Journal of Pioneering Medical Sciences
Received October 19, 2023 Accept December 26, 2023 Publish December 31, 2023.
Volume 12, Issue 4, Pages 63-67

DOI https://doi.org/10.61091/jpms202312414

Partial Purification of 5'-Nucleotidase Enzyme in Patients with Renal Failure and Kinetic Study of the Purified Enzyme in Salah al-Din

Asra'a Ismail Yaseen[1,*] and Marwa Mohammed Khalf Ahmed[1]
[1]Department of Chemistry, College of Education for Women Tikrit University, Iraq.

Corresponding author: Asra'a Ismail Yaseen (e-mail: altaiiasr@tu.edu.iq).

©2023 the Author(s). This is an open access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0

Abstract This study involved separating and partially purifying the 5'-Nucleotidase enzyme isoforms from the sera of patients with renal failure. The purification process consisted of several steps, beginning with precipitation using 30% ammonium sulfate, resulting in a purification fold of 1.2625 and an enzyme yield of 65.63%. This was followed by membrane filtration using a buffer solution, achieving a purification fold of 1.651 and an enzyme yield of 49%. The protein fraction separated from the dialysis bag was then passed through a gel filtration column, reaching a purification fold of 51.88 and an enzyme yield of 43.1%. The enzyme isoforms from the separated protein fraction were further separated using gel filtration, employing ion-exchange chromatography techniques. This resulted in three isoforms of the 5'-Nucleotidase enzyme with varying degrees of purity, the highest for isoform. at 2.297-fold. The study also included kinetic studies of the enzyme purified by gel filtration, which involved determining the Km value under the influence of varying concentrations of the enzyme's substrate, 5'-AMP, for the purified enzyme from the sera of patients with renal failure, which was found to be 12.4. The effects of temperature, pH, and reaction time on enzyme activity were also studied.

Key Words 5'-Nucleotidase Enzyme, Renal Failure, Purification

1. Introduction

Renal failure represents the condition where the kidneys become incapable of removing metabolic end products from the blood, resulting in a loss of balance and regulation of extracellular body fluids and ions [1]. The prevalence rate of renal failure is estimated to be between 8-16%, making it one of the most widespread diseases globally [2]. Historically, it was referred to as Uremic poisoning, primarily due to significant symptoms accompanying renal failure: elevated levels of urea and creatinine in the blood [3]. It signifies a substantial decline in kidney function that is irreversible, leading patients to rely entirely on alternative renal treatments. These treatments include either dialysis or kidney transplantation to avert the life-threatening Uremic Syndrome [4]. A significant indicator of deteriorating kidney function is the decline in the Glomerular Filtration Rate (GFR) [5]. Major causes leading to renal failure include pathogenic infections, genetic factors, and complications arising from surgeries that inhibit normal blood flow to the kidneys for extended periods [6]. Renal failure is classified into two main categories; 1) Acute Renal Failure (ARF) and 2) Chronic Renal Failure [7].

The enzyme 5'-NT belongs to the hydrolase class (EC 3.1.3.5). It is pivotal in controlling energy balance, regulating metabolic processes, and cell replication and division [8]. The 5'-Nucleotidase enzyme is a degradation enzyme resulting from nucleotide metabolism. It functions by removing phosphate from Deoxyribose monophosphate, and Ribonucleoside monophosphate, i.e., dephosphorylating the inorganic phosphate (Pi) esterified to the carbon atom number [5] of ribose or deoxyribose nucleoside molecules [9]. The 5'-NT enzyme is crucial in regulating nucleotide accumulations inside cells to maintain DNA composition and RNA synthesis [10]. This glycoprotein enzyme is integral to the plasma membrane in a broad and diverse group of mammalian cells [11]. It catalyzes the hydrolysis of 5'-Adenosine monophosphate (5'-AMP) into adenosine and phosphate. Adenosine participates in numerous biological processes, such as vasodilation or vasoconstriction [12], acts as a neuromuscular factor, and is also involved in immunosuppression and cell proliferation processes [13].

