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Isolation and Studying the Activity and Properties of Adenosine Deaminase (ADA) in Patients Tissues of Benign and Malignant Colon Tumors

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ABSTRACT

The present research includes determination of adenosine deaminase (ADA) activity in benign and malignant colon tumor tissues.

Patients were enrolled in the study to the surgery unit in AL-Jamhoory teaching hospital in Nineveh Governorate, samples of (6) patients (3) benign and (3) malignant colon tumors.

The results showed a significant increase in the activity of (ADA) in malignant tissues compared with benign which was (32.4 and 84.55 mU) in benign and malignant tumors respectively. Which represent about (50%) increase.

The study also included an isolation and partial purification of ADA using gel filtration chromatography from benign and malignant colon tumor tissues. the results indicated that the number of purification fold for the ADA was (10 and 12) from benign and malignant colon tumors respectively.

The approximate molecular weight of ADA by gel filtration using sephadex G-100 were (31622 Dalton, 39810 Dalton) for benign and malignant tumors respectively.

The optimum condition of ADA for the peak separated from malignant tumors showed an optimum reaction incubation time at (30) minutes, pH at (6.3), temperature at (37°C) and the substrate concentration was about (0.021mM). when we used linweaver - Burk plot, the maximum velocity (V max) and Michaelis - menten constant(Km) where found to be (31.25 mU/ml) and (10.3×10^{-3}mM) respectively. Also in this investigation we was studied the inhibiters of ADA by Zn^{+2}, Ag^{+1}, Ca^{+2}, Mg^{+2}, $Hgcl_2$, Fe^{+2} benign but not by Fe^{+3}.

Keywords: adenosine deaminase , activity, colon cancer, tissue.

عزل ودراسة فعالية وخواص انزيم أدينوسين دي امينيز في أنسجة مرضى أورام القولون الحميدة والخبيثة

الملخص

تضمن البحث الحالي قياس فعالية انزيم الادينوسين دي أمينيز في أنسجة مرضى أورام القولون الحميدة والخبيثة. جمعت العينات من المرضى المراجعين لوحدة الجراحة في مشفى الجمهوري التعليمي في محافظة نينوى، العدد الكلي للمرضى (6) كان (3) منهم يعانون من ورم القولون الحميـد و(3) مـن ورم القولـون الخبيث. أشارت النتائج الى ارتفاع معنوي للفعالية في الورم الحميد و الخبيث (32.4، 84.55 ملي وحـدة) على التوالي والذي يمثل زيادة معنوية قدرها (50%).

كما تضمنت الدراسة أيضا عزل وتنقية الانزيم باستخدام تقنية الترشيح الهلامي لأنسجة ورم القولـون الحميد والخبيث وأشارت النتائج أن عدد مرات التنقية قد بلغت (10 ، 12) مرة في النسيج الحميد والخبيـث على التوالي.

عند تقدير الوزن الجزيئي للأنزيم بتقنيـة الترشـيح الهلامـي وباسـتخدام الـسفدكس G-100 بلـغ 31622 دالتون و39810 دالتون) للورم الحميد والخبيث على التوالي.

تم تثبيت الظروف المثالية لفعالية الأنزيم في الورم الخبيث وبينت النتائج ان اعلى فعالية كانت عند 30 دقيقة وبدالة حامضية 6.3 ودرجة حرارة 37ºC و بتركيز مادة أساس (0.021 ملي مولر) وعند اسـتعمال رسم لاينوفر - برك لتعيين السرعة القصوى وثابت مكيلس- منتن وجد أنها تساوي (31.25 ملي وحدة/مل) (10.3×10^{-3} ملي مولر) على التوالي. كما تم دراسة بعض المثبطات لفعالية الأنزيم وكانـت Mg^{+2}, Ca^{+2}, Fe^{+2}، $Hgcl_2$، Ag^{+1}, Zn^{+2} لكن لم يلاحظ ذلك باستخدام Fe^{+3}.

الكلمات الدالة: أدينوسين دي أمنيز، الفعالية، سرطان القولون، النسيج.

INTRODUCTION

Colon cancer is one of the most frequent cancers in humans. Considering the knowledge of molecular mechanisms of its development, the attempt is to identify a key intracellular proteins included in rapid growth and to devise more biological approaches for the diagnostic and prognostic evaluation (Milovic and Turchanowa, 2003). The epithelial tissue homeostasis is complex, it includes many opposite processes, such as reiteration, programmed cell death and differentiation. A disturbance of these processes lead to neoplastic transformation and further tumor progression (King, 2004).

Adenosine Deaminase (ADA) (EC.3.5.4.4) is an important enzyme in the degradation of adenine nucleotidase. It is known as a key enzyme in purine metabolisim and DNA turnover and thus, in the cancer process (Gwang *et al*., 2009). It is one of five enzyme which

participate in purin salvage pathway (nucleoside phosphorylase, alkalin phosphate, nucleotidase, hypoxanthin-guanine, phosphoribosyl transferase) which lead to an increase in nucleotides synthesis required to the growth of normal and carcinogenic cells and to greater rate of nucleic acids synthesis which could be reflection of the degree of maturity of these cells (Spychala, 2000). It is also a cytosolic enzyme, which has been the object of considerable interest, mainly in human. (Cristalli *et al.*, 2001).

