------ Raf. J. Sci., Vol. 24, No.6 pp. 23-36, 2013------

Isolation of Latent Matrix Metallo Proteinase-1 (Latent Collagenase-1) from Serum and Synovial Fluid of Rheumatoid Arthritis Patient

Zahraa M. Hamoudatt **Layla A. Mustafa**

Department of Chemistry
Collage of Science
Mosul University

(Received 21 / 10 / 2012 ; Accepted 27 / 5 / 2013)

ABSTRACT

This study included the isolation of the enzyme Matrix Metalloprotrinase-1 (MMP-1; Collagenase-1) from serum of healthy individual and synovial fluid (SF) of a patient with rheumatoid arthritis using different techniques.

After precipitation of proteins using saturated ammonium sulfate, two porteinous components had been isolated by gel filtration chromatography. It was found that only the first peak has high activity of latent-MMP-1. The apparent molecular weights of MMP-1 in serum and SF using gel filtration chromatography was found to be (47752 ± 816 and 48194 ± 707) dalton respectively.

High performance liquid chromatography (HPLC) was used to show the extent of purity. The main maximum of the enzyme from serum and SF appeared at retention times (1.652 and 1.65) minutes respectively compared with the retention time of standard enzyme at (1.571) minutes. The approximate molecular weight of latent-MMP-1 by HPLC technique, in serum and SF were found (48067 and 48033) dalton respectively.

The study included, also, the effect of some material compounds on the activity of latent-MMP-1. The results revealed that the addition of ethylene diamine tetra acetic acid (EDTA), 2,4-dinitrophenol, sodium azide, potassium oxalate, mercaptoethanol (MEH), glutathione (GSH), cystein and healthy human serum decreased the activity of latent-MMP-1, while the addition of NaCl, $MgSO_4$, $CoCl_2$, $CaCl_2$, $CuSO_4$, $ZnSO_4$, $HgCl_2$ and pepsin increases the activity of enzyme.

Keywords: Matrix metalloproteinase-1, collagenase-1, Rheumatoid arthritis.

عزل أنزيم الميتالوبروتينيز-1-الكامن (الكولاجينيز-1-الكامن) من المصل والسائل الزليلي لمريض مصاب بالتهاب المفاصل الرثوي

الملخص

تضمنت هذه الدراسة عزل أنزيم الميتالوبروتينيز-1-الكامن (الكولاجينيز-1-الكامن) من مصل شخص سليم ومن السائل الزليلي لمريض مصاب بالتهاب المفاصل الرثوي باستخدام تقنيات مختلفة. وقد فصلت حزمتين رئيسيتين من البروتينات بتقنية الترشيح الهلامي للراسب البروتيني الناتج من الترسيب بكبريتات الأمونيوم. أظهرت حزمة واحدة فقط فعالية عالية لأنزيم الميتالوبروتينيز-1-الكامن.

قدر الوزن الجزيئي للأنزيم في المصل والسائل الزليلي باستخدام الترشيح الهلامي وكان بحدود (47752 ± 816) و (48194 ± 707) دالتون على التوالي.

استخدمت تقنية كروماتوكرافيا السائل ذو الأداء العالي لبيان مدى نقاوة البروتين الناتج من معرفة قمة الروغان التي تم الحصول عليها، أظهرت قمة الحزمة لأنزيم المعزول من المصل والسائل الزليلي عند زمن إحتباس (1.652 و 1.65) دقيقة على التوالي مقارنة بزمن احتباس الأنزيم القياسي (1.571) دقيقة. أظهرت النتائج أن الوزن الجزيئي التقريبي للأنزيم في المصل والسائل الزليلي و باستخدام تقنية كروماتوكرافيا السائل ذو الأداء العالي يقع بحدود (48067 و48033) دالتون على التوالي.

درس تأثير بعض المركبات الكيميائية على فعالية الأنزيم، حيث أظهرت النتائج أن إضافة رباعي حامض الخليك أثيلين ثنائي الأمين و2,4-ثنائي نايتروفينول والصوديوم أرسينيت والصوديوم أزيد وأوكزالات البوتاسيوم والميركابتو إيثانول والكلوتاثايون والسستين ومصل بشري سليم يؤدي الى تقليل فعالية الأنزيم، إلا أنه عند إضافة كلوريد الصوديوم والكالسيوم والكوبالت والزئبق وكبريتات المغنيسيوم والنحاس والخارصين والببسين تؤدي إلى زيادة فعالية الأنزيم.

الكلمات الدالة: انزيم الميتالو بروتينيز-1-، الكولاجينيز-1-، التهاب المفاصل الرثوي.

INTRODUCTION

Rheumatoid arthritis (RA) is a systemic inflammatory disease of unknown etiology (Scherer *et al.,* 2010). It usually causes damage and disability of patient's small joints by activity of the chronic and acute inflammation which is the major complication for this proteolytic disease (Yang and Wang, 2011). The destruction of cartilage joint and bone in RA is mediated by abnormal release of proteolytic enzymes which are stimulated by persistent inflammation of synovial tissue such as the matrix metalloproteinases (MMPs) (Chen *et al.,* 2012; Scherer *et al*., 2010).

Matrix metalloproteinases are family of zinc metallo endopeptidase that degrade all components of extra cellular matrix (ECM) (Philips *et al.,* 2011; Fields *et al.,* 2000; Maeda *et al.,* 1995). Interstitial collagenase-1 (collagenase-1) also known as matrix metalloproteinase-1 (E. C. 3. 4. 24. 7; MMP-1) is a member of MMPs family, which cleaves collagens type I, II and III, and resulting two triple helical fragments representing 25% and 75% of the original molecule (Daboor *et al*., 2012; Fasciglion *et al*., 2012; Fields *et al*., 2000; Maeda *et al*., 1995). Collagen is a major fibrous element of skin, bones, tends cartilage, blood vessels and teeth which are found in all multi-cellular animals (Jain and Jain, 2010). Collagens are extremely stable and highly resist to degradation by all proteinases except for MMPs (Polyakova *et al*., 2011). MMPs have been implicated in several physiological and pathological processes such as, skeletal growth and remodeling, wound healing, cancer, arthritis and multiple sclerosis (Fasciglion *et al*., 2012; Leit *et al*., 2009; Erdam *et al.,* 2002).

