Study of serum adenosine deaminase -2 (ADA-2) activity in rheumatoid arthritis

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Abstract

Prevent joint pain early detection of Rheumatoid arthritis (R.A.) is required. Adenosine deaminase-2 (ADA-2) plays a vital role in R.A. Our study aimed to assay the level of serum ADA-2 activity for R.A. patients. Purification of ADA-2 from the serum of patients and study cytotoxicity of purified ADA-2 on breast cancer (MCF-7) cell line and standard cell line (WRL-68). Activity levels of t-ADA, ADA-1, and ADA-2 were estimated. Also, isolated and purified ADA-2 from serum R.A, and finally, the cytotoxic activity of purified ADA-2 on MFC-7 and WRL -68 cells for 24h was examined using MTT assay. A significant increase (p≤ 0.0001) in ADA-t and ADA-2 activity for R.A. patients compared to healthy adults. The specific activity of the purified ADA-2 was 13 U/mg protein, with 16.3 folds of purification and 59.4% enzyme recovery. Also, ADA-2 is a single band and has a molecular weight of 105 K Da. Cell line viability showed that purified ADA-2 inhibited breast cancer cell line growth (MFC-7) at an IC50 level of 133.7 μg/ml more than affected standard cell line (WRL-68) IC50 302 μg /ml. Also, the cytotoxicity of the enzyme is higher at lower concentrations. The level of ADA-2 can be used for diagnosing R.A. disease. Purifying ADA-2 and showing its inhibitory effect on MCF-7 at low concentrations helps its use as an anti-breast cancer marker and studying the impact of anti-drugs on developing breast cancer.
1. Introduction:

The most common form of arthritis is rheumatoid arthritis (R.A.). [1]. Although the etiologies of R.A disease are still unclear; but, many factors may play an essential role in the occurrence of the disease: immunological, physiological, and hereditary [2-6]. Studies are continuing to know the causes of rheumatoid arthritis and the changes that coincide with the development of the disease, so the desired goal for patients is to reach the state of remission [7, 8]. Adenosine deaminase (ADA, E. C. 3.5.4.4) is an essential enzyme that assistance in purine turnover. ADA induce the deamination of adenosine to inosine and release ammonia [9-16]. It is considered a biomarker for immunity disease [8, 17, 18]. Many studies revealed the level of ADA activity reflects macrophage and monocyte activity in inflammatory environments, such as rheumatoid arthritis and systemic lupus system [19, 20]. It also has an essential role in severe cases and biological functions that include the differentiation and maturity of immune cell components [3, 21]. Thus adenosine deaminase may be a predictive indicator of inflammatory processes in rheumatoid arthritis [2, 22-24]. Whereas, it was found that adenosine deaminase activity in patients with rheumatoid arthritis was higher than that of osteoarthritis (O.A.), thus making it possible to use adenosine deaminase to distinguish it from other types of arthritis diseases. And the level activity of adenosine deaminase is higher in the synovial fluid of patients with rheumatoid arthritis higher than their serum, reflecting the extent of subsequent damage to the affected joint. Adenosine deaminase has two isoenzymes: adenosine deaminase-1 (ADA-1) and adenosine deaminase-2 (ADA-2). ADA-1 is available in all body cells, while ADA-2 is only found in monocytes and macrophages [25]. ADA-2 is a significant component of total ADA activity in human plasma, and ADA2 activity is substantially elevated in patients with different diseases such as diabetes, rheumatoid arthritis[26], HIV (human immunodeficiency virus), and chronic hepatitis.

Our study aimed to estimate levels of total adenosine enzyme (t-ADA), ADA-1, and ADA-2 activity in the serum for fifty Iraqi patients with rheumatoid arthritis. Also, purification of ADA-2 from serum R.A. patients and its test cytotoxicity on breast cancer cell line (MCF-7) and standard (normal) cell line (WRL-68) of ADA-2 for the serum of R.A patient.

2. Material and Methods

2.1. Population Study

This study was conducted in the period from 1st June 2019 to 1st May 2020. Fifty adults had rheumatoid arthritis from both sexes (29 females/21males). Their ages ranged from 25 to 62 years. These patients visited the rheumatology unit in AL-Kansha Teaching Hospital and those who
review the outpatient medical clinic for rheumatism and licensed by Nineveh health. These patients were met the American College of Rheumatology (ACR) and the European League Against Rheumatism (EULAR) criteria for the diagnosis of RA (ref.). At the same time, all of the patients who participated were subjected to the radiographic examination. Many criteria were excluded from this study included: Inflammatory joint diseases other than rheumatoid arthritis, such as osteoarthritis or gout. As well as the incidence of other conditions such as diabetes and cancer. Besides, 30 volunteers who were subjected to this study were considered controls. Their age was 35-60 years from both sexes (17 females, 13 males). Moreover, The Study's Protocol Was Verified By The Ethics Committee In The University Of Mosul, the college of science, And the Health Department of Nineveh. All of the participants were provided with the written form of informed consent.

