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Research Article

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Purification and kinetic studies of bovine kidney cyclooxygenases (COXs)

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Abstract: Cyclooxygenases (EC 1.14.99.1) is enzyme family that produces inflammatory prostaglandins, also known as prostaglandin H synthase (PGHS) or prostaglandin-endoperoxide synthase, is expressed in several tissues. COX activity has been detected in both brain and kidney of mice and bovine kidney (that showed the highest activity). Optimum biochemical properties of enzyme activity including incubation time, enzyme and substrate concentrations and pH were determined. Kinetic parameters of COX activity were indicated that the V_{max} and K_m values were of 66.66 $\mu\text{mole}/\text{min}$ and 125.73 μM^{-1} , respectively. COX has been purified from bovine kidney by detergent solubilization, $(\text{NH}_4)_2\text{SO}_4$ precipitation, dialysis and ion exchange chromatography on DEAE-sepharose. Three isoforms were obtained (coxI, coxII and coxIII) that showed specific activities of 592.27, 1323.3 and 1825 U/mg protein and 0.72, 1.6 and 2.2 folds purification over the crude homogenate, respectively. The purified enzyme exhibited a single protein band on Coomassie Brilliant Blue stained SDS-PAGE gel corresponding a molecular weight 70 KDa.

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Key words: Prostaglandins; Cyclooxygenase; Arachidonic acid; Inflammation.

Introduction:

Considering that most health conditions involve some levels of pain, it only makes sense to decipher the mechanism behind this feeling and find a means to alleviate it (Almada, 2000). These pains describe a complicated physiological process in the biological systems. Inflammation -pain potency- is a biological defense and repair mechanism of the innate immune system to protect against harmful stimuli, such as pathogens, damaged cells and tissues, toxic chemicals and irritants and thermal and mechanical stress (Jangbauer and Medjakovic, 2012). Inflammation and the balance between an inflammatory state and a normal one, anti-inflammatory cytokines, such as interleukins (IL-4, IL-10, IL-13), interferon- α (IFN- α), and transforming growth factor (TGF), that released by macrophages, could be controlled (Kanji *et al.*, 2011). The transcription factor nuclear factor-kappa B ((NF)-kB) regulates the expression of various genes encoding pro-inflammatory cytokines, adhesion molecules, chemokines, growth factors,

chemoattractants, such as monocytes chemoattractant protein 1 (MCP-1), cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS) (Jangbauer and Medjakovic, 2012). Prostaglandins (PGs) play myriad roles as local mediators of inflammation and as modulators of physiologic functions, such as maintenance of gastric mucosal integrity, modulation of renal microvascular hemodynamics, renin release, and tubular salt and water reabsorption (Needleman *et al.*, 1986). Although COX-2 is not expressed in the normal gastric mucosa, there has now been definitive indication for localized and regulated COX-2 expression in the mammalian kidney. The enigma concerning renal prostanoid physiology has been resolved by the identification the distribution pattern of COX-2 in the kidney (Harris *et al.*, 1994). PGs are also now recognized as mediators of inflammatory reactions in neural tissue and more recently of brain function. Constitutive COX-2 immunoreactivity and COX-2 mRNA expression have been detected in neurons and especially in

the forebrain (Yamagata et al., 1993). The present study aims to purify and determine the kinetic properties of an inflammatory prostaglandin-producing enzyme (COX) that paves the way for future more inhibitory studies.

Material and methods:

Materials:

Bovine kidney was purchased from slaughter house of Tanta University, mice's brain and kidney were supplied from animal house under the ethical committee of Faculty of Science of Tanta University, Arachidonic acid was purchased from Cayaman chemical co., N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) and Tween 40 were purchased from Acros organics co., Hematin and DEAE-sepharose were purchased from Sigma chemicals co., All of the chemicals were of high grade.

