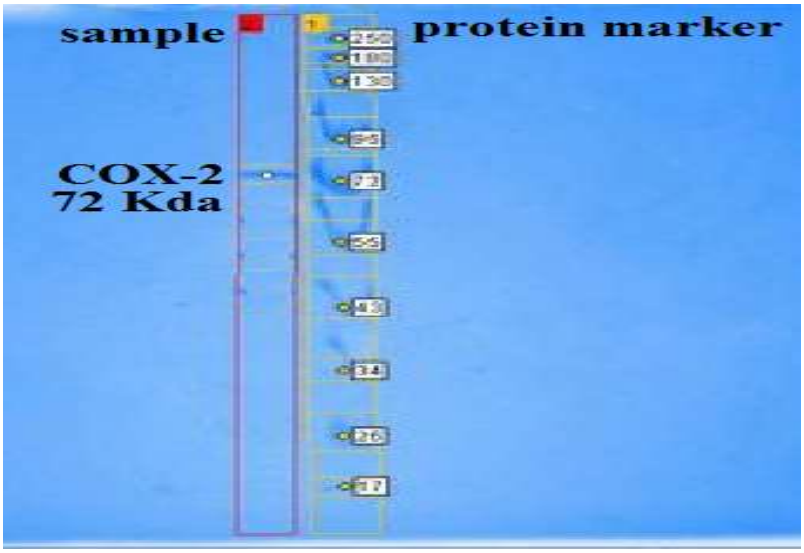


Figure (4): Cyclooxygenase-2 electrophoresis for bladder cancer patients

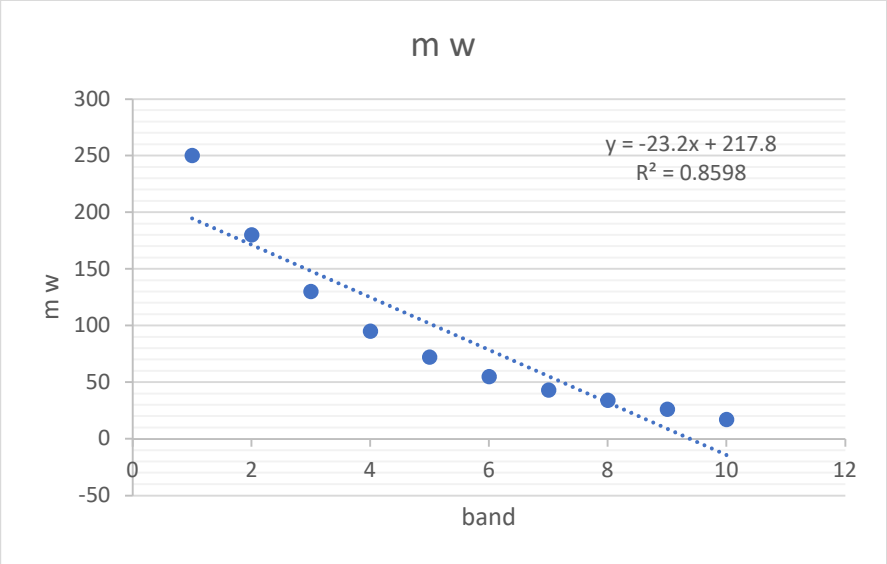
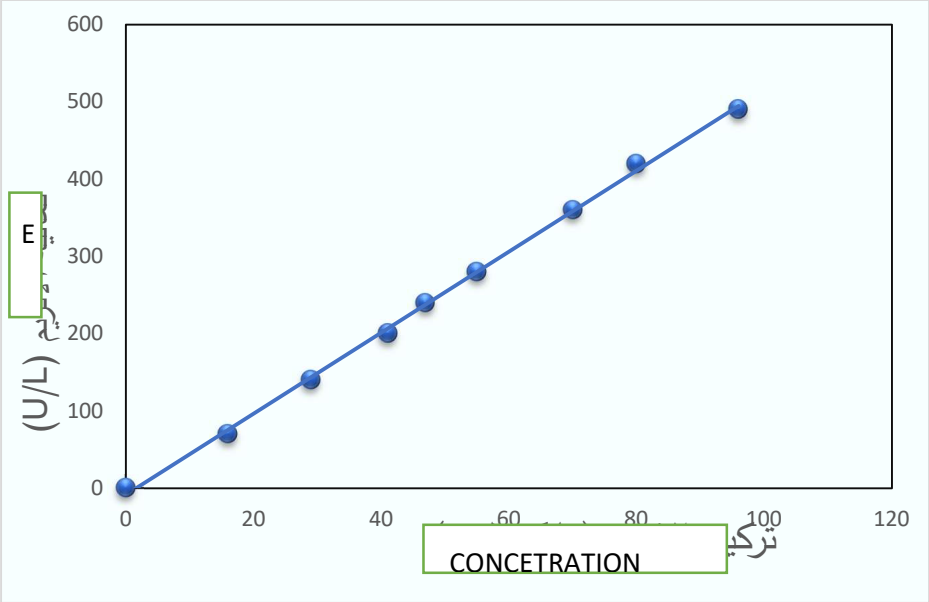