Additionally, the 5'-NT enzyme plays a role in the cell's Microfilaments system [14]. The 5'-NT enzyme is

widely distributed in vertebrate tissues and mammalian cells' plasma membrane, playing a fundamental role in nucleotide metabolism [15]. Several comprehensive studies on 5'-NT enzyme have been conducted on laboratory animals like mice, proving its presence in various body parts such as the pituitary gland, thyroid and parathyroid glands, mammary glands, kidneys, liver, lungs, brain, and the nervous system [16]. There are seven isoforms of the 5'-NT enzyme. While they operate similarly on the substrate, they differ in their polypeptide chains and amino acid content. The activity of these enzymes entirely depends on the magnesium ion (Mg^{+2}) and is inhibited by inorganic phosphate (Pi). These enzymes possess the same enzymatic activity and substrate specificity but differ in chemical, physical, and immunological properties [17].

2. Materials and Methods

A. Partial Purification of 5'-Nucleotidase from Blood Serum

The enzyme 5'-nucleotidase was purified from blood serum using the following steps:

1) Ammonium Sulfate Addition

Blood serum proteins were precipitated using graded concentrations of 1.5gm of ammonium sulfate (30%) to 5ml of blood serum over 45-60 minutes, with the serum container placed in ice and stirred continuously. The precipitate was separated using a centrifuge at 3000 rpm for 15 minutes. The precipitate was dissolved in 2.5ml of Tris HCl buffer solution (PH7.2, 50mM).

2) Dialysis (Membrane Separation)

Dialysis is one of the most essential and oldest methods used in enzyme purification. Its primary purpose is to remove the remaining ammonium sulfate used for protein precipitation. The dissolved protein from the previous step was placed in a dialysis bag, and its 5'-NT enzyme activity was measured. The bag was immersed in the Tris HCl buffer solution (50mM, PH 7.2), with the solution changed periodically overnight. The dialysis process was performed at 4°C to maintain the 5'-NT enzyme activity. After completing the dialysis, the enzyme activity was measured using the Fisk&Subbarow method.

3) Gel Filtration Chromatography

The concentrated enzyme obtained from the dialysis bag was gradually added to the surface of a sepharose 6B column in a glass column with a diameter of 1.5cm and a length of 20cm. The column was washed with ample amounts of Tris HCl buffer solution (50mM, PH 7.2) until a flow rate of 1ml/min was achieved. The eluted fractions were collected after the completion of the column run, and the 5'-NT enzyme activity and protein concentration were measured for each fraction.

4) Ion Exchange Chromatography

The concentrated enzyme from the previous steps was added to a DEAE Cellulose A- column in a glass column with a diameter of 2cm and a length of 23cm. The resin column was washed with 40ml of Tris HCl buffer solution until a flow rate of 2ml/min was achieved. The separation process began using the buffer solution (50mM, PH 7.2) containing increasing concentrations of NaCl. The eluted fractions from the column were collected in 5ml volumes. After collecting all fractions, the 5'-NT enzyme activity and protein concentration were measured.

B. Kinetic Studies of 5'-Nucleotidase

The kinetic properties of the 5'-nucleotidase enzyme were studied after separating and purifying it from blood serum using gel filtration chromatography. This included:

1) Effect of Substrate Concentration (5-Adenosine Monophosphate (5'-AMP))

The effect of different concentrations of the substrate 5'-AMP on the 5'-NT enzyme activity was studied. Concentrations of (10,20,30,40,50,60)mM were prepared to determine the optimal substrate concentration for the enzyme. The reaction rate was measured using the Fisk&Subbarow method. The relationship between reaction rate and substrate concentration was plotted to determine the maximum reaction velocity, Vmax.