A congenital defect in the enzyme causes severe combined immunodeficiency disease (SCID). ADA is an aminohydrase which participates in the purine metabolism where it degrades either adenosine or 2'-deoxyadenosine producing inosine or 2'-deoxyinosine, respectively (Kocic *et al.*, 2003).

The physiological function of ADA is critical in controlling the effects of these metabolites on immunological, neurological and vascular systems. ADA is also involved in the development of T lymphocytes band as is evident from the fact that ADA deficient animals suffer from B and T lymphopaenia (Ray and Sharma, 2002 ; Ashok *et al.*, 2008).

Serum ADA levels increase in pancreatic disorders, especially in pancreatic cnacer, it may be a serum marker for the diagnosis pancreatic cancer (Bi *et al.*, 2007). ADA is involved in some diseases such as: Behcet's disease (BD) (Calis *et al.*, 2005), tuberclosis (Kaisemann *et al.*, 2004) and Colon cancer (Milovic and Turchanowa, 2003).

The evidence of high ADA activity during rapid and stimulated growth of normal tissues is of importance in making a fully functional purine salvage pathway possible (Seiler, 2004).

The aim of present study is to isolate and study the activity and property of ADA in patients with colonic tumors and to evaluate its usefulness as possible marker of colonic tumors progression and find the relationship between ADA and colon tumors.

MATERIALS AND METHODS

1- Tissues

Tissue samples were collected immediately after operation, Patients were enrolled in the study to the surgery unit in AL-Jumhoory teaching hospital in Nineveh Governorate (6) patients (3) benign and (3) malignant colon tumors. The tissues were cut off, rinsed with normal saline, and kept frozen at (-20º C) until analyzed.

2- Tissue Homogenate Preparation

From each tissue, a weight of (1.0g) was taken and cut into small pieces and homogenized by hand homogenized using phosphate buffer (50 mM; pH 6.5) solution in a ratio of (1:10 w/v) for adenosine deaminase assay. The homogenate was sonicated, then filtered through double layer of sterile gauze and centrifuged at (10000 Xg) for (30) minutes in a cooling centrifuge. The supernatant was separated for protein and ADA activity measurements (Price and Stevens, 1989).

3- Total Protein Determination

Modified lowery method was used for protein determination in tissues homogenates was (Schacterle and Pollack, 1973). Bovine Serum Albumin (BSA) was used as a standard protein.

4- Determination of Adenosine Deaminase (ADA) Activity in Tissues

The ADA activity was determined according to Guisti method (Guisti, 1974). ADA hydrolyze adenosine to inosine and ammonia. Ammonia is determined by Berthelot reaction, where it forms an intensely blue indophenol with sodium hypochlorite and in alkaline solution. Sodium nitroprusside is the catalyst, ammonia concentration is directly proportional to the extinction of the indophenol. The reaction catalyzed by ADA is stopped at the end of the incubation period by the addition of phenol-nitroprusside solution. The activity was measured using spectrophotometer, ADA unit defined as is the amount of enzyme which forms one micromole of ammonia in one minute.

5- Purification of ADA

a- Ammonium Sulfate Precipitation:

Protein was precipitated using solid ammonium sulfate (Robyte and White, 1987). The tissue homogenate was brought to (75%) saturation with ammonium sulfate.

The supernet was separated by cooling centrifugation at (10000Xg) for (30) minutes. Protein concentration was estimated and ADA activity was measured.

b- Dialysis:

The supernat (10) ml from benign tissues and (6) ml from malignant tissues were dialyzed overnight at (4)C using sodium bicarbonate. The final volume measured and the protein concentration was estimated also ADA activity was determined (Robyte and White, 1987).

c- Gel Filtration Chromatography:

The technique was used for purifying ADA enzyme, and to determine its approximately molecular weight (Locasio *et al*., 1969). 2.0 ml of the sample was applied to a column (2 × 87 cm) containing sephadex (G-100) to (85 cm) height. Elution was carried out at flow rate (70 ml/hour), using phosphate buffer, (pH 6.5) as eluant, the fractions were collected , the protein concentration and ADA activity were estimated.

6- Factors affecting ADA activity

To determine the optimal conditions of ADA enzyme several experiments had been designed including: enzyme concentration, reaction time, temperature, pH, substrate concentration, and the effect of some metal ions on ADA activity.

RESULT AND DISCUSSION

Table (1 and 2) shows the results of the purification steps for the enzyme activity, The specific activity of ADA increased in gel filtration step compared with the crude enzyme. The specific activity of ADA in benign and malignant colon tumor tissues was (7.05 and 14.05 mU/mg protein) respectively which represent about (10 and 12) purification folds.

The activity in malignant tissues was higher (about 3 folds) than benign activity by using Gel filtration and this could be considered as a good marker for malignancy. The results of ADA purification from colon tumors were accordant with several studies that purified ADA from breast cancer and brain cancer (Al Attar, 2005; Alabachi, 2006).

The increment of ADA might be a physiologic attempt of the cancer cells to provide more substrates needed by cancer cells to accelerate the salvage pathway activity (Canbolate *et al.*, 1996). Also the purine metabolism and salvage pathway activity of purine nucleotides are accelerated in the cereous human colorectal tissue (Eroglu *et al.*, 2000).

Table 1: Partial purification of ADA from benign colon tumor tissues.