Methods:

Preparation of crude homogenates from tissues of interest

Preparation of 20% crude homogenate of mice kidney and brain and bovine kidney in 0.1 M Tris-HCl buffer pH8.0 was carried on in a blender at full speed at room temperature for 5 min. After centrifugation at 10.000 x g at 4°C for 15 min, the microsomal pellet and Tween 40 (2:1) (v/v) were suspended in the same buffer containing 1mM EDTA. The suspension was subjected to centrifugation for 1 h at 60.000 x g at 4°C and the supernatant was assayed for enzyme activity (Madhava et al., 2000).

Screening of COXs activity

The enzyme activity was assayed colorimetrically by monitoring the appearance of oxidized N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) at 600 nm (Copeland et al., (1994) ; Petrovic and Murray, 2010). The assay mixture contained Tris-HCl buffer (0.1 M, pH 8), Hematin (60 µM), EDTA (3 µM) and enzyme (10% of total volume). The mixture was incubated for 10 minutes at 25 °C and then, the reaction was initiated by the addition of arachidonic acid (200 µM) and TMPD (3.3 mM) in a total volume of 1 ml. The blank was assembled by subjecting inactive enzyme (boiled fractions) instead of active one. The activity calculated from the following equation:

$$\text{Activity} = \frac{\Delta A_{600} / 10 \text{ min}}{0.00826 \mu\text{M}^{-1}} \times \frac{\text{Assay volume}}{\text{Enzyme volume}} \div 2 = \text{U/ml}$$

Protein Determination

Protein concentration was determined either by measuring the absorbance at 280 nm (Warburg and Christian, 1941) or according to the protein dye-binding Bradford assay (Bradford, 1976) using bovine serum albumin (BSA) as a standard.

Purification of Cyclooxygenase

All steps were performed under cold condition in a 0.1 M Tris-HCl pH 8.0 (Hemler and Lands, 1976).

Ammonium sulphate precipitation of 20% crude homogenate was performed gradually by 25% then 54% ammonium sulphate saturation. Powdered (NH₄)₂SO₄ was added gradually with stirring to the crude homogenate till reach 25% saturation. Subsequently, the homogenate was centrifuged at 24000 x g for 20 min. The supernatant was carefully decanted and the pellet was resuspended in 20 ml of Tris-HCl (0.1M, pH 8.0) and recentrifuged. Combined supernatants were brought to 54% saturation by drop wise addition of saturated ammonium sulfate solution in the same buffer. The suspension was centrifuged at 24000 x g for 30 min, the supernatant was removed and the pellet was saved for desalting by dialysis.

Dialysis was performed to remove the excess of ammonium sulphate bound to protein. The sample was dialyzed overnight against Tris-HCl (0.1M, pH 8.0) in a dialysis bag with a molecular weight cut-off (MW CO) diameter of 12000 Daltons. The buffer was exchanged several times to ensure that the pellet became free of ions. Thereafter, the dialyzed pellet was centrifuged at 6000 x g and the filtrate was kept for further chromatographic purification techniques.

DEAE- sepharose column chromatography of desalted protein fraction (2 ml) that was applied to anionic exchange of DEAE-sepharose (7 x 2 cm i.d. column) that was previously equilibrated with Tris-HCl buffer (0.05 M, pH 8.0). Elution was carried out with Tris-HCl buffer (0.05 M, pH 8.0) followed by a gradient of NaCl (0.1-0.5 M) in the same buffer and fractions were collected (3 ml / 5 min). The absorbance of every tube was measured at 280 nm and the enzyme assay was carried out in protein-containing tubes.

SDS Polyacrlamide Gel Electrophoresis under denaturing conditions, SDS-PAGE was performed on 10 % (w/v) acrylamide slab gel according to the basic approach of (Laemmli, 1970 ; Goswami et al., 2003) using a Tris-glycine buffer pH 8.3. The slab gel was carried out using CLEVER SCIENTIFIC Omni PAGE Mini System at 100 V per slap gel. Samples of 20 µl containing 8 µg proteins were applied to the wells of stacking gel. Electrophoresis was carried out until the bromophenol blue marker reached the bottom of the gel. Subsequently, the gel was subjected to coomassie staining for overnight then soaked in destaining solution for overnight with shaking until the bands were properly seen. The approximate molecular weight of the visualized protein bands was determined by comparing them with the molecular weight ladder (protein marker).