2) Determination of Michaelis-Menten Constant (Km)

To determine the Km value for the substrate 5'-AMP specific to the 5'-NT enzyme, the method mentioned above was used. The Km values were determined using the Lineweaver–Burk plot (1/V vs. 1/[S]).

3) Effect of pH

The optimal pH for the 5'-NT enzyme activity was determined by altering the buffer's pH. The pH values studied were (6.4,6.8,7.2,7.6,8). The reaction mixture contained a 40mM concentration of the substrate 5'-AMP, maintained at 37°C, and the enzyme activity was measured. The relationship between enzyme activity and pH was plotted to determine the optimal pH.

4) Effect of Temperature:

The Fisk&Subbarow method was used to measure the 5'-NT enzyme activity while changing the reaction temperature. The temperatures studied were (17,27,37,47,57)°C. The reaction mixture contained a 40mM concentration of the substrate 5'-AMP.

5) Effect of Reaction Time on 5'-NT Enzyme Activity

The effect of reaction time on 5'-NT enzyme activity was studied using a 40mM concentration of the substrate 5'-AMP at time intervals of (10,20,30,40,50,60,70) minutes, maintained at 37°C in the presence of the buffer solution (50mM, PH 7.2). The relationship between enzyme activity and time was plotted to understand the time effect on enzyme reaction rate.

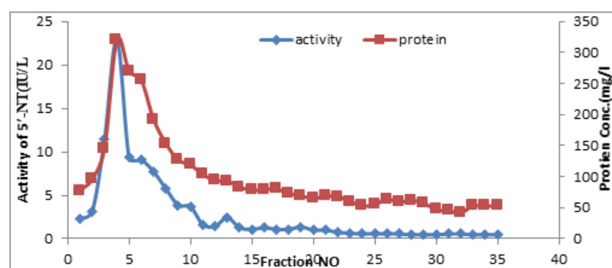

Figure 1: Partial purification of the enzyme 5’-NT from the serum of patients with kidney failure using gel filtration with Sepharose 6B

3. Results and Discussion

The partial purification of the enzyme 5’-NT and the separation of its isoenzymes from patients with kidney failure have been achieved, with the results summarized in Table 1.

Table 1 shows the various steps undertaken in the purification process and the corresponding results. Each step displays the volume eluted, the enzyme activity in IU/L, the total enzyme activity, the protein concentration in mg/L, the total protein concentration in mg, the specific activity in IU/mg, the fold increase in purification, and the yield percentage. The table shows that the purification process effectively concentrated the enzyme while reducing the protein concentration, leading to a higher specific activity as purification proceeded. The final three rows represent the specific activities of three isoenzymes of 5’-NT separated in the process. The purification process started with the crude serum showing a specific activity of 0.0381 IU/mg. As the purification steps progressed, the specific activity increased, indicating the effectiveness of the purification process. When the isoenzymes were separated, their specific activities ranged from 0.0749 to 0.0875 IU/mg, significantly higher than the starting crude serum. In terms of yield, there is a gradual decrease from 100% in the crude serum to 33.63% in isoenzyme III. This is expected as some enzyme activity is lost during the purification process. Overall, the presented results demonstrate the successful purification of 5’-NT from the blood serum of kidney failure patients and the separation of its isoenzymes. Further studies can delve deeper into the roles and significance of each isoenzyme in kidney failure conditions.

After obtaining the partially purified enzyme through gel filtration, it was passed through the DEAE-Cellulose A-50 resin column to separate the 5’-NT isoenzymes using ion exchange chromatography (Figure 1). Three isoenzymes (I, II, III) were obtained after adding 150 of the buffer. The elution process began with ascending concentrations ranging from 0.05M to 0.4M of the saline solution NaCL, achieving closely related purity levels. The purities for the isoenzymes (I, II, III) reached 1.966-fold, 2.144-fold, and 2.297-fold, respectively, with enzymatic yields of 36.5%, 34.63%, and 33.63% for each isoenzyme consecutively, as illustrated in Figure 2.