In synovial joints, MMPs are mainly secreted by fibroblasts, macrophages and chondriocytes (Chen *et al*., 2012). Synovial fluid (SF) of RA patients showed about thirty times of collagenase and elastase activity compared to normal, which degrade the joint tissues (Sandya *et al.,* 2009). MMPs are synthesized in an inactive (latent) form called zymogene or pro-MMPs. These latents of MMPs require an activation step before they become able to cleave ECM components (Sandya *et al*., 2009; Beurden and Hoff, 2005).

Latency of collagenase is removed to form the active enzyme by proteolytic enzymes such as trypsin and non proteolytic agents such as chatropic salts and organo mercurial. The conversion of latent enzyme to its active enzyme is accompanied by a decrease in its molecular weight of about (10-20) kilo dalton (Leite *et al*., 2009; Meada *et al.,* 1995; Smith *et al.,* 1989). The difference in the molecular weight of MMPs is depend on their type (serine or metalloproteinase) and their source (animal or microbial) (Daboor *et al.,* 2010). The activity of MMPs is regulated by several types of inhibitors called tissue inhibitor of metalloproteinases (TIMPs) (Sandya *et al.,* 2009; Peak *et al.,* 2005).

This study is a primary attempt to bridge the gap of knowledge about latent MMP-1 by studying its activity, purification, approximate molecular weight and the effect of some compounds on its activity.

MATERIAL AND METHODS

Collection of blood and synovial fluid

A-Blood Serum

A fresh human serum was obtained from one healthy non smoker male volunteer attending the blood bank in Nineveh Governorate, aged 38 years. Serum was kept in clean dry tube at -20°C and used as a source for the enzyme isolation (Tietz, 1994).

B- Synovial Fluid

Synovial fluid was obtained from knee joint of non smoker male patient with RA attending the rheumatology outpatient clinic at Ibin- Siena teaching hospital, aged 45 year. He has met the American College of Rheumatology (ACR) and the European League Against Rheumatism (EULAR) criteria for diagnosis of RA (Aletaha *et al*., 2010; Arnett *et al.,* 1988) and treated with non-steroidal-anti-inflammatory drugs (SIDEs), disease modifying anti-rheumatic drugs non-steroidal inflammatory drugs (DMARDs), and he was not affected by other diseases. The SF was kept in a clean dry tube at -20°C and used as a source for the enzyme isolation (Matsumoto *et al.,* 2006).

Determination of Total Protein Concentration

Total protein concentration in serum and SF was determined in each isolation's step using bovine serum albumin as a standard (Holme and Peck, 1988) according to the modified lowery method (Schacterle and Pollack, 1973).

Determination of latent –MMP-1 Activity

Latent-MMP-1 activity in serum and SF was measured in each isolation's step using a commercial Human MMP-1 ELISA kit (Kim *et al*., 2011; Peak *et al*., 2005; Erdam *et al*., 2002; Maeda *et al*., 1995), kindly donated by Ray Bio United Kingdom. This assay employs an antibody specific for human MMP-1 coated on a 96 well plate. Standards and samples were pipetted into the wells and MMP-1 present in a sample was bound to the wells by the immobilized anti body. The wells were washed and the biotinylated anti-human MMP-1 anti-body was added. After washing away the unbound biotinylated antibody, HRP-Conjugated Sterptavidin was pipetted to the wells. The wells were again washed and a 3, 3', 5, 5'-tetra methyl benzidine (TMB) substrate solution was added to the wells and a color developed in proportion to the amount of MMP-1 bound. Sulfuric acid was used to stop the reaction. the color was changed from blue to yellow and the color intensity was measured spectrophotometrically at 450 nm. Recombinant human MMP-1 was used as a standard. This kit has a limit of detection of 8 pg/ml. Samples of serum and SF were diluted with diluents buffer 1:20 and 1:40 respectively.

Protein Isolation

A- Fraction of Latent MMP-1

Proteins of serum and SF were precipitated by saturation with ammonium sulfate or by protein salting out (Robyt and White, 1987; Dioxin and Weeb, 1961). The samples of serum (30 ml) and SF (21 ml) were brought to 75 % saturation with ammonium sulfate. The addition of ammonium sulfate was gradual, a small amount was added and allowed to dissolve before making further additions. The mixtures were stirred by electrical stirror at 4ºC for 60 minutes, then left overnight in the refrigerator. The precipitate formed was then separated by refrigerated centrifuge at 10000 xg for 30 minutes.

B- Dialysis

the precipitated protein solution (22 ml) from serum and (15 ml) from SF desalted by dialysis method using a 25 mm cellulose membrane dialysis tube which is placed in a container containing sodium bicarbonate 0.1 M. Ammonium sulfate desalting was carried out three consecutive times (48 hours for each time) (Robyt and White, 1987).

C- Isolation and Partial Purification of Latent-MMP-1

Gel filtration chromatography technique was used for isolation and partial purification of latent-MMP-1 in serum and SF (Andrews 1965), using column (1.21 x 110 cm) which contains sephadex G-75 gel to (106 cm) height. The fractions were collected (2ml/5 min) at a flow rate of 24 ml/hour, using a distilled water as eluent solution. Approximate molecular weight of the partially purified latent-MMP-1 was determined from its elution volume under the same conditions of known molecular weight.

D- Freeze- Drying (Lyophilization) Technique

The porteinous fraction which was obtained from ammonium sulfate precipitation method and gel filtration column was dried by lyophilization technique to obtain solid or concentrated protein solution. The porteinous compound was kept in a deep freeze at -20°C in a tight sample tube to be used for further investigations.

- Reveres Phase -High- Performance Liquid Chromatography (RP-HPLC)

The study included using RP-HPLC technique (Shimadzu System) employing the column C_8 (RP8) which contains n-alkyl silica as a solid phase. Acetonitril 90 % solution (90:10; acetonitril: distilled water) was used as a mobile phase, at a flow rate of 2 ml/minute with UV-detector set at 280 nm (Carr, 2002). A sample from gel filtration (peak A) for serum and synovial fluid as a source of latent MMP-1 and known molecular weight proteins (as standards) were applied. This analysis was performed in the state Company for Drug Industries and Medical Appliances in Nineveh.

RESULTS AND DISCUSSION

The results of partial purification of MMP-1 from serum and SF are shown in Table (1 and 2).

