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

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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 proteinous 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, MgSO4, CoCl2, CaCl2, CuSO4, ZnSO4, HgCl2 and pepsin increases the activity of enzyme.

Keywords: Matrix metalloproteinase-1, collagenase-1, Rheumatoid arthritis.
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, tend 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 chondrocytes (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 antibody. The wells were washed and the biotinylated anti-human MMP-1 antibody 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'-tetramethyl 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 proteinous 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 proteinous compound was kept in a deep freeze at -20°C in a tight sample tube to be used for further investigations.

**- Reverse Phase -High-Performance Liquid Chromatography (RP-HPLC)**

The study included using RP-HPLC technique (Shimadzu System) employing the column C8 (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**

<table>
<thead>
<tr>
<th>Steps</th>
<th>Volume (ml)</th>
<th>Protein conc. (mg/ml)</th>
<th>Total latent MMP-1 activity (ng/ml)</th>
<th>Total specific activity of latent-MMP-1 (ng/mg)</th>
<th>Purification fold</th>
<th>Recovery %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>30</td>
<td>1470.9</td>
<td>3321</td>
<td>2.258</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>After precipitation</td>
<td>22</td>
<td>234.74</td>
<td>3090.56</td>
<td>13.165</td>
<td>6</td>
<td>93.06</td>
</tr>
<tr>
<td>After Dialysis</td>
<td>26</td>
<td>235.04</td>
<td>3494.14</td>
<td>14.866</td>
<td>7</td>
<td>105.21</td>
</tr>
<tr>
<td>Gel filtration sephadex (G-75) peak (A)</td>
<td>31.2</td>
<td>58.968</td>
<td>5143.94</td>
<td>87.232</td>
<td>39</td>
<td>154.89</td>
</tr>
<tr>
<td>Gel filtration sephadex (G-75) peak (B)</td>
<td>27.1</td>
<td>76.15</td>
<td>1722.21</td>
<td>22.615</td>
<td>10</td>
<td>51.858</td>
</tr>
</tbody>
</table>
Table 2: Partial purification of latent-MMP-1 from SF of rheumatoid arthritis patient

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

- **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 proteinous 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

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

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

Reverses 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)

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

Fig. 8: Chromatogram of latent MMP-1 standard solution (M.Wt.= 53000 dalton)
- 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-dintrophelenol, sodium arsenate, sodium azide, potassium oxalate, mercaptoethanol (MEH), glutathion (GSH), cystein and normal human serum decrease latent-MMP-1 activity while FeSO₄, BaCl₂ and ascorbic acid showed no effect.

Metallo and serine collagenase require calcium, therefore, addition of EDTA and 2, 4-dintrophelenol 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 α₂-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, MgSO₄, CaCl₂, CoCl₂, CuSO₄ and ZnSO₄. Previous studies reported that the enzyme is dependant on Ca²⁺ and Zn²⁺. Also some investigators found that Zn²⁺ simulates and acts as a cofactor. As well as Co²⁺ level reversed activity action (Hamdy, 2008; Swann et al., 1981; Fullmer et al., 1972). Mercuric compounds such as HgCl₂ 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 α₂-macroglobulin 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

<table>
<thead>
<tr>
<th>Compound (10 mM)</th>
<th>% enzyme activity in serum healthy</th>
<th>% enzyme activity in SF of RA patient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pure enzyme</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>EDTA</td>
<td>64.8</td>
<td>43</td>
</tr>
<tr>
<td>2-Mercaptoethanol(MEH)</td>
<td>88.8</td>
<td>72.82</td>
</tr>
<tr>
<td>Cystein</td>
<td>91.1</td>
<td>82.4</td>
</tr>
<tr>
<td>Glutathione(GSH)</td>
<td>79.8</td>
<td>67</td>
</tr>
<tr>
<td>Sodium arsenate</td>
<td>84.69</td>
<td>70.3</td>
</tr>
<tr>
<td>Normal human serum</td>
<td>55.1</td>
<td>43</td>
</tr>
<tr>
<td>2,4-Dinitrophenol</td>
<td>87.9</td>
<td>73.1</td>
</tr>
<tr>
<td>Sodium azide</td>
<td>81.5</td>
<td>80.6</td>
</tr>
<tr>
<td>Potassium oxalate</td>
<td>84.14</td>
<td>72.3</td>
</tr>
<tr>
<td>FeSO₄</td>
<td>101.3</td>
<td>103.6</td>
</tr>
<tr>
<td>BaCl₂</td>
<td>99</td>
<td>102</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>102</td>
<td>108</td>
</tr>
<tr>
<td>pepsin</td>
<td>120</td>
<td>113</td>
</tr>
<tr>
<td>NaCl</td>
<td>114</td>
<td>117</td>
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<tr>
<td>MgSO₄</td>
<td>125</td>
<td>133</td>
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<td>CaCl₂</td>
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<td>CoCl₂</td>
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<td>CuSO₄</td>
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<td>ZnSO₄</td>
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<tr>
<td>HgCl₂</td>
<td>155</td>
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