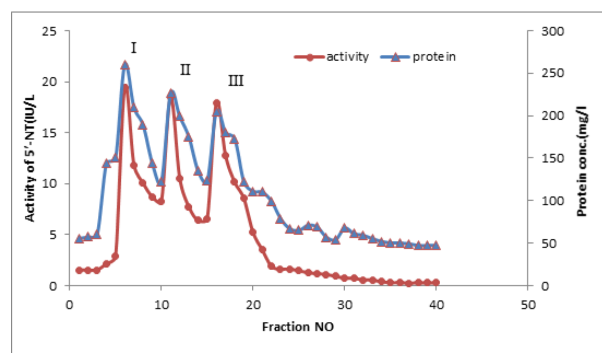

Figure 2: Separation of 5’-NT enzyme isoenzymes from the serum of patients with kidney failure using ion exchange chromatography with DEAE-Cellulose A-50

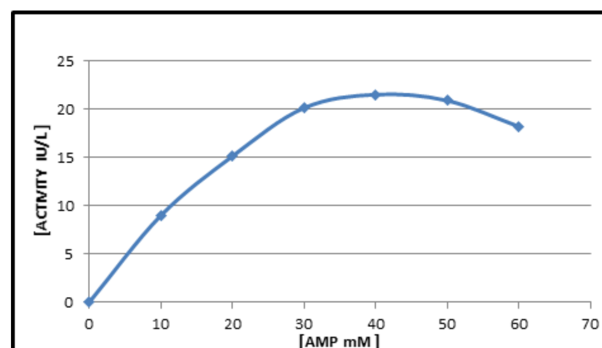

Figure 3: Relationship between the activity of the 5’-NT enzyme and different concentrations of the substrate

These results align with findings by Al-Salhi et al. [18] and Al-Taii [19] which reported the presence of three isoenzymes of 5’-NT in patients with anemia and diabetes, respectively. Kinetic Properties of the 5’-Nucleotidase Enzyme Partially Purified by Gel Filtration from the Serum of Kidney Failure Patients:

A. Optimal Concentration of the Substrate 5’-AMP and Determination of Km

The effect of the substrate concentration 5’-AMP on the enzyme reaction rate of the partially purified enzyme, separated from the Sepharose 6B gel filtration column, was studied to determine the optimal substrate concentration of 5’-AMP. The graph for the enzyme shows an increase in the 5’-NT enzyme reaction rate with increasing concentrations of 5’-AMP until reaching the maximum speed at a concentration of 40mM. Beyond this concentration, the reaction rate decreases at higher concentrations (concentrations higher than the optimal substrate concentration). As illustrated in the Figure 3, the enzyme purified from the gel filtration step conforms to the Michaelis-Menten equation, where the resulting plot is hyperbolic. This observation is consistent with the findings by Al-Tai [20] and Brouwer et al. [21].

The Lineweaver-Burk linear method was employed to calculate the Km value of the 5’-NT enzyme purified by gel fil-

STEP	Elute (ml)	Activity (IU/L)	Total Activity (IU)	Protein Conc. (mg/L)	Total Protein Conc. (mg)	Specific activity (IU/mg)	Degree of purification (Fold)	Yield %
Crud serum	5	32	0.16	840	4.2	0.0381	1	100
Ammonium sulphate	3.5	30.12	0.105	623	2.181	0.0481	1.2625	65.63
Dialysis	3	26.09	0.0783	415	1.245	0.0629	1.651	49
Gell filtration	3	22.95	0.0689	320	0.969	0.0718	1.885	43.1
lightgray(Ion exchange) DEAE-Cellulose A50								
Isoenzyme I	3	19.46	0.0584	260	0.78	0.0749	1.966	36.5
Isoenzyme II	3	18.47	0.0554	226	0.678	0.0817	2.1444	34.63
Isoenzyme III	3	17.94	0.0538	205	0.615	0.0875	2.297	33.63