The data in Table 1 and 2 show that the recovery of MMP-1 increased in peak A 154.89% and 189% than crude extracts of serum and SF respectively. The increased enzyme activity in SF of patient with RA to about 5 times more than the enzyme activity in serum of healthy due to the presence of cytokines derived from inflammatory cells such as interleukin-1, which can modulate the amount of collagenase synthesized. This result is similar to the finding of several studies (Kim *et al*., 2011; Peak *et al*., 2005; Tchetverikov *et al*., 2005; Maeda *et al*., 1995).

Table 1: Partial purification of latent-MMP-1 from serum healthy individual

Steps	Volume (ml)	Protein conc. (mg/ml)	Total latent MMP-1 activity (ng/ml)	Total specific activity of latent-MMP-1 (ng/mg)	Purification fold	Recovery %
Serum	30	1470.9	3321	2.258	1	100
After precipitation	22	234.74	3090.56	13.165	6	93.06
After Dialysis	26	235.04	3494.14	14.866	7	105.21
Gel filtration sephadex (G-75) peak (A)	31.2	58.968	5143.94	87.232	39	154.89
Gel filtration sephadex (G-75) peak (B)	27.1	76.15	1722.21	22.615	10	51.858

Table 2: Partial purification of latent-MMP-1 from SF of rheumatoid arthritis patient

Steps	Volume (ml)	Protein conc. (mg/ml)	Total latent MMP-1 activity (ng/ml)	Total specific activity of latent-MMP-1 (ng/mg)	Purification fold	Recovery %
Synovial fluid	21	483	11036.97	22.85	1	100
After precipitation	15	149.1	9180	61.569	3	83.18
After Dialysis	20	165.6	10354.6	62.528	3	93.82
Gel filtration sephadex (G-75) peak (A)	28.3	18.961	20860.5	1100.179	48	189
Gel filtration sephadex (G-75) peak (B)	30.2	43.49	4990.25	114.75	5	45.21

- Fractionation of Total Protein

Fractionation of total proteins, resulting from ammonium sulfate saturation after dialysis and lyophilization for serum and SF, by gel filtration chromatography produced two porteinous compounds A and B (Fig.1 and 2). Peak A was obtained with high MMP-1 activity (164.87 and 737.12) ng/ml from serum and SF respectively while peak B with low MMP-1 activity in both serum and SF was neglected at this stage.

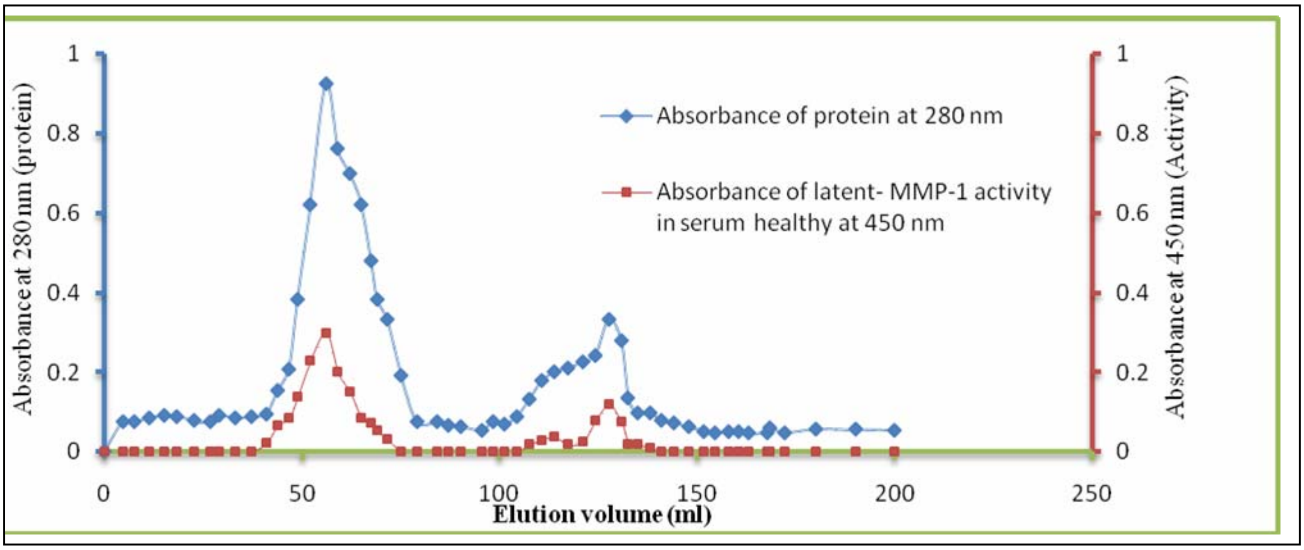


Fig. 1: Elution profile of latent-MMP-1 in serum healthy using G-75, column (1.21 x 110 cm) to height (106 cm). Each fraction was (2 ml/5 minutes) at a flow rate of 24 ml/hour

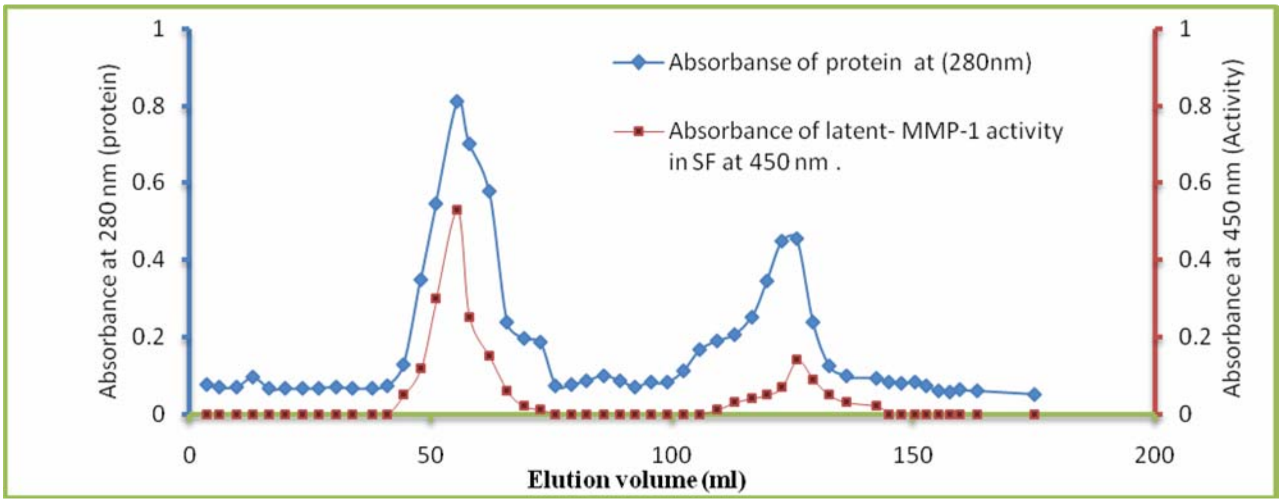


Fig. 2: Elution profile of latent-MMP-1 in SF of RA patient using G-75, column (1.21 x 110 cm) to height (106 cm). Each fraction was (2 ml/5 minutes) at a flow rate of 24 ml/ hour

