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ISOLATION AND DETERMINATION OF THE MOLECULAR WEIHT OF AMINOACYLASE-1 FROM THE SERUM OF HEPATOCELLULAR CARCINOMA AND ITS KINETICS STUDIED BY GEL FILTRATON CHROMATOGRAPHY

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Abstract:
Hepatocellular carcinoma (HCC) is one of the most common primary liver cancers in the world. Early diagnosis of HCC is difficult because symptoms often don't appear until the tumor has spread and reached an advanced stage. Aminoacylase-1 (ACY1) is an enzyme belonging to the zinc-containing aminoacylase family. ACY1 is expressed in the liver, brain, kidneys, and some other tissues. ACY1 deficiency causes rare metabolic disorders that increase the excretion of N-acetylated amino acids in the urine. ACY1 is closely associated with HCC due to its active role in regulating metabolism and its influence on the growth and spread of cancer cells. ACY1 and its analogs are used as a biomarker for detecting tumors in cancerous tissues, as a potential therapeutic target, and as an inhibitor that prevents growth and spread in hepatocellular carcinoma.

Aminoacylase-1 enzyme was purified from the serum of hepatocellular carcinoma patients. Ammonium sulfate (65%) was used for precipitation. Dialysis was performed to remove residual salts. The enzyme was isolated by ion exchange and gel filtration methods. A single isoform of the enzyme was obtained with a specific activity (94.36 U/mg) four times that of crude serum. The molecular weight of ACY1 was estimated by gel filtration and electrophoresis and was approximately (45000 Daltons). The kinetics and optimal conditions for the ACY1 enzyme were studied. The optimal substrate concentration was (7.5 mM), the value (Km=2.5 mM), the maximum velocity (Vmax=0.084 U/mL), the value (pH=7.5), the incubation period was (25 minutes), and the temperature was (37C°).

Keywords: Aminoacylase-1; Electrophoresis; Hepatocellular carcinoma; Kinetic study; Ion exchange; Optimum conditions; Purification; Risk factors.

Introduction

Hepatocellular carcinoma (HCC)

Hepatocellular carcinoma (HCC) is one of the most common primary liver cancers worldwide [1]. HCC ranks sixth among cancer types and third in terms of cancer deaths worldwide [2]. HCC is difficult to diagnose in its early stages because there are no symptoms or signs that confirm the infection. HCC is diagnosed in its advanced stages after the tumor has spread or invaded locally because it is a malignant disease [3]. HCC is diagnosed by several techniques and tests, including: magnetic resonance imaging of the liver, ultrasound imaging, computed tomography, alpha-fetoprotein test and the modern protein technology SomaScan [4].

Nine modifiable primary risk factors for hepatocellular carcinoma have been identified: viral infections (hepatitis B, hepatitis C, Clonorchis sinensis), lifestyle factors (smoking, alcohol, and aflatoxin B1), and metabolic factors (nonalcoholic fatty liver disease, type 2 diabetes, and obesity) [1,2].

Treatment techniques for HCC before surgery, including immunotherapy and liquid biopsy therapy, are among the most important strategies used to reduce tumor recurrence. Long-term treatment results for patients reach (70%) of cancer recurrence rates five years after surgery. Studies have confirmed that the cause of recurrence is immune evasion, biological activity, and environmental factors that promote tumor return. Liver transplants for patients are very rare due to the scarcity of donors [5]. Radiation therapy and radiofrequency ablation are used in cases where surgical ablation is difficult [6]. Transarterial chemotherapy is an effective standard treatment for patients with unresectable stage B hepatocellular carcinoma. Biological excision is better and more accurate than surgical excision, because knowing and studying the behavior and microenvironment and the tumor's ability to regenerate is more important and accurate than just the size or number of the tumor. Following transarterial chemotherapy in HCC, rare but serious biliary and arterial complications may occur if not addressed promptly, and in severe cases, these complications can be fatal [3].

Liver failure is one of the most serious complications that threaten the patient's life as a result of removing part of the liver during treatment for hepatocellular carcinoma. The remaining part of the liver is unable to meet the body's vital and metabolic needs, resulting in coagulation disorders, dysfunction of multiple organs, elevated bilirubin levels in the blood, and ascites, leading to increased mortality rates [7].

Aminoacylase-1 (ACY1)

Aminoacylase-1 (ACY1) is a zinc-containing enzyme (EC 3.5.1.14) belonging to the aminoacylase family. ACY1 catalyzes the hydrolysis of N-acetylated proteins, removing acetyl groups and converting them into their corresponding free amino acids and fatty acids, as shown in Figure 1. ACY1 is expressed in the liver, kidneys, brain, and some other tissues [8]. ACY1 is found in the cytoplasm, Dual units and high homogeneity [9]. There are four types of Aminoacylase: (1) acylase-1, also called (N-acylamino acid amidohydrolase), (2) acylase-2

(EC 3.5.1.15), also called (N-acyl-L-aspartate amidohydrolase), (3) acyllysine deacylase (EC 3.1.5.17), also called (N"-acyl-L-lysine amidohydrolase), and (4) acylase-3 [10].