Table 1: Summary of Partial Purification and Isoenzyme Separation of 5'-NT in Patients with Kidney Failure

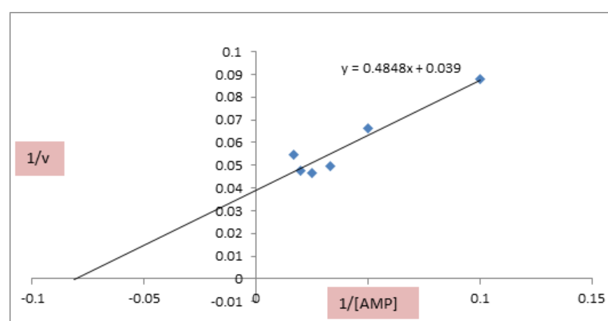

Figure 4: Lineweaver-Burk plot for calculating the Km of 5'-NT enzyme purified from patients with kidney failure

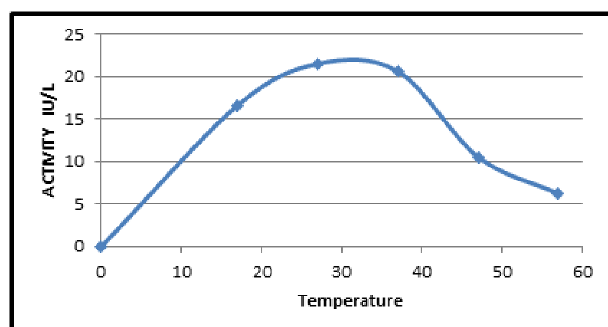

Figure 5: Effect of temperature on the reaction rate of the partially purified 5'-NT enzyme

tration. The Km value was determined using the Lineweaver-Burk method, as illustrated in Figure 4. The Km value was 12.4 millimolar for the enzyme purified using the gel filtration technique. Meanwhile, in a study by Khorsheed et al. [22] on the 5'-NT enzyme in anemia patients, they found a different value for the Km.

B. Effect of Temperature on the Activity of the 5'-NT Enzyme

The results showed that the reaction rate of the purified 5'-NT enzyme increases with rising temperature, reaching its peak at 37°C. Figure 5 illustrates the effect of temperature on the enzyme purified using gel filtration. This is consistent with findings presented by Al-Taii in 2006 [20].

C. Effect of pH on the Reaction Rate of the 5'-NT Enzyme

Upon plotting the relationship between varying pH levels of the buffer solution (Tris-HCl) and the enzymatic reaction rate

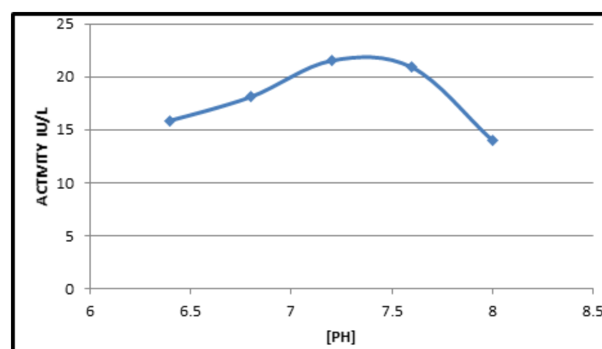

Figure 6: Effect of pH on the reaction rate of the partially purified 5'-NT enzyme

of purified 5'-NT, it was observed that the reaction rate of the purified enzyme increased with the rise in pH, reaching its maximum speed at the optimal pH of 7.2. After this point, the enzyme's activity declined as the pH continued to rise, as illustrated in Figure 6. These results align with findings reported by Al-Taii in 2006 [20].

