Original Article

Determination of Molecular Weight and Partial Purification of Cyclooxygenase-2 Enzyme and its Kinetic Study in Male Colorectal Cancer Patients

Ali Rahman Nama

ABSTRACT

Objective: To purify cyclooxygenase-2 from the serum of male colorectal cancer patients.
Study Design: Combined clinical and experimental in vitro study
Place and Duration of Study: This study was conducted at the College of Science, Kirkuk University, Kirkuk, Iraq from 15th December 2025 to 30th April 2026.
Methods: Sedimentation techniques, hemodialysis, ion-exchange chromatography column separation, and gel filtration were used. The resin material (DEAE cellulose) was positively charged.
Results: Two analogs of the enzyme were obtained. Cyclooxygenase-2 activity was higher in peak A than in peak B, with specific activity values of 4.94 U/mg and 2.17 U/mg respectively. The approximate molecular weight of the cyclooxygenase-2 from (peak A) was estimated by gel filtration using Sefadex G-100 gel and was 71302 dalton. Optimal conditions were estimated after kinetic study of purified cyclooxygenase-2: velocity maximum (Vmax=1 U/mL), Michaelis-Menten constant (Km=2.5 mM, pH=7.5), temperature 38 °C and time period 70 seconds.
Conclusion: The success of the proposed purification protocol in isolating highly purified cyclooxygenase-2 enzyme and maintaining its kinetic stability from complex clinical samples. The inferred kinetic parameters (K m, Vmax) contribute to a deeper understanding of the catalytic behavior of the human enzyme, supporting future research into the mechanisms of pharmacological inhibition.

Key Words: Electrophoresis, Gel filtration, Inhibitors, Ion exchange, Optimum conditions, Risk factors

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INTRODUCTION

Colorectal cancer includes cancer of the colon and rectum. It is the second most malignant cancer leading to death, and the most common type of cancer after lung cancer and breast cancer.[1] Colorectal cancer originates from polyps that develop in the mucous membrane of the colon, which turn into malignant tumors if they are not removed.[2] Adenomatous polyps in the colon begin with a slow, malignant transformation process that takes years. The size of the polyps increases as the tumor grows, and the risk increases when they exceed 1-2 cm.[3] The tumor spreads in the colon wall to distant organs via the lymphatic system or bloodstream.[4]

Lecturer, Department of Biochemistry, General Directorate of Kirkuk Education, Kirkuk, Ministry of Education, Iraq.

Correspondence: Ali Rahman Nama, Lecturer, Department of Biochemistry, General Directorate of Kirkuk Education, Kirkuk, Ministry of Education, Iraq.
Contact No: +9647704043566
Email: alirahman@uokirkuk.edu.iq

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Oxidative stress and chronic inflammation in the intestines, resulting from impaired integrity of the intestinal mucosa, are key factors causing colon damage and colorectal cancer.[5]

Cyclooxygenase-2 (COX-2) enzymes play a vital role in colorectal cancer. COX-2 enzyme levels increase when the cascade of inflammatory reactions begins in the platelets, which activates thromboxanes, leading to tumor initiation, growth, and spread.[6] In chronic inflammatory bowel diseases such as ulcerative colitis and colorectal cancer, COX-2 levels are elevated due to the production of prostaglandin-promoting cytokines. Prostaglandin production promotes tumor growth and development, causing reprogramming of stem cell genes, inhibition of programmed cell death, angiogenesis, and proliferation of cancer cells.[7] Disruption in the metabolism of polyunsaturated fatty acids in the liver leads to an increase in the production of oxylipins. Oxylipins are derived from arachidonic acid, particularly prostaglandins, causing an increase in COX-2 hyperactivity, forming inflammatory oxylipins that are the main cause of the inflammatory response caused by oxidative stress.[8]

In colorectal cancer, oxidative stress contributes through lipid peroxidation, DNA damage, and protein

modification leading to the production of reactive oxygen species (ROS) with weakened antioxidant defenses. High levels of ROS contribute to genetic mutations, alter the tumor microenvironment, and activate inflammatory pathways, thereby promoting the survival and proliferation of cancer cells.[5]

Colorectal cancer is closely associated with two types of risk factors. Modifiable factors related to habits and lifestyles, and these factors can be controlled by individuals, such as (obesity, smoking, alcohol, diet, socioeconomic and environmental lifestyle, and psychological stress). Non-modifiable factors that are beyond the control of individuals include (gender and race, family history, age, chronic diseases, benign tumors in the intestines, intestinal microbiota).[9]