The apparent molecular weight (MWt.) of the partially purified latent-MMP-1 peak A in serum and SF was determined by gel filtration (Andrews, 1965) and found to be in the range of (47752 ± 816) and (48194 ± 707) dalton respectively by comparing them with known molecular weight proteins which are listed in Table (3) and Fig. (3). These results were in good agreement with other findings (Vater *et al*., 1978).

Table 3: Elution volume of known molecular weight materials and samples from (serum and SF) using G-75

Material	Molecular weight (dalton)	Elution volume (ml)
BSA	67000	40
α-amylase	58000	46
Egg albumin	45000	58
Pepsin	36000	70
Latent-MMP-1/Serum	47752	55.6
Latent-MMP-1/SF	48194	55.1

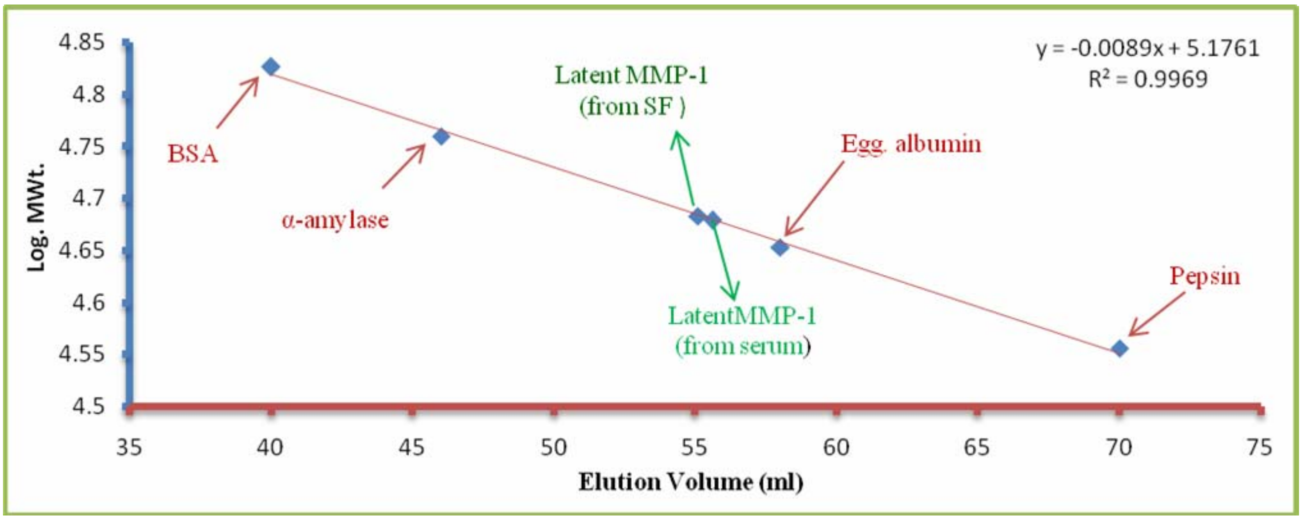


Fig. 3: Standard curve for determining the molecular weight (MWt.) of Latent-MMP-1 from serum and synovial fluid using Sephadex G -75

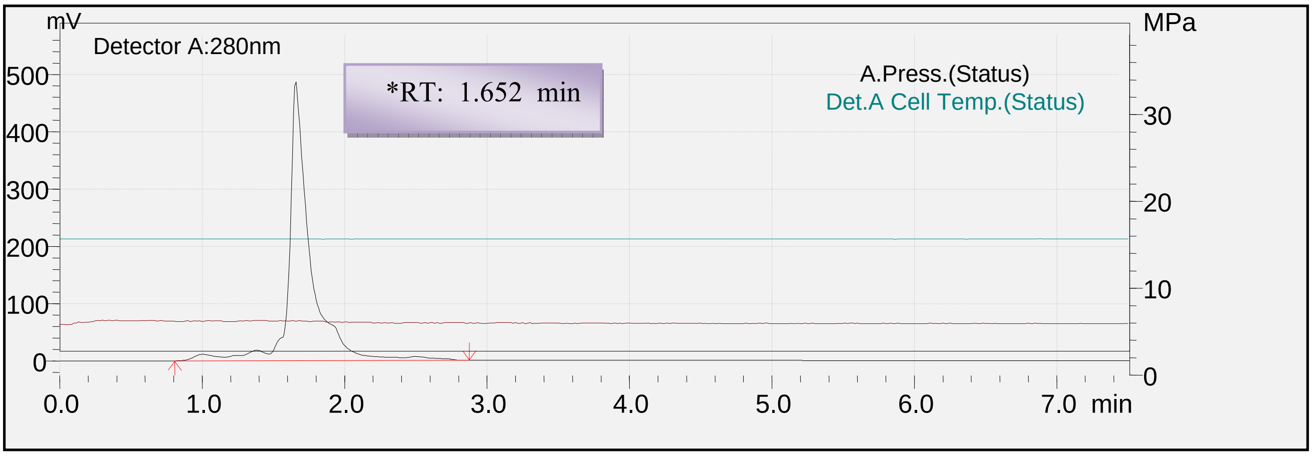
- Reveres Phase High- Performance Liquid Chromatography (RP-HPLC)

Reveres Phase High- Performance Liquid Chromatography technique (RP-HPLC) was used to check the purity of the isolated latent matrix metalloproteinase-1 (peak A) from serum and synovial fluid.