D. Impact of Reaction Time on the Efficacy of the 5'-NT Enzyme

The influence of reaction time on the efficacy of the purified enzyme was examined by incubating the enzyme with its substrate at a temperature of 37℃ for varying time intervals (10, 20, 30, 40, 50, 60, 70 minutes) at pH 7.2. The results demonstrated an increase in enzyme activity with prolonged incubation, with the enzyme reaching its peak activity at 37℃ after a 30-minute incubation period. After this 30-minute period, the enzyme's activity began to decline, as depicted in Figure 7, for the enzyme purified using gel filtration. This phenomenon could be attributed to the enzyme's thermosensitive nature, where prolonged exposure to heat may disrupt the bonds between amino acids.

4. Conclusion

This research successfully separated and partially purified the 5'-Nucleotidase (5'-NT) isoenzymes from the sera of patients with renal failure. The study employed a multi-step purification process, including ammonium sulfate precipitation, dialysis , gel filtration, and ion-exchange chromatography. These methods significantly improved the enzyme's purity and specific activity, as evidenced by the increasing

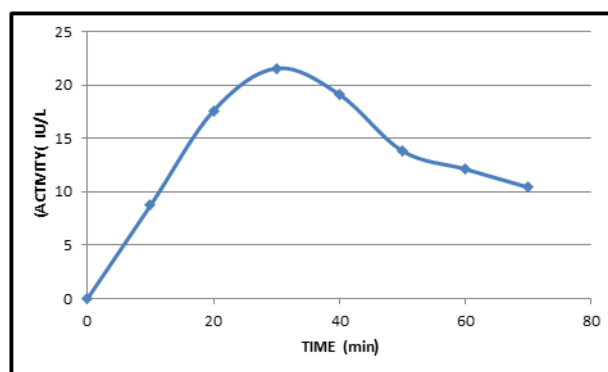

Figure 7: Effect of time on the reaction rate of the partially purified 5'-NT enzyme

purification folds and specific activities across the steps. The separation of three distinct isoenzymes, highlights the complexity and diversity of this enzyme in renal failure conditions. Kinetic studies on the purified enzyme revealed crucial insights into its behavior under various conditions. The determination of the Km value for the enzyme with its substrate (5'-AMP) provided a deeper understanding of its affinity towards the substrate in renal failure patients. Temperature, pH, and reaction time studies further elucidated the optimal conditions for the enzyme's activity, contributing valuable information to the field of biochemical pathology. Overall, the findings underscore the importance of the 5'-NT in renal failure and offer a foundation for future research into the roles and therapeutic potential of its isoforms in kidney-related diseases. The successful purification and detailed kinetic analysis of the enzyme pave the way for more targeted studies, potentially leading to new diagnostic markers or treatment approaches for renal failure.

Conflict of Interest

The authors declare no conflict of interests. All authors read and approved final version of the paper.

Authors Contribution

All authors contributed equally in this paper.

References

[1] Belayneh, K. (2018). Current Management of Chronic Kidney Disease: Literature Review. Urology and Nephrology, 6(2), 555684.

[2] Khalaf, W. S., Hussein, W. N., & Ahmed, A. H. (2019). Tikrit Journal of Pure Science, 24(4), 47-51.

[3] Aguilera, P., Jhun, P., Bright, A., & Herbert, M. (2015). Why does your dandruff smell like urine. Annals of Emergency Medicine, 65(3), 342-344.

[4] Al-Taiee, T. A. K. (2019). Study of the Anti-Diuretic Hormone (ADH) on End Stage Renal Failure Disease (ESRD) Pre-Hemodialysis in Iraqi Patients. Ibn Al-Haitham Journal for Pure and Applied Science, 32(2), 30-37.

[5] Zinman, B., Wanner, C., Lachin, J. M., Fitchett, D., Bluhmki, E., Hantel, S., et al. (2015). Empagliflozin cardiovascular outcomes and mortality in type 2 diabetes. New England Journal of Medicine, 373(22), 2117-2128.