Kinetic Studies of Cyclooxygenase

The effect of time, enzyme concentration, substrate concentration and different pH values of reaction on COXs catalytic activity was tested.

Effect of time: Enzyme assay – as previously described - was carried out at different time points ranging from 5 to 40 min.

Effect of enzyme concentration: Enzyme assay was carried out using different enzyme concentrations 0.41, 0.82, 1.23, 1.64 and 2µg/ml.

Effect of substrate (Arachidonic acid) concentration: The effect of substrate concentrations in the range 50 to 500 μM were on enzyme activity was investigated.

Michael's constant (K_m value): The kinetic parameter, K_m , indicates the affinity of cyclooxygenase towards its substrate, Arachidonic acid. It was determined using the Lineweaver–Burk reciprocal plot graphic method.

Optimum pH: Different buffer systems of pH values between 3.8 and 11 were assembled to detect the optimum pH of the enzyme.

Results and discussion:

Cyclooxygenase, a key enzyme involved in the biosynthesis of prostaglandin (Smith *et al.*, 1996). It plays an important role in inflammation and variety of other disorders (Cummings *et al.*, 1977). With the discovery of inducible form of cyclooxygenase, COX-2 (Xie *et al.*, 1991), it has been postulated that PGs, which contribute to inflammatory process, are derived exclusively from COX-2, on the other hand, many of the "house-keeping" effects of COX appear to be mediated by COX-I (Madhava *et al.*, 2000).

In the present study, we evaluated the expression of cyclooxygenase in different tissues isolated from different animals (Table 1). The results showed that the screened activity of the mice kidney (40.78 U/ml) was higher than that of mice brain (24.8 U/ml). Comparatively, the activity of bovine kidney COX, was the highest (54.14 U/ml) in the crude homogenate with a specific activity of (826.95 U/mg protein). Rome and Lands showed that COX has been expressed in sheep seminal vesicles with a 60-fold purification following solubilization; however bovine seminal vesicle showed only 10-fold purification after solubilization (Miyamoto *et al.*, 1974). On the other hands, COX expression in the kidneys of rabbits exhibited a specific activity of 3.12×10^{-3} $\mu\text{mole}/\text{min}/\text{mg}$ protein (Wu Yu Sheng *et al.*, 1982)

COX purification has been improved by starting with ammonium sulphate precipitation that helped to concentrate the crude homogenate before introducing to dialysis. The dialyzed materials were applied to a DEAE-sepharose column that resolved the enzyme into three different isoforms designated as coxI, coxII and coxIII (Fig 1). Quantitative comparisons of the enzyme activity at different stages of purification are shown in (Table 2). Closely related purified isoforms, coxI, coxII and coxIII, showed different percentage of recovery as 13.4, 11.2 and 10.3 with increasing in specific activities as 592.27, 1323.3 and 1825 U/mg protein, respectively. Other study showed that the highest enzyme activity had been detected in seminal vesicles with a specific activity of (11600 U/mg protein) in the DEAE-cellulose eluant that also represented

10% recovery from the crude homogenate (Hemler and Lands, 1976).

SDS-polyacrylamide gel electrophoresis was performed on enzyme fractions – considered three different isoforms – after DEAE-sepharose chromatography, for each fraction, a single band appeared within a range of 68 – 70 KDa (Fig 2), while a single band of 72 KDa was reported in the case of UV induced human COX-2 (Zhang *et al.*, 2006). Likewise, recombinant human COX-1 and COX-2 assumed a molecular mass of 70 KDa (Vezaa *et al.*, 1996).