The results in Fig. (4 and 5) show a sharp peak for serum and SF manifested with retention times (RT) (1.652 and 1.65) minutes respectively. This technique was also used to determine the approximate molecular weight of peak (A) as a source of latent MMP-1 from serum and SF by comparing them with the known molecular weight proteins which were listed in Fig. (6, 7,8 and 9).

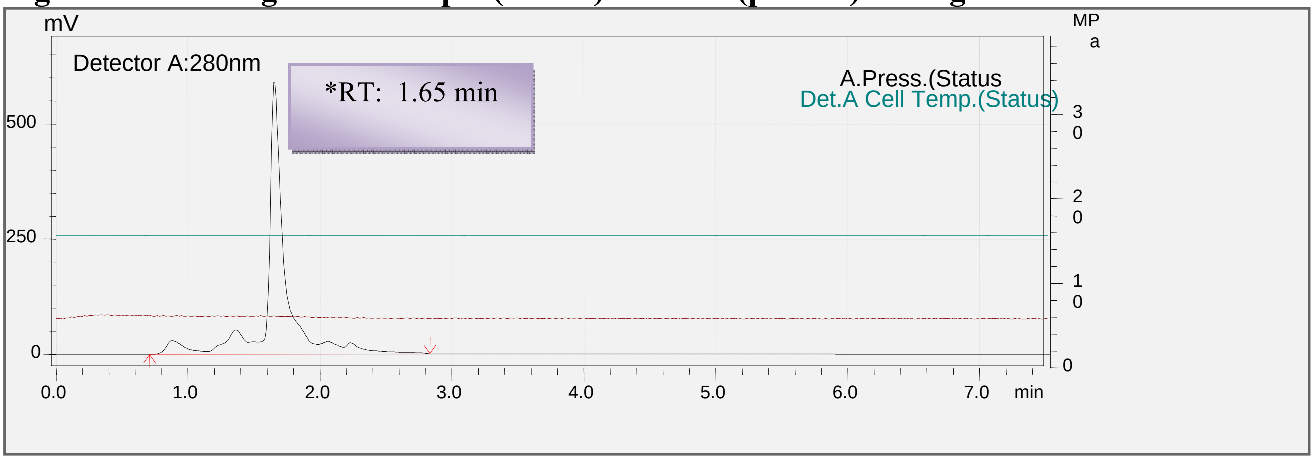
The results show a sharp peak appearance of serum and SF between retention times of standards egg albumin and latent- MMP-1 which indicates that the molecular weight of enzyme in serum and SF were found (48067 and 48033) dalton respectively.

These results are in good agreement with other findings (Vater *et al*., 1978; Maeda *et al.*, 1995; Daboor *et al.*, 2012). This enzyme is converted by trypsin to an active form with a molecular weight of about (33000) dalton. When the enzyme is mixed with an inhibitor the active enzyme formed an inactive complex again with molecular weight between (45000-49000) dalton (Vater *et al.*, 1978).



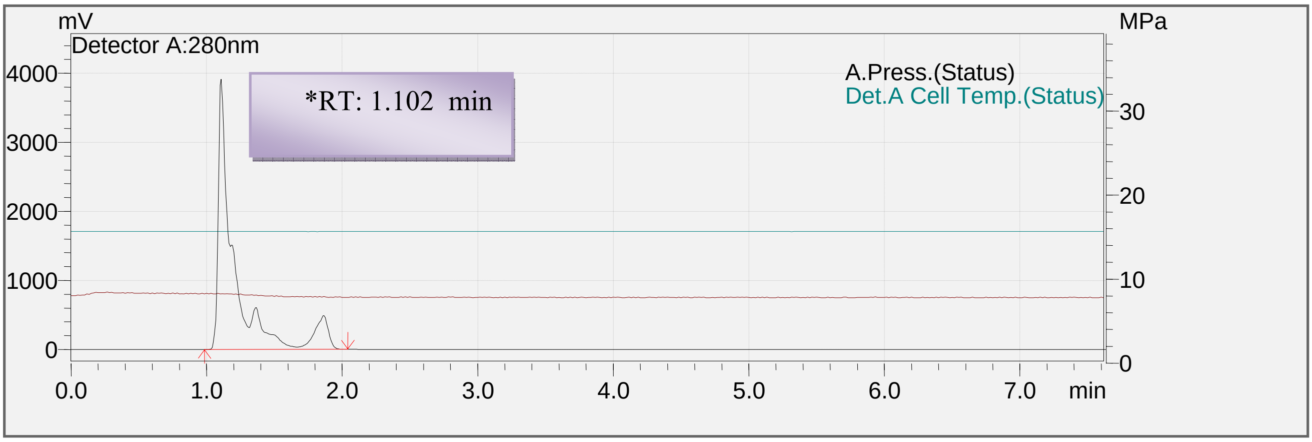
*RT: retention time

Fig. 4: Chromatogram of sample (serum) solution (peak A) from gel filtration



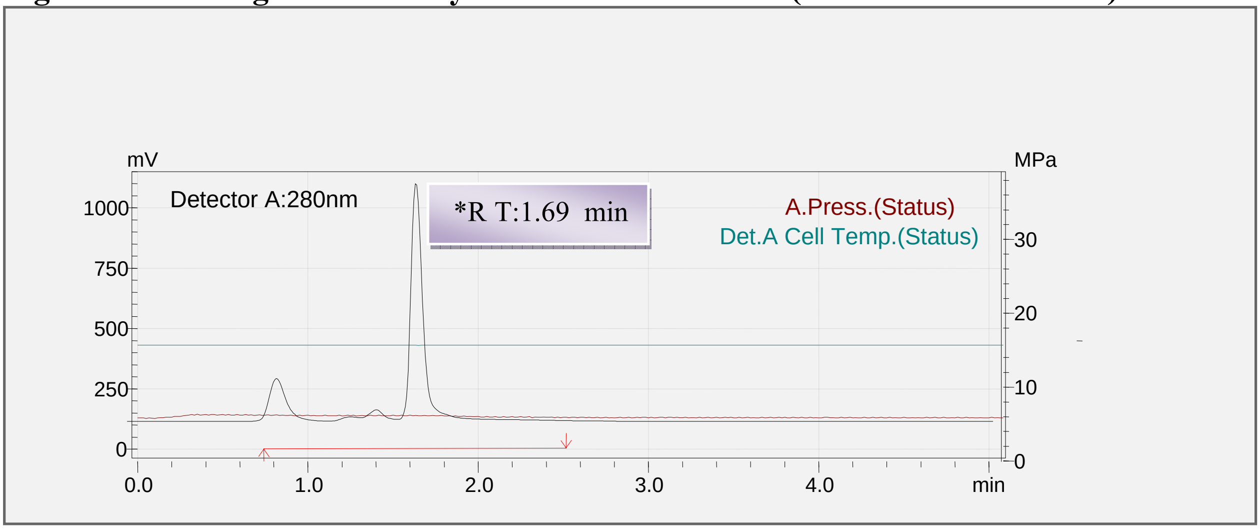
*RT: retention time

Fig. 5: Chromatogram of sample (synovial fluid) solution (peak A) from gel filtration