Steps	**Volume (ml)**	**Total protein (mg)**	**Total activity (mU)***	**Specific activity (mU/ mg)**	**Purification fold**	**Recovery %**
Crude homogenate	30	45	32.4	0.72	1	100
High speed supernatant	28	31.36	28.5	0.91	1	88
After precipitation	10	8.1	19.8	2.4	3	61
Dialysis	8	4.51	17.85	3.3	5	55
Gel filtration sephadex (G-100)	24.5	1.25	8.81	7.05	10	27

- Enzyme unit: the amount of enzyme which forms one micromole of ammonia in one minute.

Table 2: Partial purification of ADA from malignant colon tumor tissues.

Steps	**Volume (ml)**	**Total protein (mg)**	**Total activity (mU)***	**Specific activity (mU /mg)**	**Purification fold**	**Recovery %**
Crude homogenate	30	69.3	84.55	1.22	1	100
High speed supernatant	27	49.41	72.63	1.47	1	86
After precipitation	6	9.36	41.31	4.41	4	49
Dialysis	5	6.6	36.3	5.5	5	43
Gel filtration sephadex (G-100)	21	1.8	25.3	14.05	12	30

- Enzyme unit: the amount of enzyme which forms one micromole of ammonia in one minute.

The total activity of ADA (8.81mU) with (27%) recovery for benign colonic tumors tissue obtained at elution volume (124)ml, while the total activity of ADA (25.3 mU) with (30%) recovery for malignant colonic tumors tissue obtained at elution volume (130)ml as shown in Fig. (1 and 2) respectively.

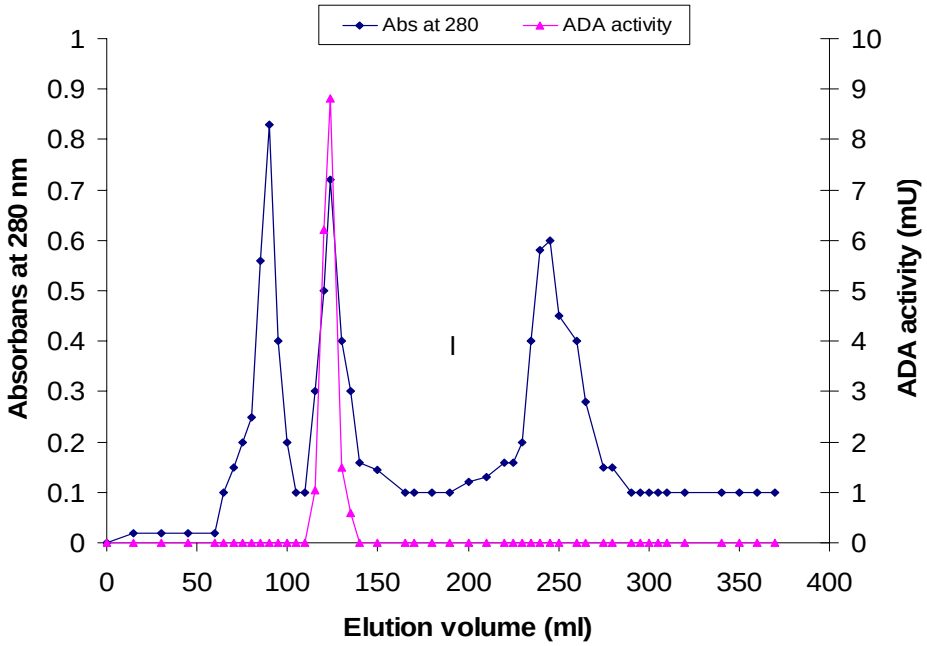

Fig. 1: Elution profile of ADA enzyme from benign colon tumor tissues using Sephadex G-100, column (2×87 cm), flow rate (70 ml/hr).

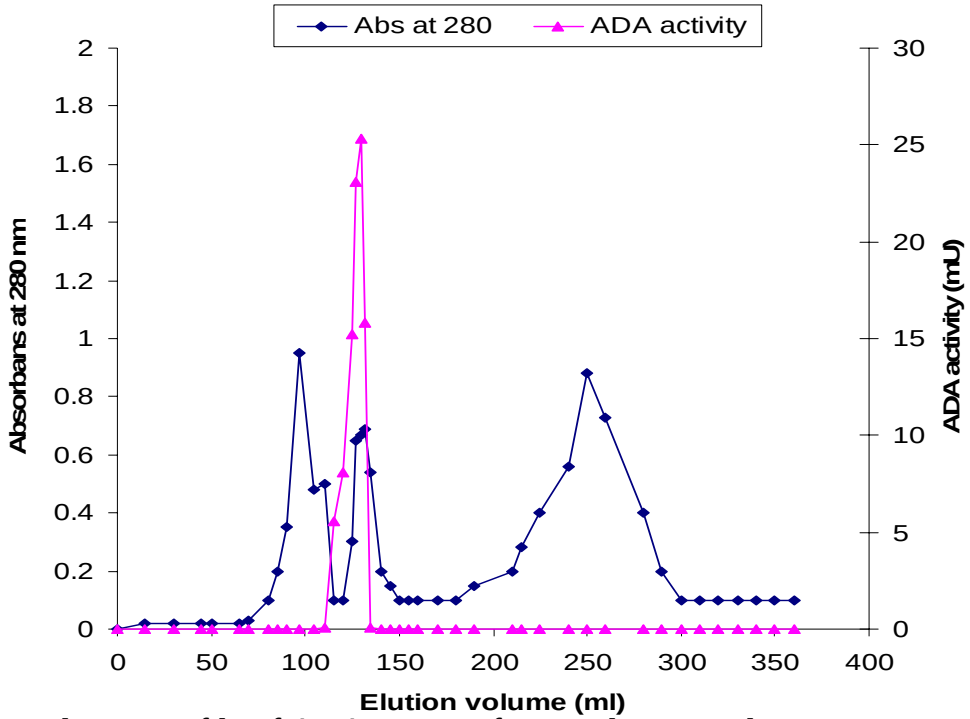

Fig. 2: Elution profile of ADA enzyme from malignant colon tumor tissues using Sephadex G-100, column (2×87 cm), flow rate (70 ml/hr**).**