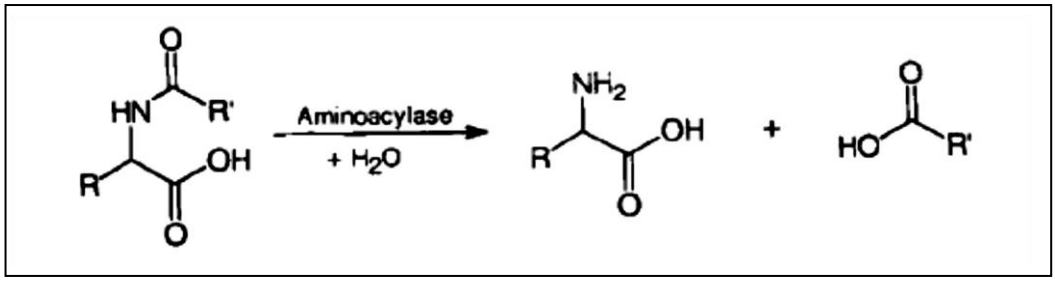

Figure (1) The process of hydrolysis and removal of acetyl from substrates by aminoacyl for typical substrates. R = alkyl, aromatic, or alkyl groups, and R' = methyl or chloroethyl groups [10].

ACY1 deficiency is a rare metabolic disorder resulting from genetic mutations in the ACY1 genes. This disorder leads to increased excretion of N-acetylated amino acids in the urine, such as alanine, glycine, valine, leucine, methionine, and serine [11]. The ACY1 enzyme performs various biological roles: it acts as a tumor suppressor, detoxifier, promotes cell growth, inhibits programmed cell death, and activates various compounds in cancer cells. ACY1 levels are reduced in cancer cells [9].

ACY1 acts on short-chain acylated substrates of aliphatic amino acids, with the exception of the substrate (N-acetyl aspartate), which is hydrolyzed by Aminoacylase-2 into the corresponding free amino acids and acetic acid [8]. The easily hydrolyzable substrates of the ACY1 enzyme with good absorbance are N-(cinnamolyl), N-[3-(2-furyl)acryloyl], and N-(thenyl-acryloyl). Preferred ACY1 substrates for the amino acids N-acetyl-S-aryl- and N-acetyl-S-aralkyl-L-- exist. Several competitive inhibitors of the ACY1 enzyme exist from the N-hydroxy-3-aminoheptanoate class of amino acids, such as N-hydroxy-3-aminoheptanoate and N-hydroxy-3-aminoheptanoate. There are other compounds that act as inhibitors of the ACY1 enzyme, such as (N-(tributyloxycarbonyl)-L-X) compounds, where X represents (valine, leucine, methionine, isoleucine). The compound para-toluenesulfonyl)-L-phenylalanine- is also a competitive inhibitor of the ACY1 enzyme [10].

The relationship between hepatocellular carcinoma and the enzyme Aminoacylase-1

Aminoacylase-1 enzyme is expressed in the liver, where it converts N-acetyl amino acids into free amino acids [12]. The ACY1 enzyme is closely related to HCC due to its active role in regulating metabolism and its effect on the growth and spread of cancer cells. ACY1 gene expression levels are reduced in many HCC cases, leading to impaired metabolic reprogramming, cancer cells rely on it to meet their nutritional needs and grow faster [13]. The reason for the decrease in ACY1 levels is impaired mitochondrial function with a decrease in energy consumption and an increased incidence of diet-induced metabolic disorders [12]. Studies have confirmed that ACY1 acts as an inhibitor of the growth and spread of cancerous

tumors, because it prevents cancer cells from dividing, migrating, and growing abnormally. The ACY1 enzyme is involved in regulating cellular and biological signaling pathways that cancer cells depend on for proliferation and spread [13].

The ACY1 enzyme and its analogs are used as a biomarker for detecting tumors in cancerous tissues. Changes in amino acid metabolism are a distinctive indicator for detecting liver tumors, and by tracking the activity of ACY1 and its receptors, the extent of disease progression and severity can be assessed [14,15]. ACY1 acts as a potential therapeutic target for liver cancer due to its pivotal role in tumor physiology, making it important as a diagnostic marker for tumors, especially when the mechanism of regulation of the ACY1 enzyme in amino acid metabolism is understood. Then ACY1 acts as a targeted therapy in depriving cancer cells of essential nutrients, growth, and spread, and thus inhibiting the tumor [14].

Methods and Materials

Sample Collection

Serum samples were collected from patients with hepatocellular carcinoma (HCC) during their visits to Azadi Teaching Hospital. For both genders (males and females), their ages ranged (40-60 years). It was confirmed through reports, laboratory tests, and doctors' diagnoses that they had hepatocellular carcinoma. It was also confirmed that they did not have any other chronic diseases. Patient consent was obtained before blood was drawn from them as ethical consent as part of the job requirements.