There are two symmetrical forms from cyclooxygenase enzymes, namely cyclooxygenase-2 (COX-2) and cyclooxygenase-1 (COX-1). These two enzymes share overlapping catalytic functions, but they differ significantly in physiological roles, gene expression patterns, and genetic structure.COX-2 is considered a crucial factor in tumor growth and a therapeutic target for many types of cancer in humans and animals.[10]

The protective efficacy of COX-2 in colorectal cancer has been established by taking treatments that reduce or inhibit COX-2 expression. Excessive COX-2 secretion is directly correlated with malignancy and disease progression, making COX-2 a promising therapeutic target. Non-steroidal anti-inflammatory drugs and selective COX-2 inhibitors have shown chemo-preventive results in the treatment of adenomas.[10,11]

COX-2 inhibitors work as preventatives against chronic inflammatory bowel disease and colorectal cancer. Elevated COX-2 levels are diagnostic vital signs and detection of early-stage colorectal cancer. COX-2 is related to inflammation and tumor development, It has a crucial role in initiation, progression, and transformation of colorectal cancer into adenocarcinomas and subsequently into malignant tumors.[10]

The purpose is to characterize both the disease and the enzyme, and their intrinsic relationship. The approximate molecular weight of the purified COX-2 enzyme is determined. The enzyme's kinetics were studied, the values of both the Michaelis-Menten constant (Km) and the maximum velocity (Vmax) were calculated, and the factors affecting the activity of cyclooxygenase-2 and the optimal conditions for its operation were studied.

METHODS

This combined clinical and experimental in vitro study was conducted at College of Science, Kirkuk University, Kirkuk, Iraq from 15th December 2025 to 30th April 2026 vide letter No. 3131/QM/Approval/kdjkjrr dated 1st December 2025. Blood samples were taken from 15 male patients with colorectal cancer attending Azadi Teaching Hospital in Kirkuk Governorate. The patients were aged 50-70 years. Ethical consent was obtained from the patients before blood samples were drawn as part of the work requirements. Initially, it was confirmed that the patients did not have chronic heart disease with high blood pressure, diabetes, etc, and that they did not consume alcohol or smoke. Three milliliters of blood were drawn from a vein and placed in sealed, dedicated tubes (gel tubes). The samples were placed in the water bath and incubated for 15 minutes at 37°C. The blood serums were separated by centrifuge for (20 minutes) at a speed of (3000 rpm). Finally, it was frozen in specially prepared plastic tubes at -20°C until partial purification for cyclooxygenase-2.

Colorimetric methods were used to measure COX-2 activity.[12] Tetramethylphenylenediamine (TMPD) is oxidized by hydrogen peroxide reduction, yielding a blue compound,, and the absorbance is measured at (λ=610 nanometers).Estimating total protein concentration was modified Lowry method was used to estimate the total protein amount at (λ=280 nm).[13]

Highly soluble neutral salts, such as ammonium sulfate were used in the protein precipitation process during purification.[14] Ammonium sulfate is added gradually to (15 mL) of crude blood serum. The mixture is stirred for one hour using a magnetic stirrer, at a temperature of (4°C). The mixture was left in the refrigerator for 24 hours to ensure better precipitation. The separation was done by centrifugation for (25 minutes) at a speed of (6000 xg) to separate the filtrate from the precipitate. Total protein and Cyclooxygenase-2 activity were measured for both the filtrate and the sediment. The COX-2 enzyme activity was highest in the precipitate after the addition of distilled water. The solution was stored at -20°C in preparation for use in subsequent purification steps.

The dialysis process is carried out at (4°C) to remove the remaining ammonium sulfate. The dialysis bag was filled with the proteinaceous precipitate solution. The bag was then completely submerged in a (500 mL) glass flask containing (0.1 M) ammonium bicarbonate solution. The flask was placed on a magnetic stirrer, and the solution was stirred for (24 hours). The solution was replaced every (6 hours). COX-2 activity and total protein content are estimated, and the solution is kept at a temperature of (-20°C) until it is used in subsequent steps.[15]

The purification column with dimensions (2.5 cm × 50 cm) was used with the positively charged resin diethyl aminoethyl cellulose (DEAE cellulose).[16] The column was washed using a buffer solution (trishydroxy-methylaminomethane) with a pH of (pH=8) and a concentration of (0.1M). The flow rate was set to (1 mL/minute). (9 ml) of the sample solution resulting from the dialysis step was injected gently from the top of the column. The samples were collected at the

bottom of the column in test tubes. The total protein amount was measured at the absorbance (280 nm) with COX-2 activity at (610 nm) in all separated parts.