Molecular Weight Determination

The apparent molecular weight were estimated for ADA from benign malignant colon tumor tissues using sephadex G-100 column (2 × 87) cm calibrated with known molecular weight proteins which were listed in Table (3). The apparent molecular weight of ADA enzyme was determined from the standard curve which was represented by Fig. (3). It has been found that the apparent molecular weight of ADA enzyme for benign and malignant were (31622 Dalton, 39810 Dalton) respectively.

Table 3: Elution volumes of known molecular weight materials on sephadex G-100.

Material	Molecular weight (Dalton)	Elution volume (ml)
Blue dextran	2000000	65.8
BSA	67000	101
Egg albumin	45000	121
Trypsin enzyme	23000	134
Insulin hormone	5734	285
Tryptophan	204	436

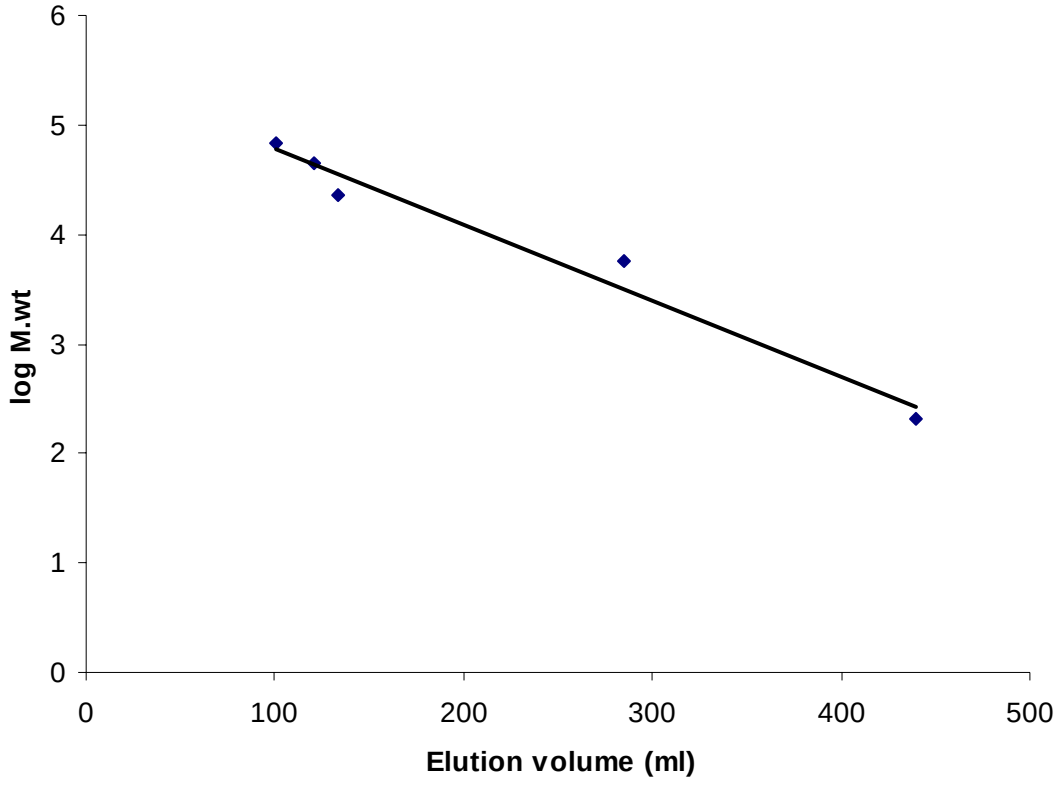

Fig. 3: Standard curve for determination the molecular weight of ADA using sephadex G-100

Other studies showed the range of molecular weight of ADA in serum bronchial asthma patients was (35000-50000 Dalton) (Fajou *et al*., 2001). The molecular weight of ADA has also been determined by gel-filtration chromatography, which estimated to be (25000 ± 3000 Dalton) from human brain tumor tissue (Al- Abachi, 2006).

Factors affecting ADA activity

To determine the optimal conditions of ADA enzyme in malignant colon tumor tissues, the following experiments had been designed including:

1- Effect of enzyme concentration on ADA activity

A sequence of experiments has been done using different quantities of enzyme, higher activity obtained at (0.2 µg/ml) of the enzyme, Fig. 4. The activity is essentially the measurement of the initial velocity under conditions that make it the maximum velocity. The activity should be proportional to the amount of enzyme added. That is, if two or three times as much enzyme is added the activity should be two or three times as great (Robyt and White, 1987).