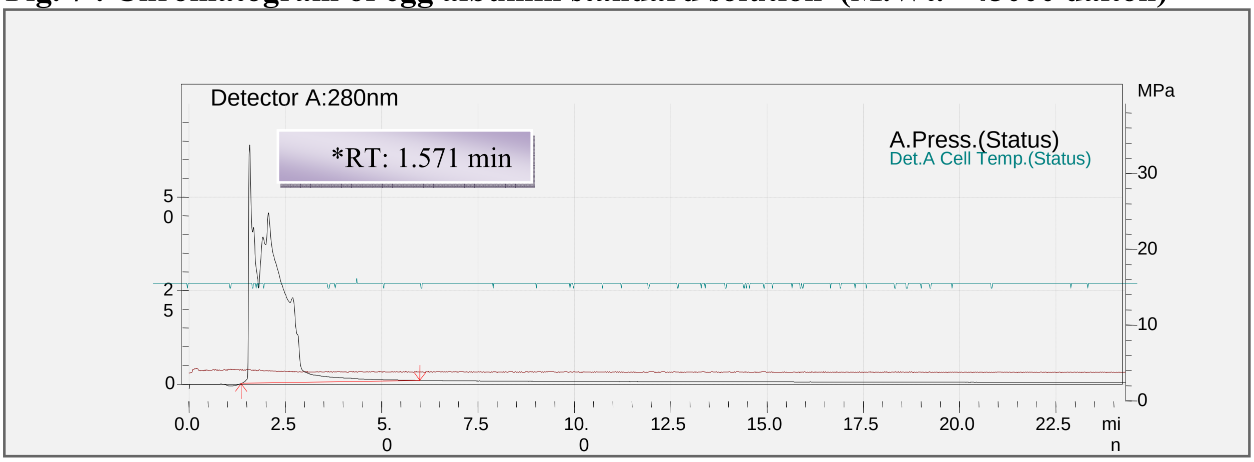
*RT: retention time

Fig. 6: Chromatogram of α-amylase standard solution (M.Wt.= 58000 dalton)



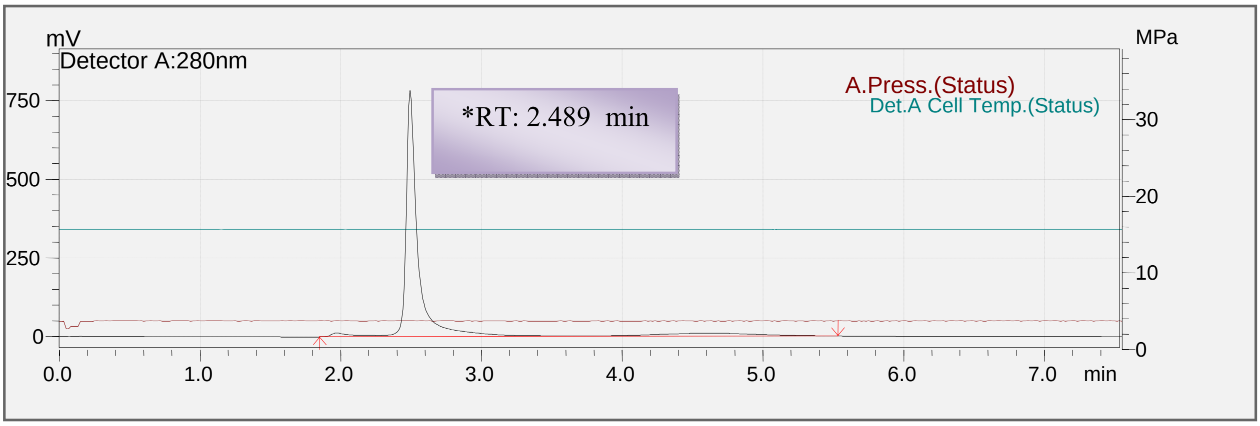
*RT: retention time

Fig. 7 : Chromatogram of egg albumin standard solution (M.Wt.= 45000 dalton)



*RT: retention time

Fig. 8: Chromatogram of latent MMP-1 standard solution (M.Wt.= 53000 dalton)



*RT: retention time

Fig. 9: Chromatogram of of pepsin standard solution (M.Wt.= 36000 dalton)

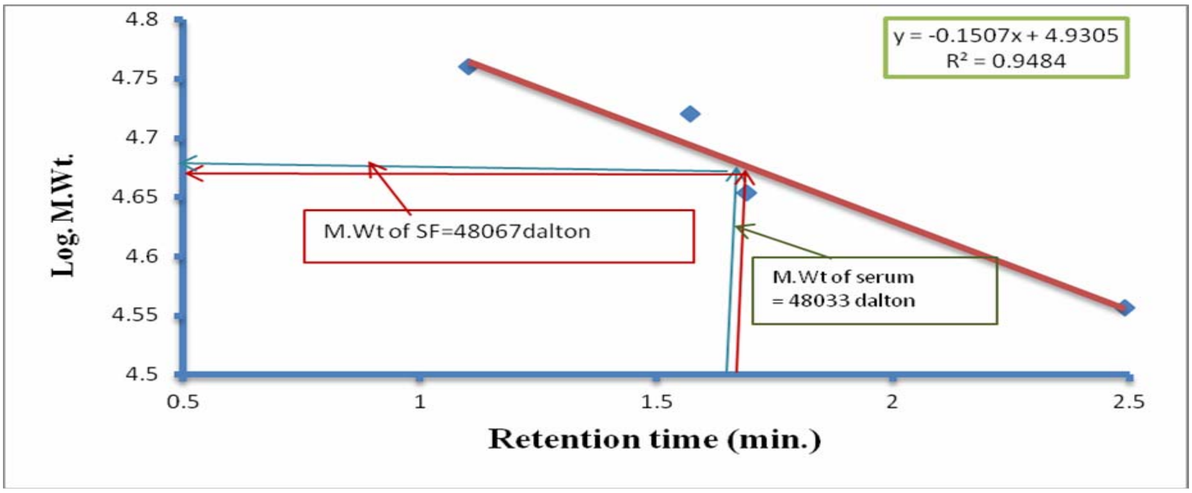


Fig. 10: Standard curve for determining the molecular weight (MWt.) of Latent-MMP-1 from serum and synovial fluid using using HPLC