Enzyme assay was carried out repeatedly at different conditions to determine the optimum ones. In this context, when different time periods were applied in the enzyme assay, it showed that activity increased from zero time till it reached its maximum after 20 min. beyond this, the enzyme activity remained constant as time increases (Fig 3). Different enzyme (COX) and substrate (arachidonic acid) concentrations under other stable assay conditions showed increasing enzyme activity directly (Fig 4 and 5, respectively). The affinity of COX towards arachidonic acid (Michaelis-Menten constant (K_m)) was determined and showed a value of 125.73 μM . The linear nature of Line weaver-Burk plots Shows that the maximum reaction velocity (V_{max}) value is 66.66 $\mu\text{mole product}/\text{minute}/\text{ml}$ (Fig 6) (Lineweaver and Burk, 1934). Sheng and his colleagues indicated that the K_m value for the enzymatic conversion of arachidonate to PGE_2 in rabbit's kidney was 10-20 μM with a V_{max} of 3.44×10^{-3} $\mu\text{mole}/\text{mg protein}/\text{min}$ that determined at 80 μM arachidonic acid. Optimum pH was identified by assaying the enzyme activity over a wide range of pH. The highest activity was recorded by Tris-HCl buffer, pH 8.0 (Fig 7). However, in a previous study the highest activity of COX was reported in 0.1 M phosphate buffer, pH 7.8 (Riengold, 1981).

Conclusion:

Three different isozymes of cyclooxygenase COXI, COXII and COXIII with a molecular weight of 70 KDa, were observed in purification folds from DEAE-sepharose column chromatography with 0.72, 1.6 and 2.2 fold purification over the crude one, respectively. K_m value of 125.73 μM and V_{max} value of 66.66 $\mu\text{mole product}/\text{minute}/\text{ml}$ were determined.

Table (1): COXs activity in different organs of different animals.

Animal/ Organ	Activity (U/ml)
Mice brain	24.8
Mice kidney	40.78
Bovine kidney	54.14

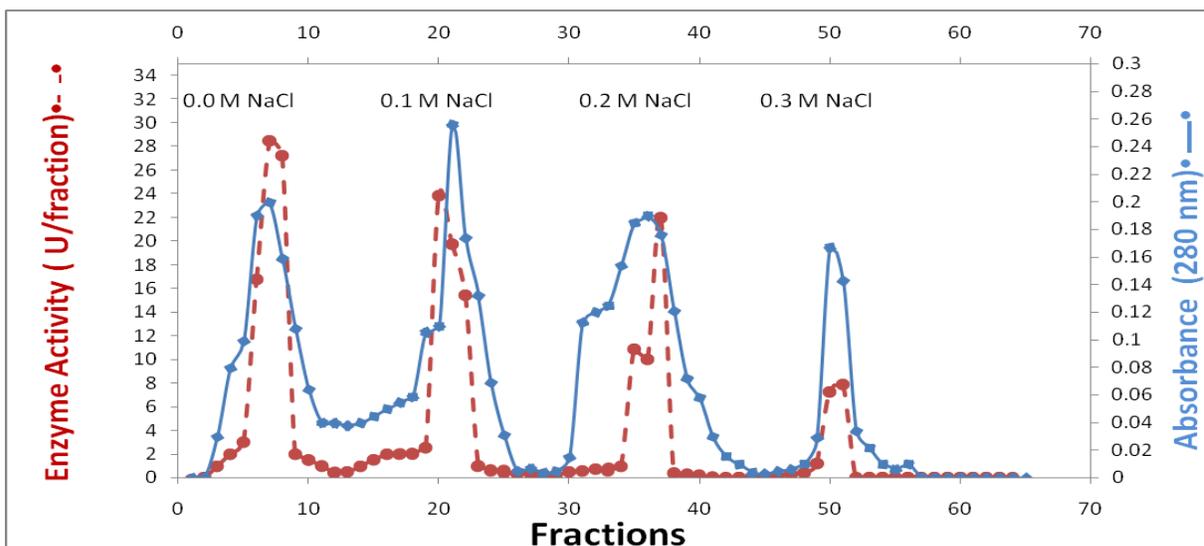
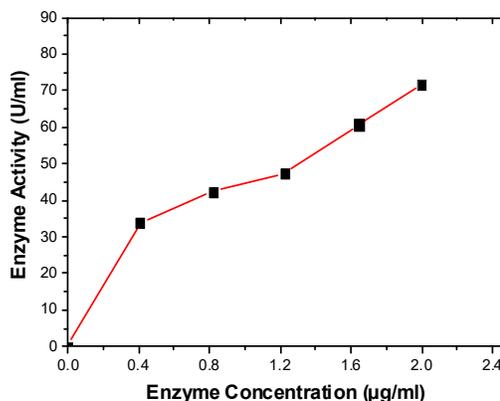