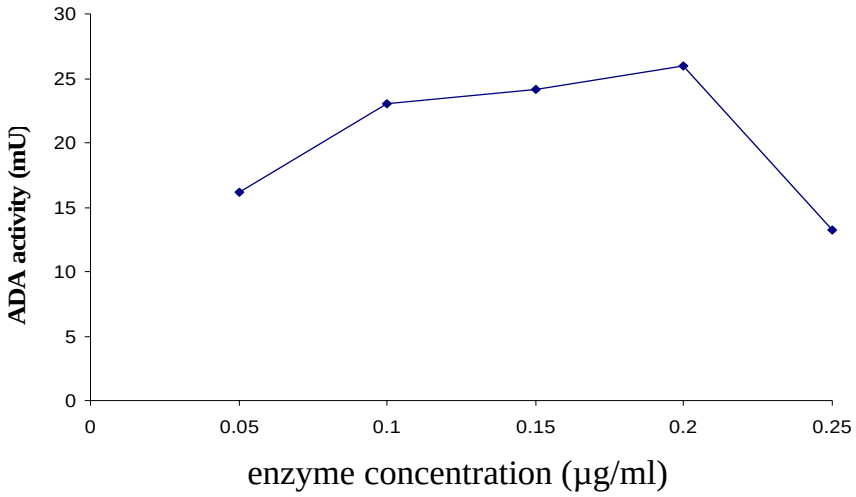

Fig. 4: Effect of enzyme concentration (protein) on ADA activity

2- Effect of reaction time on ADA activity

Maximum ADA activity observed after (30) min of the reaction as shown in figure (5), this results was with agreement with (Kalantari *et al*., 2010). The activity of an enzyme is the amount of reaction that a certain amount of enzyme will produce in a specified period of time. The activity is determined by measuring the amount of product produced or the amount of substrate used up per unit of time under high concentrations or saturating conditions of substrate. (Robyt and White, 1987).

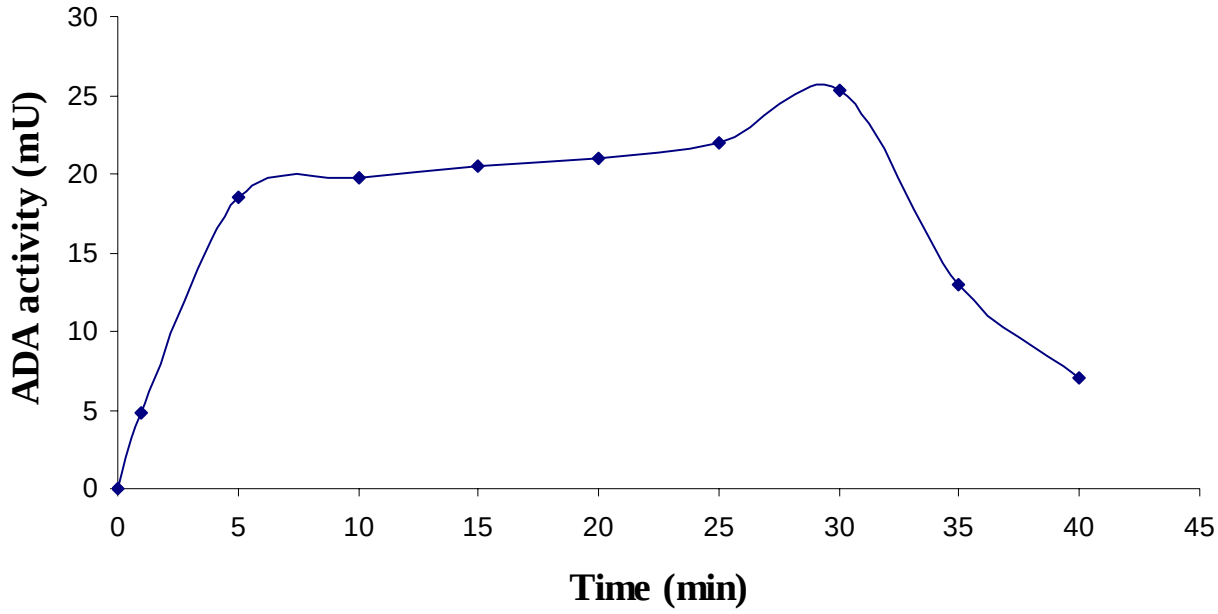

Fig. 5: Effect of reaction time on ADA activity

3- Effect of temperature on ADA activity

It seems that by increasing temperature lead to increase ADA activity, the maximum activity of the enzyme showed at (37 ºC) followed by decreasing in enzyme activity as shown in figure (6) so that (37 ºC) used as optimal temperature to estimate ADA activity in the following experiments. Other studies showed that optimum temperature of ADA in some liver diseases was also (37 ºC) (Raczynska *et al*., 1996).