Figure (5): Molecular weight estimation curve of the enzyme cyclooxygenase-2 by electrophoresis for bladder cancer patients.

3-3- Study the effect of some factors on the effectiveness of the cyclooxygenase-2 enzyme

The factors affecting the effectiveness of the enzyme cyclooxygenase-2 were studied for the purpose of identifying the effectiveness of this enzyme accurately and finding the optimal conditions in which it works. The lyophilized protein package obtained by the ion exchange method was used as a source of the enzyme, as described in the following paragraphs.

1- Effect of enzyme concentration: The effectiveness of the COX-2 enzyme was measured at different concentrations of the enzyme partially purified from the blood serum of bladder cancer patients. The studied concentrations ranged between (0-120 microliters), and Figure (6) shows the relationship between the effectiveness of Enzyme and enzyme concentration holding other factors constant, as it has been shown that the speed of the enzymatic reaction increases directly with increasing enzyme concentration, meaning that the speed of the enzyme-catalyzed reaction is directly proportional to the enzyme concentration when the base material is abundant in the surroundings. The reason for this is due to the fact that with increasing enzyme concentration, the number of active sites increases. Which allows the binding of the base material, thus shortening the reaction time and increasing its speed (Murray et al., 2012).



- Effect of pH: The activity of the enzyme (COX-2) was estimated in the imidazole buffer solution with a concentration of 0.1 molar while changing the pH of the buffer solution in a range (1-12) for the purpose of finding the highest activity of the enzyme, and the results shown in Figure (7) indicate The highest activity of the enzyme was at pH (7.5).

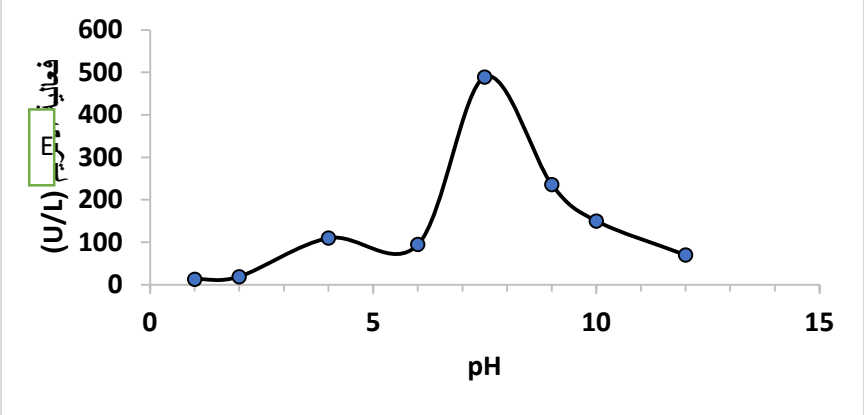


Figure (7): The effect of pH on the activity of the cyclooxygenase-2 enzyme.