[6] Collins, A. J., Foley, R. N., Herzog, C., Chavers, B. M., Gilbertson, D., Ishani, A., Kasiske, B. L., Liu, J., Mau, L. W., McBean, M., Murray, A., St Peter, W., Guo, H., Li, Q., Li, S., Li, S., Peng, Y., Qiu, Y., Roberts, T., Skeans, M., ... Agodoa, L. (2010). Excerpts from the US Renal Data System 2009 Annual Data Report. American journal of kidney diseases : the official journal of the National Kidney Foundation, 55(1 Suppl 1), S1–A7. https://doi.org/10.1053/j.ajkd.2009.10.009

[7] Mukhtar, N., Hamad AL, N. J., & Kareem, M. F. (2012). Physiological Study of Chronic Renal Failure According to Peripheral Neuropathy. Medical Journal of Babylon, 9(2), 320-331.

[8] Abdullah, K. K., Shakir, S. D., AL-Samarraie, M. Q., & Mustafa, M. A. (2022, November). A comparative study of effect of Cymbopogon citrates aqueous extract and rosuvastatin on experimentally induced hyperlipidemia in local rabbits. In AIP Conference Proceedings (Vol. 2394, No. 1). AIP Publishing.

[9] Zimmermann, H. (1992). 5'-Nucleotidase: molecular structure and functional aspects. Biochemical Journal, 285(2), 345-365.

[10] Zachowski, A., Evans, W. H., & Paraf, A. (1981). Biochimica et Biophysica Acta, 664, 121-126.

[11] Harvey, R. A. (2011). Lippincott Illustrated Reviews: Biochemistry (5th ed., p. 299).

[12] Newby, A. C., Luzio, J. P., & Hales, C. N. (1975). Biochemical Journal, 146, 625-633.

[13] Arch, J. R. S., & Newsholme, E. A. (1978). Essays in Biochemistry, 14, 82-123.

[14] Fischer, D., Van Der Weyden, M. B., Snyderman, R., & Kellcy, W. N. (1976). Journal of Clinical Investigation, 58, 399-407.

[15] Sidorov, V. P. (1992). Factors affecting the frequency of exploratory thoracotomies in lung cancer. Grudnaia Khirurgiia, 285(2), 84-87.

[16] Sunderman, F. W. (1990). The clinical biochemistry of 5'-nucleotidase. Annals of Clinical and Laboratory Science, 20(2), 123-139.

[17] Walldén, K. (2008). Structural Studies of Human 2'-Nucleotidases. ISBN 978-91-7155-718-6. Stockholm.

[18] Al-Salhi, F., Al-Ghali, I. Y., & Khorsheed, S. H. (2009). Partial purification of 5-nucleotidase isoenzymes from the sera of anemic patients. Rafidain Journal of Science, 20(1), 81-92.

[19] Al-Taii, A. (2015). Estimation of 5- nucleotidase activity from saliva of diabetic patients with partial purification of its isoenzymes. Tikrit Journal of Pure Science, 20(2), 1813-1662.

[20] Al-Taii, A. I. Y. (2006). A bio-chemical study of 5'- nucleotidase isoenzymes partially purified from the sera of anemic patients (Master's Thesis). Tikrit University, College of Education for Women. Iraq.

[21] Brouwer, C., Vogels-Mentink, T. M., Keizer-Garritsen, J. J., Trijbels, F. J., Bokkerink, J. P., Hoogerbrugge, P. M., Van Wering, E. R., Veerman, A. J., & De Abreu, R. A. (2005). Role of 5-nucleotidase in thiopurine metabolism: Enzyme kinetic profile and association with thio-GMP levels in patients with acute lymphoblastic leukemia during 6-mercaptopurine treatment. Clinica Chimica Acta, 361(1-2), 95-103.

[22] Khorsheed, H. S., Al-Salihi, F. G., & Yaseen, A. (2009). Partial purification of 5'-nucleotidase isoenzymes from blood sera of anemic patients. Rafidain Journal of Science, 20(2), 81-92.