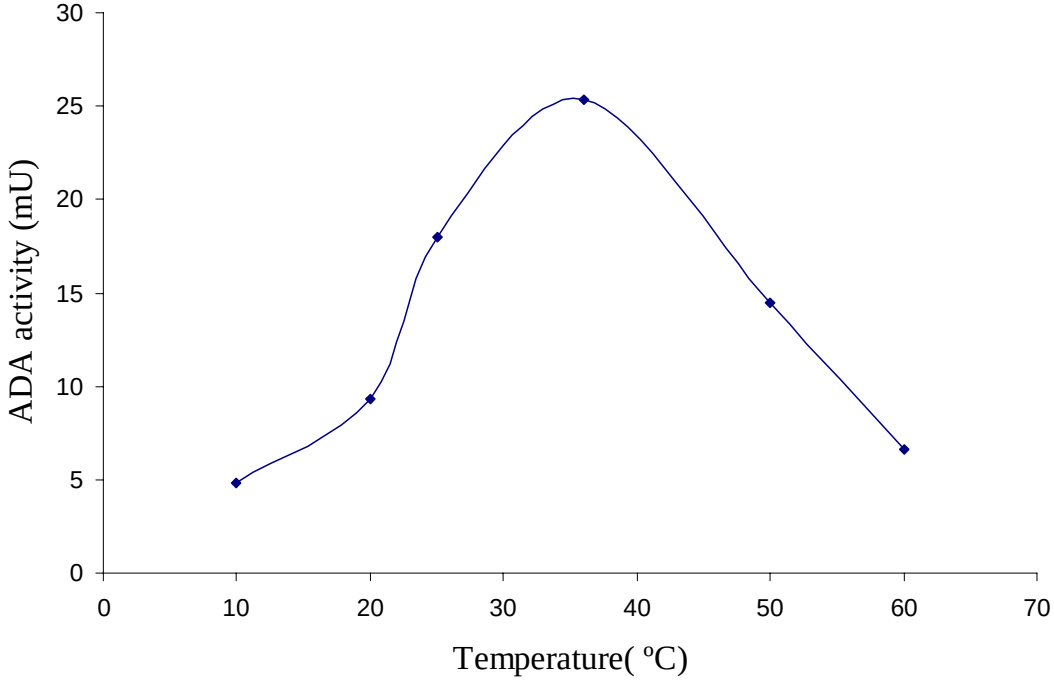

Fig. 6: Effect of temperature on ADA activity

4- Effect of pH on ADA activity

The influence of pH upon the ADA activity was investigated using the sodium phosphate buffer solution with different pH (5.5-7.5) as shown in Fig. (7). We show that the ADA activity was maximum at 6.3, thus in all further experiment incubation were carried out at this point. The velocity of enzyme-catalyzed reactions depends on pH. Enzymes have pH optima and often give bell-shaped curves of velocity versus pH, although other shapes have been observed. The pH optimum for different enzymes varies depending on the nature of the catalytic groups. (Robyt and White, 1987).

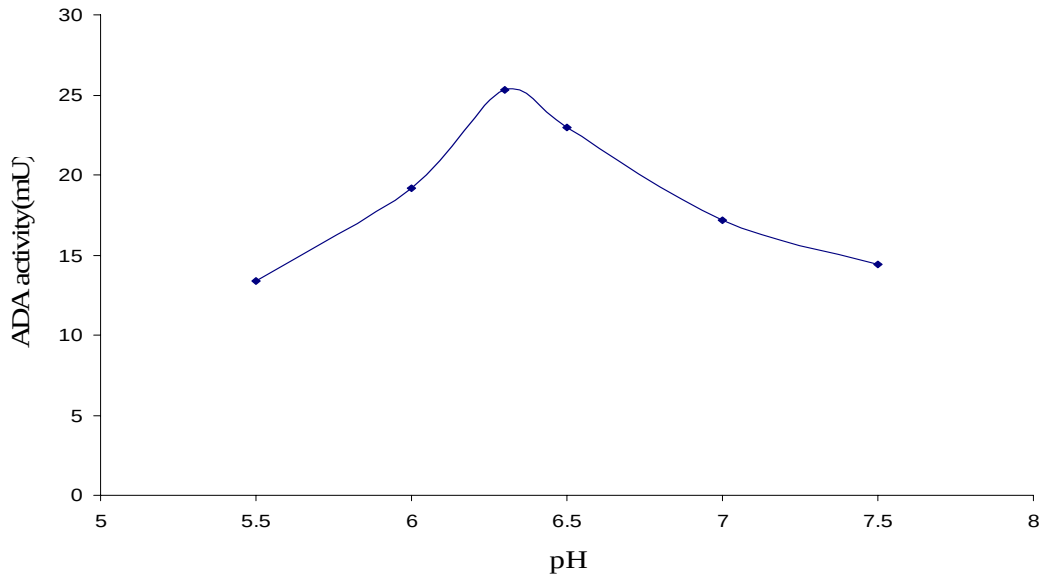

Fig. 7: Effect of pH on ADA activity

5- Effect of substrate concentration on ADA activity

The activity of the enzyme was measured in the presence of different concentrations of adenosine as a substrate. It was found that the maximum activity of the enzyme was obtained by using (0.021 mM) of adenosine Fig. (8).

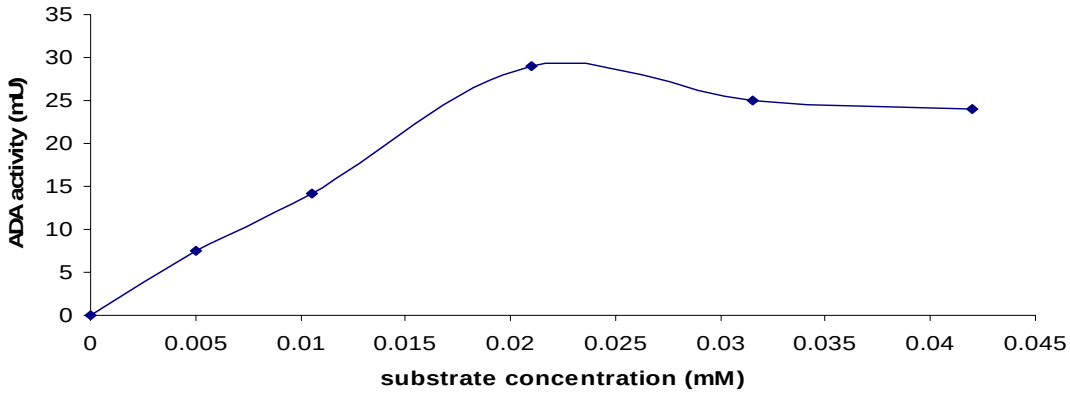

Fig. 8: Effect of substrate concentration on ADA activity

Lineweaver-burk plot as shown in Fig. (9). The maximum velocity (Vmax) and Michaelis Minten constant (Km) were found to be (31.25)mU/ml and (10.3×10^{-3}) mM respectively.