2.2. Samples Collection:

Blood samples were collected from the patient's vein in a clean, dry, and tightly covered plastic Plan tube in a volume of (5ml). The tubes were placed in a Centrifuge for (15) minutes at a speed of 3000 xg after being incubated in a water bath at 37°C for 10 minutes. The serum was then extracted from the clotted part of the blood and stored at -20 °C until use.

2.3. Determination of Adenosine Deaminase Activity

Total adenosine deaminase (t-ADA) activity level, adenosine deaminase-1 (ADA-1), and adenosine deaminase-2 (ADA-2) were assayed according to the method of Giusti [27]. Briefly, ADA activity was assayed by the phenol-hypochlorite colorimetric method, evaluating the amount of ammonia liberated in the catalyzed deamination of adenosine from the absorbance of the assay mixture at 630 nm. ADA2 activity level was measured in the presence of selective ADA1 inhibitor, erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA) (0.1mM). Total ADA activity was measured in the absence of EHNA, and ADA1 activity was then calculated by subtracting the ADA2 from the total ADA activity [28].

2.4 Protein Assay

Protein concentration was determined according to the Bradford method (1976), using the Coomassie blue dye G-250. The standard curve of bovine serum albumin (BSA) was used at different concentrations to find the concentration of the unknown protein [29].
2.5. Purification of Adenosine Deiminase-2 (ADA-2)

2.5.1 Precipitation of ADA-2

The protein was precipitated, through a gradual addition of ammonium sulfate, at a saturation rate of 65% to 50 ml of serum for rheumatoid arthritis patients. At a temperature of 4 °C, the addition was slow, using slow stirring, and using a magnetic stirrer. Leave the precipitate for 24 hours at a temperature of Celsius. Then the precipitate was separated from the filtrate using a refrigerated centrifuge at 300xg for 20 minutes [30]. The precipitate was dissolved with an appropriate volume of sodium phosphate in a solution of sodium phosphate at pH = 6.5

2.5.2 Ion Exchange Chromatography:

DEAE-Cellulose column was used to pass the enzyme through (2.5 cmx20 cm). NaCl solution with a gradual concentration was used for sequential filtration (0.1-1 M). Each fraction had a flow rate of 1 ml/fraction. Using a U.V. Spectrophotometer, the absorbance of each fraction was estimated at 280 nm. The activity of adenosine deiminase-2 was measured in each fraction. Adenosine deiminase-2-active fractions were pooled and held for further purification steps [31].

2.5.3 Gel Filtration Chromatography:

The partially purified enzyme from the ion exchange step was applied to the Sephadex G-200 column (2.5 cm x 35 cm). Elution achieved at a rate of 1 ml/fraction. At 280 nm, the optical density of each fraction was measured. The enzyme activity in the peaks was measured, and the active fractions were collected[32, 33].

2.6 Determine the Purity and Molecular Weight of Adenosine Deaminase (ADA-2)

The purity of adenosine deiminase -2 (ADA-2) was measured using electrophoresis on an SDS polyacrylamide gel (8cmx 8cm) in the presence of standard proteins with molecular weights in the range of 50,000 to 100,000. (10000-225000 Dalton). After electrophoresis, the distances of protein migrated to the anode were measured to assess the enzyme molecular weight [34].

2.7 The Cytotoxic Effect of Adenosine Deiminase-2 (ADA-2)

2.7.1 Cancer Cell Lines Used

In this study, the MCF-7 breast cancer cell line and, for comparison purposes, the WRL-68 standard hepatocyte cell line were used, which were obtained from the University of Malaya Kuala Lumpur / College of Medicine /Department of Pharmacy /Center For Natural Products Research
And Drug Discovery/ Malaysia. The cancer streak cells were maintained and developed and examined at the CAC Center for Research and Biotechnology in Baghdad.