Gel filtration technique was used to estimate the molecular weight of cyclooxygenase-2 approximately.[17] The proteins with known standard molecular weights ranging from 204 to 2,000,000 Daltons were used. A separation column measuring (2 cm in diameter and 100 cm in height) was filled with Sefadex G-100 gel to a height of (90 cm). After filling, the gel (stationary phase) was washed with buffer solution (mobile phase).[18] The protein material was rinsed at a flow rate of (5 mL/5 minutes), or at a rate of (60 ml/hour)The standard calibration curve for the volumes of protein aceous materials was plotted with the logarithm of their molecular weights. The enzyme's approximate molecular weight was determined from the resulting linear equation, from the calibration curve. The purified enzyme is kept until the kinetic study and the factors affecting it are carried out. The data was analyzed through SPSS-25.

RESULTS

The partial separation and purification of COX-2 in the serum of male patients with colorectal cancer was shown in Table 1. After the precipitation process using ammonium sulfate, an increase in the specific activity of COX-2 was observed from (1.20-1.40 U/mg). Fold increased from (1-1.17), and the yield ratio became (87.34%). The remaining ammonium sulfate salt was removed from the precipitation process by dialysis. The specific activity increased after hemodialysis to (2.01 U/mg), the folding increased to (1.67), and the yield ratio became (90.34%). Two bands, (band A) and (band B), of COX-2 enzyme symmetrical were obtained using ion-exchange chromatography (Fig. 1)

The kinetics of COX-2 was studied to determine the optimal conditions and solutions with different pH values 5-8.5 were prepared. The optimal pH was found to be 7.5. The optimal time for the highest peak COX-2 activity at different time intervals (10-110 seconds) was (70 seconds). The optimal temperature at different temperatures 30-46°C was 38°C. Different concentrations of the substrate (hydrogen peroxide) were prepared, ranging from 1-12 mM. COX-2 activity was measured at these concentrations, and it was observed that COX-2 activity increased with increasing substrate concentration. A high substrate concentration leads to an increased rate of enzyme reaction. Until the active sites of the COX-2 enzyme become saturated with the substrate. When the rate of enzyme reaction reaches its maximum speed (Vmax), it remains constant no matter how much the substrate concentration increases (Fig. 2)

The Lineover-Berke plot was used between COX-2 activity and substrate concentration (Fig. 3). The value of velocity maximum was determined (Vmax = 1 U/mL), and the Michaelis-Menten constant was (Km=2.5 mM). Km is defined as the substrate concentration when the COX-2 enzyme activity (velocity) is half the velocity maximum (0.5 Vmax).

The purified COX-2 enzyme was passed through (peak A) on the gel separation column. The highest COX-2 activity was obtained through the highest peak at the elution volumes 171 mL (Fig. 4).

Table No. 1: Steps for purifying cyclooxygenase-2 enzyme in the serum of male colorectal cancer patients

Purification steps	Volume (mL)	Total protein (mg)	Activity of COX-2 (U/mL)	Total activity (U*)	Specific activity (U/mg)	Fold	Yield %
Serum	15	11.1	0.89	13.35	1.20	1	100
Ammonium sulphate (50%)	11	8.31	1.06	11.66	1.40	1.17	87.34
Dialysis	9	6.01	1.34	12.06	2.01	1.67	90.34
Ion Exchange DEAE-Cellulose (A50)							
Pack A	12	2.65	1.09	13.08	4.94	4.10	97.98
Pack B	10	4.65	1.01	10.1	2.17	1.55	86.62

Table No. 2: Estimation of elution volumes from the titration curve of standard proteins with the logarithm of their molecular weights

Materials	Molecular Weight	Elution Volume	Log Molecular Weight
Blue dextrin	2000000	64.3	6.30
Urease	48000	97.4	6.68
Bovine serum albumin	67000	135.9	4.83
Egg albumin	45000	177.7	4.68
Pepsin	36000	200.1	4.56
Insulin	5750	325.3	3.76
Tryptophan	204	372.7	2.31

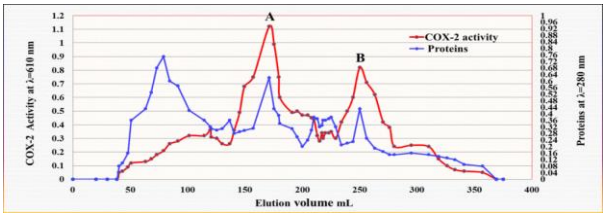

Figure No. 1: Ion-exchange chromatography for purification of cyclooxygenase-2 enzyme in the serum of male colorectal cancer patients