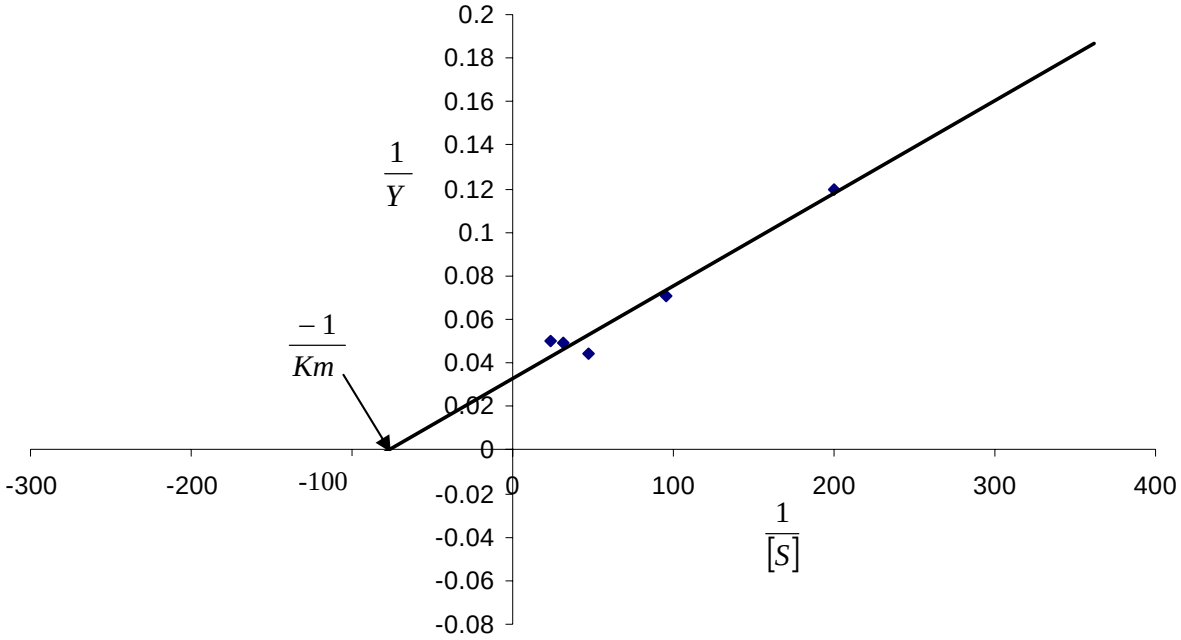

Fig. 9: Linweaver-Burk plot on ADA activity

Other studies found Km value for ADA enzyme separated from Calf thymus equal to (0.07 mM) using adenosine as substrate (Oconnell and Keller, 1994).

From all the above experiments we can conclude the optimum conditions for ADA enzyme isolated and partially purified from malignant colon tumor tissues as shown in table (4).

Table 4: Optimum conditions to evaluate ADA activity partially purified from malignant colon tumor tissue.

Enzyme concentration (μg/ml)	0.2
Reaction time (min)	30
pH	6.3
Temperature (ºC)	37
Substrate concentration (mM)	0.021

6- Effect of some metal ions on ADA activity

It was observed, from table (5), that ADA activity increased by adding (1mM) of ferric ion (Fe^{+3}) to the reaction solution, while the recent ions (Zn^{+2}, Ag^{+1}, Ca^{+2}, Mg^{+2}, $Hgcl_2$, Fe^{+2}) reduced ADA activity. Our results refer that Fe^{+3} may activate the enzyme while the other ions inhibit it. These inhibitors ions will diminish the activity of enzyme that will be in higher levels in patients with colonic tumors. These results are in correspondent with those previously obtained by (Jolly *et al*., 2005), Who submitted that these inhibitor ions can be used as oncological therapy to control tumor activity.

It has been reported that ADA inhibitors will inhibit the. growth of various types of cancer cells by increasing adenosine level. (Barry and Lind, 2000).

Table 5: Effect of some metal ions on ADA activity**.**

Metal ion	ADA activity (mU)	Inhibition %
Non	25.3	100
Zn^{+2}	19.82	85.5
Ag^{+1}	12.9	55.8
Ca^{+2}	11.5	49.7
Mg^{+2}	8.41	36.4
$Hgcl^{2}$	7.22	31.2
Fe^{+2}	6.36	27.5
Fe^{+3}	29.5	1.27

REFERENCES

Al Abachi, S. Z. M. (2006). " Enzymatic and biochemical changes in blood serum and tissues of benign and malignant brain tumors", Ph.D. Thesis, Mosul University, College of Science, Chemistry department, Iraq.

Al Attar, H. Y. (2005). "An enzymatic and harmonic study of the blood and tissues of benign and malignant breast tumors in the city of Mosul and some of the northern region", Ph.D. Thesis, College of Science, University of Mosul, Iraq.