2.7.2 Cytotoxic Assay (MTT (Methylthiazolyldiphenyl-Tetrazolium) Assay)

The cytotoxic effect of adenosine deiminase-2 on the cell lines was performed by using MTT (Methylthiazolyldiphenyl-tetrazolium) assay:

MTT solution (10 ml) was applied to each well of a 96-well plate, which was then incubated for 4 hours at 37o C with the testing sample (The solution became yellow). After that, each well was filled with 200 μl of DMSO (dimethylsulfoxide) and shaken for 5 minutes (The DMSO solution became purple). With an ELISA reader, the absorbance of the colored solution obtained from living cells was red at 575 nm after complete solubilization of the dye. For each group of replicates, the mean absorbance was determined. Cell Viability % = [Absorbance of treated sample/Absorbance of non-treated sample] × 100 (In all experiments, non-treated cultures contained only the medium [35]

2.8 Statistical Analysis

The results that appeared in this study were expressed as mean ± standard deviation (S.D.). The results were analyzed using a statistical program (SPSS, version 25). An independent T-test was used for comparison between two groups; when the p-value ≤ 0.05, it is considered significant.

3. Results

3.1 Study The Level of t-ADA, ADA-1 and ADA-2 in Patients with Rheumatoid Arthritis

The results showed in Table 1 that the average age of patients with rheumatoid arthritis was 44.3 ± 17.2 years and 47.2 ± 19.7 years. The average age of a healthy person is 45.3 ± 18.4 years.

Table 1: Characterization of rheumatoid arthritis patients

<table>
<thead>
<tr>
<th>Cases</th>
<th>Control adults, no.= 30</th>
<th>R.A. patients, no.=50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>45.3±18.4</td>
<td>44.3±17.2</td>
</tr>
<tr>
<td>Sex (F\M)</td>
<td>17\13</td>
<td>29\21</td>
</tr>
</tbody>
</table>

F: Females; M: Males; no. : number; R.A.: Rheumatoid arthritis.
The results showed (Fig. 1) that the level activity of t-ADA and ADA-2 in serum of patients with rheumatoid arthritis (53.3±16.4 U/ml and 42.7±14.2 U/ml; respectively) was significantly increased (p≤ 0.0001) when compared with healthy adults (21.6 ± 7.6 U/ml and 11.42 ± 2.4 U/ml; respectively). In contrast, there was no significant difference in the level of ADA-1 activity between rheumatoid arthritis patients (10.9 ±2.3 U/ml, p=0.89) and healthy subjects (10.4 ±2.3 U/ml).

![Figure 1: Levels activity of total adenosine deaminase (t-ADA), adenosine deaminase-1 (ADA-1, and adenosine deaminase -2 (ADA-2) in the serum of patients with rheumatoid arthritis and controls adults](image)

3.2 Purification of Adenosine Deiminase-2 (ADA-2)

Three purification steps were summarized in Table 2 to purify adenosine deaminase-2 produced from serum of R.A. patients under optimal conditions. The elevated enzyme-specific activity (1.31U/mg protein) of precipitation for adenosine deaminase activities was achieved at 65 % ammonium sulfate. Purification using DEAE-cellulose chromatography increased enzyme activity even further. The enzyme activity and unique activity for serum ADA-2 are depicted in the graph Fig 2. below. According to the results, the first peak in a washing step with fractions 5 to 1 included enzyme-specific activity (1.25U/ml). The enzyme-specific activity of the second peak (eluted at 0.4 NaCl) at fractions 60 to 71 reached 6.73 U/ml. The third protein peak protein with 0.5M NaCl at fractions 76 to 82 hadn't ADA-2 activity. Thus, it was neglected had adenosine deiminase activity reached 3 U/ml.
Table 2: Purification steps for adenosine deiminase-2 (ADA-2) produced from serum patients with rheumatoid arthritis.

<table>
<thead>
<tr>
<th>Purification steps</th>
<th>Volume (ml)</th>
<th>ADA-2 activity (U/ml)</th>
<th>Protein Concentration (mg/ml)</th>
<th>Specific Activity (U/mg)</th>
<th>Total Activity (U)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude enzyme</td>
<td>50</td>
<td>40</td>
<td>50</td>
<td>0.8</td>
<td>2000</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Ammonium sulfate precipitation 65%</td>
<td>35</td>
<td>47</td>
<td>36</td>
<td>1.31</td>
<td>1645</td>
<td>1.64</td>
<td>88</td>
</tr>
<tr>
<td>Ion exchange chromatography DEAE-cellulose/Washing/fraction no.(19-23)</td>
<td>5</td>
<td>25</td>
<td>20</td>
<td>1.25</td>
<td>125</td>
<td>1.56</td>
<td>6.25</td>
</tr>
<tr>
<td>Ion exchange chromatography DEAE-cellulose/Elution/fraction no, (60-71)</td>
<td>11</td>
<td>101</td>
<td>15</td>
<td>6.73</td>
<td>1111</td>
<td>8.4</td>
<td>55.6</td>
</tr>
<tr>
<td>Gel filtration/Sephadex G-200/ Elution volume at (40-52) ml</td>
<td>12</td>
<td>99</td>
<td>7.6</td>
<td>13</td>
<td>1188</td>
<td>16.3</td>
<td>59.4</td>
</tr>
</tbody>
</table>
Gel filtration chromatography technique was the final step in the purification of adenosine deaminase-2 produced by sera patients with R.A. After purification process by ion-exchange purification step; specific activity of purified adenosine deaminase-2 reached 13 U/mg with 16.3 purification fold and 59.4% overall yield, Fig. 3.