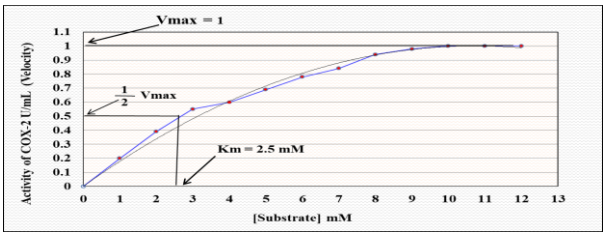

Figure No. 2: Impact of substrate concentration on the reaction rate of purified cyclooxygenase-2 activity

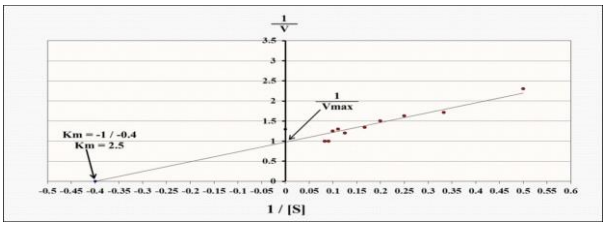

Figure No. 3: Lineover-Berke plot to find Vmax and Km values for cyclooxygenase-2 enzyme activity

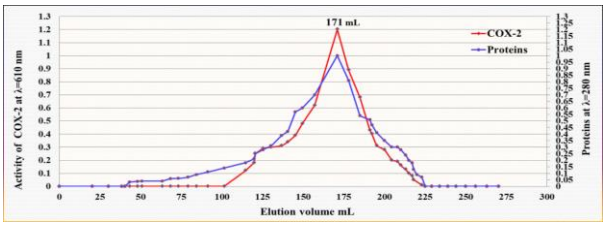

Figure No. 4: Gel filtration chromatography for partial purification of cyclooxygenase-2 enzyme in the serum of male colorectal cancer patients

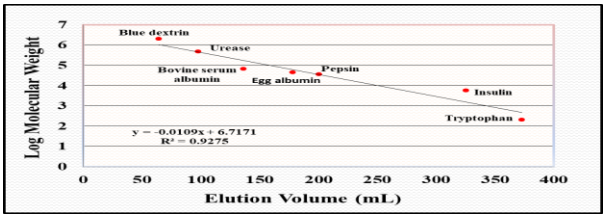

Figure No. 5: Standard calibration curve using gel chromatography for estimating the approximate molecular weight of cyclooxygenase-2

The molecular weight of the purified enzyme was estimated passing proteins of known molecular weights (204–2,000,000 Daltons) through a gel filtration column. In Table 2, the elution volumes were obtained for the known molecular weights.

The relationship between the logarithms of the known molecular weights of proteins and their elution volumes was plotted using a calibration curve. The equation of the straight line shown in figure 5 was obtained.

DISCUSSION

Two bands, band A and band B, of COX-2 enzyme analogs were identified using ion-exchange technique. The specific activity of band A (4.94 U/mg) was higher than that of band B (2.17 U/mg). Their yield rates were (97.98%) and (86.62%) respectively. The COX-2 activity in (band A) was higher than in (band B). The approximate molecular weight was estimated from (band A) with the highest enzyme activity. The specific activity of purified COX-2 in (band A) increased fourfold compared to raw blood before purification. Gel filtration chromatography was used to determine particle sizes and molecular weights in solution.[19] Finally, the molecular weight of COX-2 was estimated using electrophoresis, and it was found to be 71 kDa.[20]

In the gel filtration technique, the volume of pure COX-2 (171 mL) from figure 4 is substituted into the straight-line equation, and we obtain the approximate value of the molecular weight of COX-2, which is (71302 Daltons).In electrophoresis technique, a distinct protein band appeared after travelling a certain distance towards the positive electrode from the starting point. From this, the approximate molecular weight of the COX-2 enzyme was estimated to be (71 kDa).[20] This study is consistent with several previous studies in which the molecular weight of cyclooxygenase-2 enzyme (69-72 kDa).[21-23]

CONCLUSION

Colorectal cancer originates from polyps that develop in the colonic mucosa. It transforms into a malignant tumor if not removed. Cancer is associated with several risk factors, including obesity, smoking, alcohol consumption, diet, family history, and the microbiome. Cyclooxygenase-2enzymes play a vital role in colorectal cancer. Oxidative stress and chronic inflammation in the intestines and liver, triggered by pro-inflammatory cytokines, lead to the production of prostaglandins. The inferred kinetic parameters (K m, Vmax) contribute to a deeper understanding of the catalytic behavior of the human enzyme, supporting future research into the mechanisms of pharmacological inhibition.

Author's Contribution:

Concept & Design or acquisition of analysis or interpretation of data:	Ali Rahman Nama
Drafting or Revising Critically:	Ali Rahman Nama

Final Approval of version:	The above author
Agreement to accountable for all aspects of work:	The above author

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