Effect of Temperature: The effectiveness of the enzyme was estimated at different temperatures ranging from (9-78 °C), by increasing the temperature by 10 °C each time. It was found that the gradual increase in temperature led to an increase in the effectiveness of the enzyme gradually to the point of (40 °C), after which the enzyme activity decreased clearly, as shown in Figure (8). This is due to the protein nature of enzymes, which are sensitive to temperature changes, and the degree of their activity is determined within a narrow range of temperatures. Therefore, it is

observed that the rate of reaction speed catalyzed by the enzyme increases as the temperature increases, and when temperatures higher than the optimum temperature are used. The reaction rate decreases due to distortion or denaturation of the nature of the enzyme as a result of the disintegration of hydrogen bonds and the forces responsible for maintaining the tertiary structure of the protein, and thus the enzyme loses its effectiveness (Murray et al., 2012).

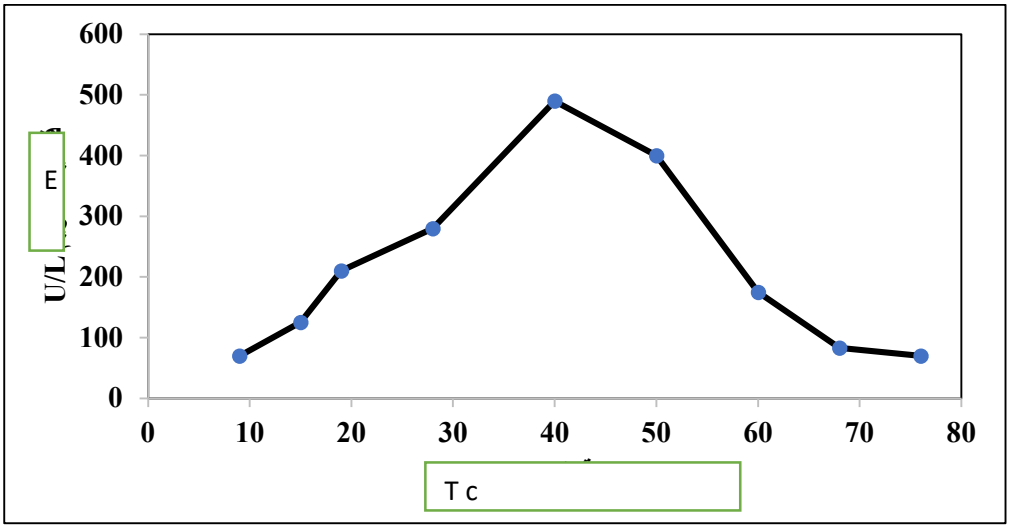


Figure (8): The effect of temperature on the activity of the cyclooxygenase-2 enzyme.

4- Effect of substrate concentration: The activity of the enzyme was measured in the presence of different concentrations of substrate to determine the relationship between the activity of the enzyme (COX-2) and the concentration of substrate (H2O2), as substrate concentrations ranged between (0.1-0.95) millimolar. / liter, and the enzyme concentration was fixed at (40) microliters / ml, and Figure (9) shows that increasing the concentration of the substrate works to increase the speed of the enzymatic reaction until it reaches a value after which there is no increase in the speed of the enzymatic reaction, and thus it is possible to determine the concentration of the substrate necessary for it to occur. The maximum speed (Vmax) of the enzyme, and this is identical to what was proven by researchers Michaelis and Menten, who confirmed that using low concentrations of the substrate makes the active sites of the enzyme not saturated with the substrate, and therefore the speed of the enzymatic reaction depends on the concentration of the substrate, but when the concentration of the substrate increases The base is widely used, so that the active sites of the enzyme become saturated with the base material, so the speed of the enzymatic reaction does not depend on the concentration of the base material (Nelson and Cox, 2005). Figure (9) shows that the saturation of the enzyme with the base material was at the concentration (0.6 mM). Of hydrogen peroxide, which gave the maximum speed of activity of the cyclooxygenase-2 enzyme, which reached 490 U/L.