The advanced ADA-2 purification steps (from crude to purify enzyme) in SDS-PAGE chromatography for adenosine deaminase-2 of rheumatoid arthritis serum are shown in Fig4. A. The
results showed that the purified ADA-2 has a molecular weight of 105 KDa compared to standard proteins Fig. 4 B.

3.3 Cytotoxic Activity Of Adenosine Deiminase-2 (ADA-2) Using The MTT Assay

The MTT assay was used to determine the toxic effect of purified adenosine deaminase-2 (ADA-2) from the serum of rheumatoid arthritis patients on the MCF-7) cancer cell line to the normal cell line WRL-68. As percentage survival of cells MCF-7) and WRL-68, respectively, in a dependent manner. Use different concentrations of purified ADA-2 and incubate it with cancer and standard cell lines. It was found that ADA-2 has a toxic effect on the MCF-7) cell line. This effect increases at low concentrations of 25 µg/ml and decreases with increasing concentration and IC50, an inhibitory concentration of half of the cell growth. The ADA-2 for the purified was IC50 133.7 µg/ml for the cancer cell line, while the ADA-2 was not inhibited for the normal cell line WRL-68,
IC50 302 μg/ml. The results also showed that at a concentration of 25 μg / ml, the survival rate of cancerous and normal cells was observed at 35% and 80%, respectively, after incubation with purified ADA-2 for 24 hours. However, (Figure 5 and Table 3).

**Figure 5:** Cytotoxic activity of purified adenosine deaminase-2 (ADA-2) on hepatocellular carcinoma cell line using MTT test after 24 h and 37°C.

**Table 5:** Effects of different concentrations of purified adenosine deaminase-2 (ADA-2) on the growth of breast cancer cell line (MCF-7) and standard cell line (WRL-68) in incubation for 24 hours.

<table>
<thead>
<tr>
<th>Concentration of purified adenosine deaminase-2 (ADA-2) µg/ml</th>
<th>MCF-7</th>
<th>WRL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>400</td>
<td>65.51</td>
<td>2.39</td>
</tr>
<tr>
<td>200</td>
<td>70.99</td>
<td>0.94</td>
</tr>
<tr>
<td>100</td>
<td>85.80</td>
<td>3.86</td>
</tr>
<tr>
<td>50</td>
<td>91.47</td>
<td>3.65</td>
</tr>
<tr>
<td>25</td>
<td>95.06</td>
<td>1.12</td>
</tr>
</tbody>
</table>
4. Discussion

According to the findings, the levels of t-ADA and ADA-2 activity in the serum of patients with rheumatoid arthritis were significantly higher when compared to healthy adults, according to the findings. This result is also consistent with [8, 36]. In contrast, no significant difference in ADA-1 activity was found between rheumatoid arthritis patients and healthy subjects. This finding is consistent with previous research [3, 21]. Several studies have found that adenosine deaminase in the serum of R.A. patients is related to the severity of the disease [2, 8, 22, 36]. Sari et al., 2003 and Hameed et al., 2019 demonstrated a link between serum ADA activity and its isoenzymes and the severity of R.A. They also discovered that total ADA and ADA2 associated with disease activity in R.A. patients [8, 36]. While Cordero et al., 2001 concluded that there was no relationship between ADA activity level and disease activity in R.A. patients; also, Erer et al., 2009 concluded that there was no relationship between ADA activity level and disease activity R.A. patients [37, 38]. Differences in outcomes maybe because most of these studies were conducted with small sample size and did not depict the patient's appearance and medications in detail [2, 8, 22, 37, 38]. As a result, the elevated level of adenosine deaminase-2, which accounts for 80% of total adenosine deaminase activity, is due to the rise in total adenosine deaminase activity (t-ADA), making measuring adenosine deaminase-2 activity a valid predictor for a rheumatoid arthritis diagnosis. The results show that as the purification stages progress, the particular activity of adenosine deaminase-2 increases. Since ADA-2 is a single band with a molecular weight of about 105 K Da, these findings are conclusive[28, 39, 40]. The isolation and purification of adenosine deaminase-2 give us a clear picture of its properties and distinguishes it from the total adenosine and its analogs adenosine deaminase-1.