Table (2): Purification table of bovine kidney COXs. Steps followed as 25% then 54% Amm. sulphate precipitation, dialysis and column chromatography on DEAE-sepharose with gradient NaCl buffers.

Step	Total protein (µg)	Total activity (Units)	Specific activity (U/mg protein)	Recovery (%)	Fold purification
Crude homogenate (20%)	1028.8	850	826.85	100	1
(NH₄)₂SO₄ precipitation:					
25 % fold	201.6	225.44	1118.25	26.52	1.35
54 % fold	253.4	174.7	689.42	20.55	0.83
Combined folds	454.86	410.94	903.44	48.34	1.1
Dialysis of combined folds	391.2	257.55	658.35	30.3	0.8
DEAE Sepharose:					
coxI (0.0 M NaCl)	192.48	114	592.27	13.4	0.72
coxII (0.1 M NaCl)	72	95.28	1323.3	11.2	1.6
coxIII (0.2 M NaCl)	48	87.6	1825	10.3	2.2

Figure (1): Elution profile of bovine kidney COXs through DEAE-sepharose column (7x2 cm) equilibrated with 0.05M Tris-HCl buffer pH 8.0 with flow rate 3 ml/5 min.



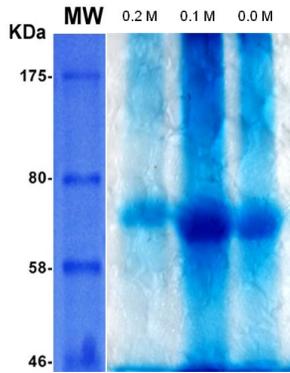


Figure (2): SDS-PAGE of purified bovine kidney COXs fractions on 10% acrylamid gel at 100 V per slap showing three different isozyemes of nearby molecular weights.

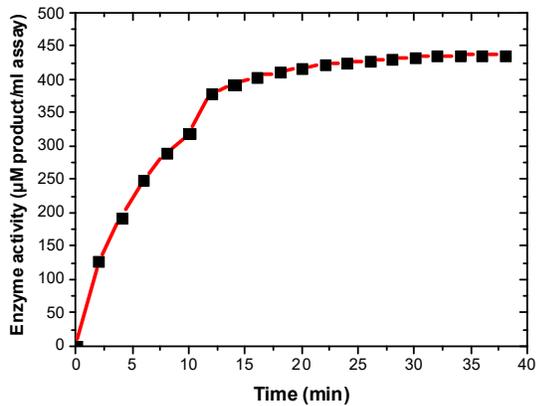


Figure (3): Effect of time on bovine kidney COXs activity showing increasing in activity by the time till reaction reach maximum.

Figure (4): Effect of enzyme concentration on bovine kidney COXs activity showing increasing activity by increasing enzyme concentration.