- The Effect of Various Material Compounds on Latent MMP-1 Activity

In order to evaluate the effect of some material compounds on partially purified latent-MMP-1 activity *in vitro*, series of experiments were performed and the results were listed in Table 4.

The results in Table (4) revealed that ethylene diamine tetra acetic acid (EDTA), 2, 4-dintrophenol, sodium arsenate, sodium azide, potassium oxalate, mercaptoethanol (MEH), glutathion (GSH), cystein and normal human serum decrease latent-MMP-1 activity while $FeSO_4$, $BaCl_2$ and ascorbic acid showed no effect.

Metallo and serine collagenase require calcium, therefore, addition of EDTA and 2, 4-dintrophenol leads to a decrease in the enzyme activity because EDTA acts as a chelating agent (Daboor *et al.*, 2010; Hamdy, 2008; Swann *et al.*, 1981; Fullmer *et al.*, 1972; Hook *et al.*, 1971). Moreover, the enzyme activity declined when cystein, GSH and sodium arsenate were added. This was attributed to that these compounds act specifically on disulfide bonds (Daboor *et al.*, 2010; Hook *et al.*, 1971). Mercaptoethanol (MEH) causes irreversible activation of enzyme activity by reduction of its thiol functional group (Hook *et al.,* 1971). Also human serum causes a decline in enzyme activity, that α_2-macroglobulin is a major serum protein, which makes divers functions included, inhibition of the collagenase activity and binding of growth factor, cytokines disease therefore adding serum to the partial purified causes decline enzyme activity (Vater *et al.*, 1978).

On the other hand, addition some compounds to the partially purified enzyme (peak A) which were listed in Table 4 showed an increase in the enzyme activity. This could be explained to that the enzyme activity is depends on metal compounds such as NaCl, $MgSO_4$, $CaCl_2$, $CoCl_2$, $CuSO_4$ and $ZnSO_4$. Previous studies reported that the enzyme is dependant on Ca^{+2} and Zn^{+2}. Also some investigators found that Zn^{+2} simulates and acts as a cofactor. As well as Co^{+2} level reversed activity action (Hamdy, 2008; Swann *et al.,* 1981; Fullmer *et al.*, 1972). Mercuric compounds such as $HgCl_2$ causes dissociation of collagen-inhibitor complex, resulting in free enzyme. Pepsin also manifested the increase of the enzyme activity which might bind to an inhibitor protein such as α_2-macroglubuline complex (Daboor *et al*., 2010).

Table 4: Effect of some material compounds on partially purified latent- MMP-1 activity *in vitro* from serum healthy and synovial fluid (SF) of rheumatoid arthritis (RA) patients

Compound (10 mM)	% enzyme activity in serum healthy	% enzyme activity in SF of RA patient
Pure enzyme	100	100
EDTA	64.8	43
2-Mercaptoethanol(MEH)	88.8	72.82
Cystein	91.1	82.4
Glutathione(GSH)	79.8	67
Sodium arsenate	84.69	70.3
Normal human serum	55.1	43
2,4-Dinitrophenol	87.9	73.1
Sodium azide	81.5	80.6
Potassium oxalate	84.14	72.3
$FeSO_4$	101.3	103.6
$BaCl_2$	99	102
Ascorbic acid	102	108
pepsin	120	113
NaCl	114	117
$MgSO_4$	125	133
$CaCl_2$	135	14o
$CoCl_2$	113	119
CuSO4	110	122.2
$ZnSO_4$	131	144
$HgCl_2$	155	175

REFERENCES

Aletaha, D.; Neogi, T.; Sliman, A.J. (2010). Rheumatoid arthritis classification criteria an American College of Rheumatology/European League Against Rheumatism collaborative initiative. *Ann. Rheum. Dis.,* **69**,1580-1588.

Andrews, P. (1965). The gel filtration behavior of proteins related to their molecular weight over a wide range. *J. Biochem.* **96**, 595-606.

Arnett, F.C.; Edworthjy, S.M.; Bloch, D.A. (1988). The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis. Rheum*., **31**, 315-324.

Beurden, P.A.M.; Hoff, J.W. (2005). Zymographic techniques for the analysis of matrix metalloproteinases and their inhibitors. *J. Bio. Techniques*, **38**(1), 73-83.

Carr, D. (2002). " The Handbook of Analysis and Purification of Peptides and Proteins by Reversed Phase HPLC". 3rd ed., Vydac, Hesperia, CA, USA. pp.30-39.

Chen, Y.; Nixon, N.B.; Dawes, P.T.; Mattey, D.L. (2012). Influence of variations across the MMP-1 and -3 genes on the serum levels of MMP-1 and -3 and disease activity in rheumatoid arthritis. *Genes Immunity*, **13** , 29- 37.

Daboor, S.; Buduge, S.M.; Ghaly, A.E.; Brooks, S.L.; Dave, D. (2010). Extraction and purification of collagenase enzymes. Acritical review. *Am. J. Biochem. and Biotech*,**6** (4), 239-263.

Daboor, S.M.; Budge, S.M.; Ghaly, A.E.; Brooks, M.S.; Dave, D. (2012). Isolation and activation of collagenase from fish processing waste. *Advances in Biosci. and Biotech*., **3**,191-203.

Dioxin, M.; Weeb, E.C. (1961). "Tools of Biochemistry". T.G. Copperol: 370. John Wiley and sons. Inc,. 370 p.