The advanced ADA-2 purification steps (from crude to purify enzyme) in SDS-PAGE chromatography for adenosine deiminase-2 of rheumatoid arthritis serum are shown in Fig4. A. The results showed that the purified ADA-2 has a molecular weight of 105 KDa compared to standard proteins Fig. 4 B. These results correspond to previous studies [28, 39].The inhibitory effect of ADA-2 purified from serum of patients with rheumatoid arthritis varied according to its different concentrations, possibly due to the effect of cancer cell receptors with different concentrations of the purified ADA-2 enzyme, which has a role in apoptosis [41]. The results of our study where it was found that the higher the concentration of the purified enzyme, the lower the rate of inhibition of cancer cells, that is, the inverse relationship between the concentration and the rate of inhibition, and this condition is called antagonistic in the effect of dosing (Hormesis) Hormotic effect, and this is a common biological phenomenon in toxicology and pharmacology, which is characterized by an inverse relationship between Low-concentration doses with high-concentration doses. This
phenomenon is used in the effect of low doses of some toxic compounds or pollutants in treating some incurable diseases such as Alzheimer's and cancer, as low doses of some compounds can kill cancer cells without affecting normal cells [42]. This may be due to the release of specific components, such as cytokines, from cancer cells, which may be inhibited by the enzyme adenosine d-aminase-2. Its inhibitory effect increases at low concentrations. Arshad et al. (2014) indicated that the percentage of cell inhibition of the cancer cell line MCF-7 decreased with increasing the concentration of methanolic extract of Prosopis cineraria leaves. In their study of plant extracts, the inhibitory effect was based on lower concentrations more than high concentrations, and this depends on several factors, including the concentration of the substance and the molecular weight, as the higher the concentration of the substance is low, the easier that penetration into the outer membrane in the cell wall, but not to reduce the dilution that makes it lose its toxic potency[43].

Conclusions

The rise in total adenosine deaminase activity (t-ADA) is attributed to the high level of adenosine deaminase-2, which represents 80% of total adenosine deaminase activity, calculating activity level of adenosine deaminase-2 a reliable indicator for a rheumatoid arthritis diagnosis. The purified enzyme also showed its toxic effect on the MCF-7 cancer cell line. The toxicity and inhibitory effect of breast cancer cell line growth increase with low concentrations of purified ADA-2. Purifying ADA-2 and showing its inhibitory effect on MCF-7 at low concentrations helps in its use as an anti-breast cancer marker and studying the impact of anti-drugs on developing breast cancer.

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7. References:


دراسة فعالية مصل الأدينوسين دي أمينيز - 2 (ADA-2) في مرضى التهاب المفاصل الرتئوي

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قسم الكيمياء، كلية العلوم، جامعة الموصل، العراق

المستخلص

الوقاية من أمراض المفاصل مطلوب الكشف المبكر عن التهاب المفاصل الروماتويدي. يلعب ADA-2 دورًا حيويًا في هده دراسة إلى قياس مستوى نشاط مصل ADA-2 لمرضى التهاب المفاصل الروماتويدي. تنقية -2 من مصل المرضى ودراسة السمية الخلوية ل-2 ADA ونافذة الخلية القياسية (MCF-7) (WRL-68) ومن ثم تقديم مستويات نشاط ADA-1 وADA-2. أيضا، تم عزل ADA-2 من مصل و - WRL-68 لمدة 48 ساعة باستخدام مقايضة رأسي. R.A. زادت كميةADA-2 في نشاط 0.0001 (p≤0.0001) نسبة 59.4٪ وـ 16.3 مره و ADA-2 من مصل R.A. نسبة 105 K Da في نطاق 5.93 و 13 وحدة / مجم بروتين مع تنقية. أظهرت صفوي شديد خاصية ADA-2 عبارة عن نطاق أحادي وله وزن جزيئي قدره 105 K Da. عند مستوى IC50 133.7 ميكروغرام / مل أكثر من خط المنفي ومع نمو خلايا سرطان الثدي (MCF-7) بالنسبة للإنصات للإضطرابات التي تؤثر على سرطان الثدي، يمكن استخدام مستوي ADA-2 لتشخيص R.A. منخفضة في استخدام كعلامة مضادة لسرطان الثدي ودراسة تأثير الأدوية المضادة على الإصابة بسرطان الثدي.