Ashok, K. J.; Pinto, G. J.; Kavitha, A. K.; Palathra, M. J. (2008). The diagnostic and prognostic value of serum adenosine deaminase levels in head and neck cancer. *J. Clinic. Diagnostic Res.*, **3**, 833-837.

Barry, C. P. ; Lind, S. E. (2000). Adenosine-mediated killing of cultured epithelial cancer cells. *Cancer Res*., **60**(7), 1887-1894.

Bi, M.; Koklu, S.; Meric, Y.; Basar, O.; Yilmaz, G.; Yuksel, O.; Yildirim, E.; Ozturk, Z. (2007). Serum adenosine deaminase levels in pancreatic diseases. *Pancreat*, **7**, 5-6.

Calis, M.; Ates, F., Yazici, C.; Kose, K.; Kirnap, M.; Demir, M.; Borlu, M.; Evereklioglu, C. (2005). Adenosine deaminase enzyme levels, their relation with disease activity, and the effect of colchicine on adenosine deaminase levels in patients with Boheet's disease. *Rheumatol Int*., 452-456.

Canbolate, O.; Durak, I.; Cetin, R.; Kavutcu, M.; Ozturk, S. (1996). Activities of adenosine deaminase, 5' –nucleotidase, guanase and cytidine deaminase enzymes in cancerous and non-cancerous human breast tissues. *Breast Cancer Res. and Treatment*, 37(2), 189-193.

Cristalli, G.; Costanzi, S.; Lambertucci, C.; Lupidi, G.; Vittori, S.; Volpini, R.; Camaioni, E. (2001). Adenosine deaminase: functional implications and different classes of inhibitors. *Med. Rev.*, **21**, 105-128.

Eroglu, A.; Canbolat, O.; Demirici, S.; Kocaoglu, H.; Eryavuz, Y. ; Akgul, H. (2000). Activities of adenosine deaminase and 5`-nucleotidase in cancerous and noncancerous colorectal tissues. *Med. Oncol*., **17**(4), 319-324.

Fajou, I.; Hassan, H.; Abdullah, B. (2001). Bronchial asthma and serum adenosine deaminase activity in Iraqi patients. *I. Fac. Med., Baghdad*, **43**(2), 289-295.

Guisti, G. (1974). "Adenosine Deaminase", In method of enzymatic Analysis, 2nd edn., Bergameyer, H.U. New York Academic Press.

Gwang, L.; Sang, S. L; Kwang, Y. K.; Dong, W.K.; Sangdun, C.; Hong, K. J. (2009). Isolation and characterization of a novel adenosine deaminase inhibitor, IADA-7, from Bacillus sp. J-89. *J. Enzyme Inhibition & Med. Chem*., **24**(1), 59-64.

Jolly, P. , Kelley, W., and Orkin, S. (2005). Adenosin Deaminase Inhibitors. *J. Med Chem*., **118**: 428, 431.

Kaisemann, M.; Kritski, A.; Pereira, M.; Trajman, A. (2004). Pleural fluid adenosine deaminase detection for the diagnosis of pleural tubercnlosis. *J. Bras. Pneumol*, **30**, 549-556.

Kalantari, S.; Nejad, S. Z. M.; Tavirani, M. R.; Moghaddamnia, S. H.; Shabani, A.; Mostafavi, M. (2010). A study on the activity and thermal stability of adenosine deaminase in the presence of spermine. *J. paramedical Sci*., **1**(1) ISSN 2008-496X. also found at www.SID.ir.

King, M. W. (2004). The medical biochemistry page. Cited in: (http://www.Indstate.edu/theme/mwking/home.html).

Kocic, G.; Stanojevic, G.; Nagorni, A.; Brankvic, B., Pavlovic, D.; Jevtovic, T. (2003). Diagnostic importance of adenosine deaminase activity for progression and invasion of human colon tumors. *Medicine and Biology*, **10**(2), 76-78.

Locasio, G.; Tigier, H.; Del, C.; Battle, A. (1969). Estimation of molecular weights of proteins by agarose gel filtration . *J. Chromato*., **40**, 453-457.

Milovic, V. ; Turchanowa, L. (2003). Polyamines and colon cancer. *Biochem. Soc. Trans*., **31**, 381-383.

Oconnell, M.A. ; Keller, W. (1994). Purification and properties of double-standard RNA-specific adenosine deaminase from calf thymus. *Proc. Noah. Acad. Sci*., **25**(91), 10596-10600.

Price, N. C.; Stevens, L. (1989). " Fundamental of Enzymology", 2nd edn., Oxford University Press, New York.

Raczynska, J.; Jonas, S.; Krawczinski, J. (1996). Diagnostic value of adenosine in some liver diseases. *Clin. Chem. Acta*., **13**, 151-154.

Ray, I. ; Sharma, R., (2002). Dietary regulation of adenosine deaminase activity in stomach, small intestine and spleen of mice. *Indian J. Biochem. Biophysics*, **39**, 419-421.

Robyte, J.F. ; White B.J. (1987). "Biochemical Technique, Theory and Practice", Wads Worth Inc., Belmont, California, U.S.A.

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.

Seiler, N. (2004). Catabolism of polyamines . *Amino acids*, **26,** 217-233.

Spychala, J. (2000). Tumor-promoting functions of adenosine. *Pharmacol. Ther*., **87**(2-3), 161-173.