Erdam, S.P.H.; Serdar, M.; Dinc, A.; Simsek, I.; Turan, M. (2002). Comparison of synovial MMP-1 and TIMP-1 levels in patients with various inflammatory arthiritis: is there any difference between rheumatoid arthritis, behcet s disease and familial Mediterranean. *Clin Rheumatol*. **21**, 511-515.

Fasciglione, G.F.; Gioia, M.; Tsukada, J.l.; Iundusi, R.; Tarantino, U.; Coletta, M.; Pourmotabbed, T.; Marini, S. (2012). The collagenolytic action of MMP-1 is regulated by the intraction between the catalytic domain and the hinge region. *J. Biol. Inorg. Chem*. **17**, 663-672.

Fields, J.L.L.; Tuzinski, K.A.; Shimokawa, K.; Nagase, H. (2000). Hydrolysis of triple-helical collagen peptide models by matrix metalloproteinases. *J. Biol. Chem*., **275**(18), 13282-13290.

Fullmer, H.M.; Taylor, R.E.; Guthrie, R.W. (1972). Human gingival collagenase: purification, molecular weight and inhibitor studies. *J. Dent. Supplement*. **51**, 349-355.

Hamdy, H.S. (2008). Extracellular collagenase from Rhizoctonia Solani: production, purification and characterization. *Indian J. Biotechnol*. 7, 333-340.

Holm, D.J. ; Peak, H. (1988). "Analytical Biochemistry". John Wiley and Sons, Inc., New York, USA.

Hook, C.W.; Brown, S.I.; Iwanij, W.; Nakanishi, I. (1971). Characterization and inhibition of corneal collagenase. *Ophthalmol. Visual Sci.* **10**, 496-503.

Jain, R.; Jain P.C. (2010). Production and Partial characterization of collagenase of Streptomyces exfoliates CFS 1068 using poulry feather. *Indian J. Experimental Biology*. **48**,174-178.

Kim, K.S. ; Choi, H.M.; Lee, Y.A.; Choi, I.A.; Lee, S.H. ; Hong, S.J.; Yang, H. I. ; Yoo, M. C. (2011). Expression of gelatinases MMP-2 and MMP-9 and colagenases MMP-1 and

MMP-3 with VEGF in synovial fluid of patients with arthritis. *Rheumatol Int.*, **31**, 543-547.

Leite, S. R. A. (2009). Inhibitors of collagenase, MMP1. *Ecl. Quim., Sao Paulo*, **34**(4),187-102.

Maeda, S.; Sawai, T.; Uzuki, M.; Takahashi, Y.; Omoto, H.; Seki, M.; Sakurai M. (1995). Determination of interstitial collagenase (MMP-1) in patients with rheumatoid arthritis. *Ann. Rheum. Dis.*, **54**, 970-975.

Matsumoto, T.; Tsurumoto, T. (2006). Interleukin-6 levels in synovial fluids of patients with rheumatoid arthiritis correlated with the infiltration of inflammatory cells in synovial membrane. *Rheumatol Int.* **26**, 1096-1100.

Peak, N.J.; Khawaja, K.; Myers, A.; Jones, D.; Cawston, T.E.; Rawan, A.D.; Foster, H.E. (2005). Levels of matrix metalloproteinase (MMP-1) in paired sera and synovial fluids of juvinale idiopathic arthiritis patients: relationship to inflammatory activity, MMP-3 and tissue inhibitor of metalloproteinases-1 in alongitudinal study. *Rheumatology* **44**,1383-1389.

Philips, N.; Auler, S.; Hugo R. and Gonzalez S. (2011). Beneficial regulation of matrix metalloproteinases for skin healt. Enzyme Research, Review Articale. **10**: 1-4.

Polyakova, V.; Loeffler, I.; Hein, S.; Miyagawa, S.; Piotrowska, I.; Dammer, S.; Risteli, J.; Schaper, J.; Kostin, S. (2011). Fibrosis in endstage human heart failure: severe changes in collagen metabolism and MMP/TIMP profiles. *International J. Cardiology*, **151**,18-33.

Robyt , J.F.; White, B.J. (1987). "Biochemical Techniques Theory and Practice". Wads Worth Inc., Monterey, California, USA., pp. 235-236, 263, 269.

Sandya, S.; Achan, M.A.; Sudhakaran, P. R. (2009). Multiple matrix metalloproteinases in type II collagen induced arthritis. *Indian J. Clinical Biochem*., **24**(1), 42-48.

Schacterle, G.R.; Pollack, R.L. (1973). A simplified method for the quantitative assay of small amounts of protein in biological material. *Anal. Biochem*., **51**,654-655.

Sherer, S.; Souza, T.B.; Paoli, J.; Brenol, C.V.; Xavier, R.M.; Brenol, J.C.T.; Chies, J.A. (2010). Matrix metalloproteinase gene polymorphisms in patients with rheumatoid arthritis. *Rheumatol INT*., **30**, 369-373.

Smith, Z.G.; Nagase, H.; Woessner, J.F. (1989). Purification of nutral proteoglycan-degrading metalloproteinase from human articular cartilage tissue and its identification as stromelysin matrix metallop roteinase-3 . *J. Biochem*. **258**,115-119.

Swann, J.C.; Reynolds, J.J.; Galloway, W.A. (1981). Zinc metallo properties of active and latent collagenase from rabbit bone. *J. Biochem*.**195**, 41-49.

Tchetverikov, I.; Lohmander, L.S.; Verzijl, N.; Huizinga, T.W.J.; Tekoppele, J.M.; Hanemaaijer, R.; Degroot, J. (2005). MMP and activity levels in synovial fluid from patients with joint injury, inflammatory arthritis and osteoarthritis. *Ann. Rheum. Dis*, **64**, 694-698.

Tietz, N.W. (1994). "Text Book of Clinical Chemistry". W. B. Sanders Company, USA, A. Division of Harcourt Brace and Company, Philadelpha.

Vater, C.A.; Mainardi, C.L.; Harris, E.D. (1978). Activation in vitro of rheumatoid collagens from cell cultures. *J. Clin. Invest*. **62**, 987-992.

Yang, X.; Wang, Y. (2011). Role of siglecs on the leucocytes during the process of the joint's inflammation in rheumatoid arthritis. *Medical Hypotheses*, **77**,1051-1053.