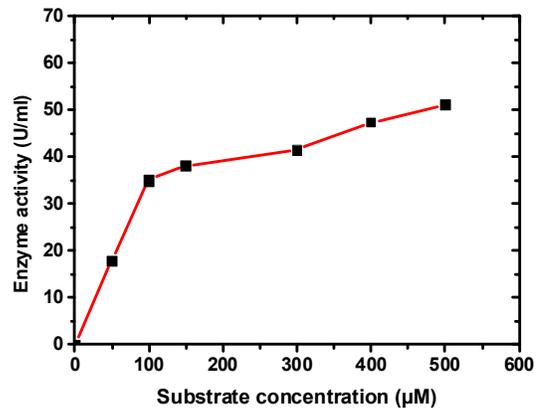


Figure (5): Effect of substrate concentration on bovine kidney COXs activity showing increasing activity by increasing substrate that shows slightly stability.

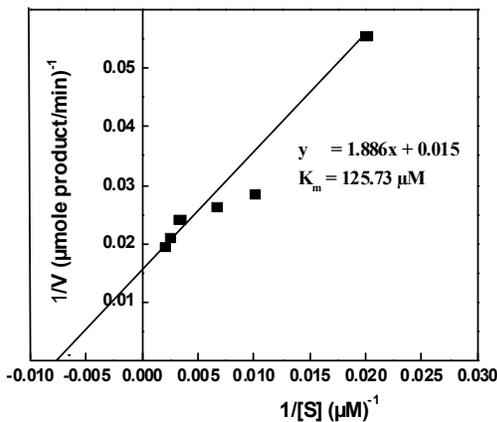


Figure (6): Lineweaver-Burk plot of COXs reaction velocity to Arachidonic acid concentration.

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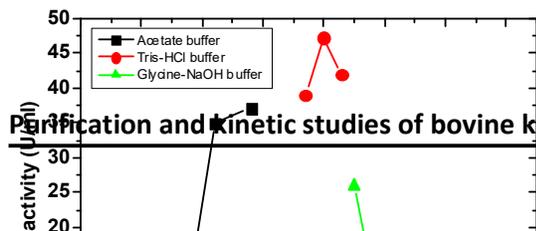
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: الملخص

تقوم العديد من الأنسجة بتكوين إنزيمات السيكلوأوكسجيناز ذات الرقم التصنيفي (1.14.99.1) و هي المسنولة عن تخليق مركبات البروستاجلاندين المسببة للإلتهابات. و بمعانة نشاط الإنزيم في كلاً من خلايا المخ و الكلاوى لفئران التجارب و خلايا كلاوى الأبقار و جد أن خلايا كلاوى الأبقار هي الأعلى نشاطاً. كما قد تم دراسة الخواص الكيميائية و الحركية للإنزيم عن طريق دراسة تأثير معامل الوقت و تركيز كلاً من الإنزيم و الركيزة و مع ثبوت العوامل الأخرى. كما تم تعيين ثابت ميكاليس و مونتني K_m بقيمة 125.73 ميكرو مول لتر⁻¹ و الذي يعبر عن قيمة الركيزة التي تصل بالتفاعل إلى منتصف الحد الأقصى V_{max} بقيمة 66.66 وحدة نشاط لكل مليلتر. و تم دراسة تأثير محاليل منظمة مختلفة بمختلف تركيز أيون الهيدروجين pH. قد تمت عملية تنقية الإنزيم من خلايا كلاوى البقر بداية بالإستخلاص ثم بترسيب البروتينات بكبريتات الأمونيوم، ثم عملية الترشيح أو غسيل الراسب. ثم عملية الفصل الكروماتوجرافي العمودي على مادة الداى إيثيل أمينو إيثيل سيفاروز و قد تحقق وجود ثلاث مشتبهات من الإنزيم COXIII، COXII، COXI بنشاط 1825، 1323.3، 592.27 وحدة نشاط لكل مجم بروتين ما يمثل 0.72، 1.6، 2.2 ضعفاً من النقاوة عن

Figure (7): Effect of different buffers with different pH on bovine kidney COXs activity that shows optimum pH in TRIS-HCl system.

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المستخلص الخام، على الترتيب. و بقياس الوزن الجزيئي للمشتبهات الثلاثة وجد أنهم على نفس الوزن 70 كيلو دالتون.