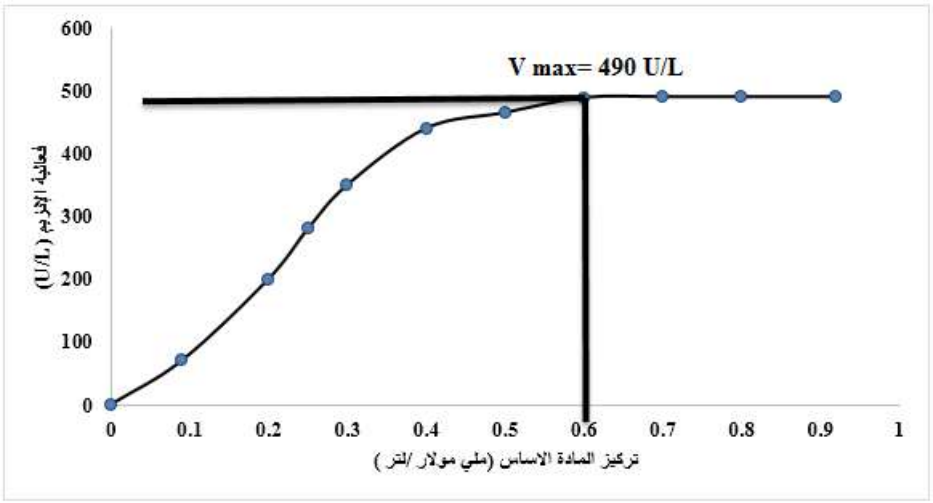


Figure (9): The effect of matrix Concentration of substrate of the cyclooxygenase-2 enzyme.

5- Effect of reaction time: The speed of the enzymatic reaction was studied at different times, ranging from (0-10) minutes, by increasing the reaction time by two minutes each time. The results shown in Figure (10) indicated that the highest effectiveness of the enzyme was at The fifth minute, then the reaction rate stabilized, and this is due to the saturation of the active sites of the enzyme with the matrix or to a decrease in the concentration of the matrix (Murray et al., 2012).

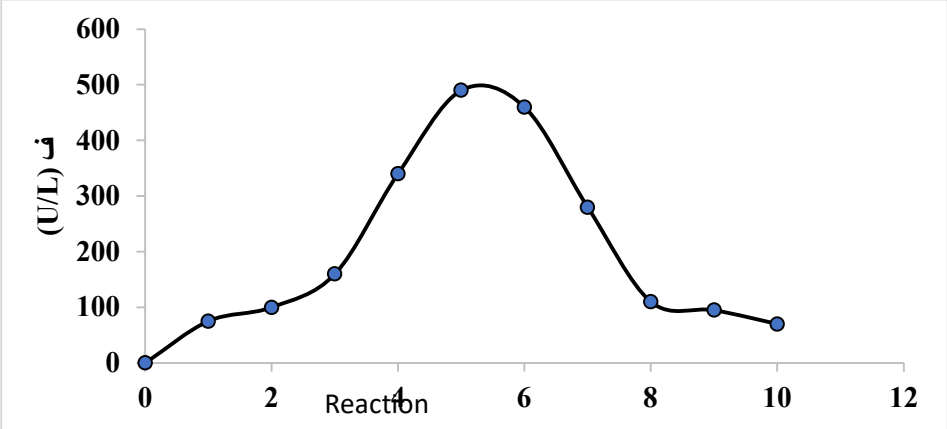


Figure (10): The effect of reaction time on the activity of the cyclooxygenase-2 enzyme

6- Summary of the optimal conditions for measuring the effectiveness of the Cyclooxygenase-2 enzyme: Table (2) shows a summary of the optimal conditions for measuring the effectiveness of the Cyclooxygenase-2 enzyme purified from the blood serum of bladder cancer patients.

Table (2): Optimal conditions for the COX-2 enzyme partially purified from the blood serum of bladder cancer patients.

Reaction time)minute(	Base material concentration)millimolar(	temperature)Celsius(	pH)(	Enzyme concentration (µl)(
5	0.6	40	7.5	100

Conclusion: We conclude from the above that the molecular weight of the cyclooxygenase-2 enzyme in bladder cancer patients reached 72 kDa, and that the optimal conditions for measuring the effectiveness of the cyclooxygenase-2 enzyme are 5 minutes for reaction time, 7.5 for pH, a temperature of 40°C, and 0.6 mM. Base concentration and 100 µl for enzyme concentration.

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