Estimation of Aminoacylase-1 (ACY1) and Total Protein Activity:

ACY1 activity was determined spectroscopically using the Peterson method [16]. This method is based on the hydrolysis of the substrate (L-Nacetyl-L-methionine). Ninhydrin was used in the Rosen method [17] to determine the L-methionine produced by the hydrolysis. Absorbance was measured at a wavelength of 570 nm. Total protein concentrations were estimated using the Lowry method [18].

Steps for Isolating Aminoacylase-1 from the Serum of Patients with Hepatocellular Carcinoma

1- First Step (Precipitation): Ammonium sulfate, a highly soluble neutral salt, was used [19]. Add ammonium sulfate to a saturation level of (65%) to (20 ml) of raw blood serum. The sample was left at a temperature of (4C°) for (24 hours). The components were separated by centrifugation at (5000 rpm). The enzyme activity and total protein concentration of the filtrate and precipitate were measured. The concentrated sample was kept at (-20 C°) for use in subsequent purification steps.

2- **The second step (dialysis):** Dialysis is carried out at (4C°) to get rid of the salt residues from the precipitation step [20]. (16 ml) of the concentrated sample was placed in a semi-permeable bag, and then the bag was immersed in a (750 mL) flask containing a buffer

phosphate solution (0.1M) and (pH=7.1). The solution was moved by a magnetic motor (24 hours), and the solution was replaced every (6 hours). ACY1 activity and total protein are then determined.

3- Third step (ion-exchange chromatography): The positively charged resin diethylaminoethylcellulose (DEAEcellulose) was used in an ion-exchange column with dimensions of (2cm × 45cm) [21]. (12 mL) of the concentrated sample was added to a height of (40 cm) of the column. The flow rate was set at (60 mL/hr). ACY1 activity was measured in all separated fractions at (570 nm) with the total protein concentration at (280 nm).

4- Fourth step (gel filtration chromatography): (10 ml) of the concentrated sample resulting from the ion exchange step was placed into a gel filtration column with dimensions (2 cm × 100 cm) [22]. Sephadex G-100 gel was used and the flow rate was set at (1 ml/min). ACY1 enzyme activity and total protein concentrations were measured.

5- The fifth step (kinetic study and optimal conditions): The purified ACY1 enzyme is kept at (-20 C°) to study the enzyme's kinetics and optimal conditions and how to extract the Michaelis-Menten constant (Km) and maximum velocity (Vmax) values.

Estimation of the molecular weight of Aminoacylase-1

1- By gel filtration chromatography: The gel filtration technique is used with standard proteins of known molecular weight in the range of (204-2000000 Daltons) to find the molecular weight approximating the ACY1 enzyme [23]. The separation column was washed with the buffer solution several times, then the standard proteins were added to it, and the flow rate of the column was set to (5 ml/5 minutes). The activity of ACY1 was then measured at each fraction until it reached its peak, at which point elution volume was used in the straight-line equation. A calibration curve is plotted for all standard proteins with the logarithm of their molecular weights.

2- By electrophoresis: ACY1 is separated by electrophoresis using sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE). Negatively charged proteins migrate towards the positive electrode. Smaller proteins move faster and deeper through the gel pores compared to larger proteins [24].

Results and discussion

Table (1) shows the results of the partial isolation of Aminoacylase-1 enzyme from the serum of hepatocellular carcinoma patients. The precipitation process using neutralized ammonium sulfate was employed at a saturation level of (65%). The proteins were precipitated after adding ammonium sulfate to (10 ml) of crude serum. The specific activity of ACY1 increased to (26.64 U/mg) compared to crude serum, and the yield percentage was (83.45%).

European Science Methodical Journal
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Table (1) Partial purification of Aminoacylase-1 enzyme from serum of hepatocellular carcinoma patients

No.	Purification steps	Volume (ml)	Total protein (mg)	Activity of ACY1×10-3 (U*/mL)	Total activity (U)	Specific activity (U/mg)	Fold	Yield %
1	raw Serum	20	5.43	6.72	134.4	24.75	1	100
2	ammonium sulphate (65%)	16	4.21	7.01	112.2	26.64	1.08	83.45
3	Dialysis	12	3.33	9.7	116.4	34.95	1.41	86.61
4	Ion Exchange DEAE-Cellulose A50	10	2.08	11.1	111	53.37	2.16	82.59
5	Gel Filteration Sephadex G25 (One Peak)	8	1.17	13.8	110.4	94.36	3.54	98.43

In the dialysis step, the remaining salts were removed from the protein precipitation. As a result, ACY1 activity (9.7 U*/mL), specific activity (34.95 U/mg), fold (1.41) and yield percentage (86.61%) all increased, respectively. A distinct peak (One isoenzyme) of ACY1 appeared at the elution volume (137 mL), as shown in Figure (2).

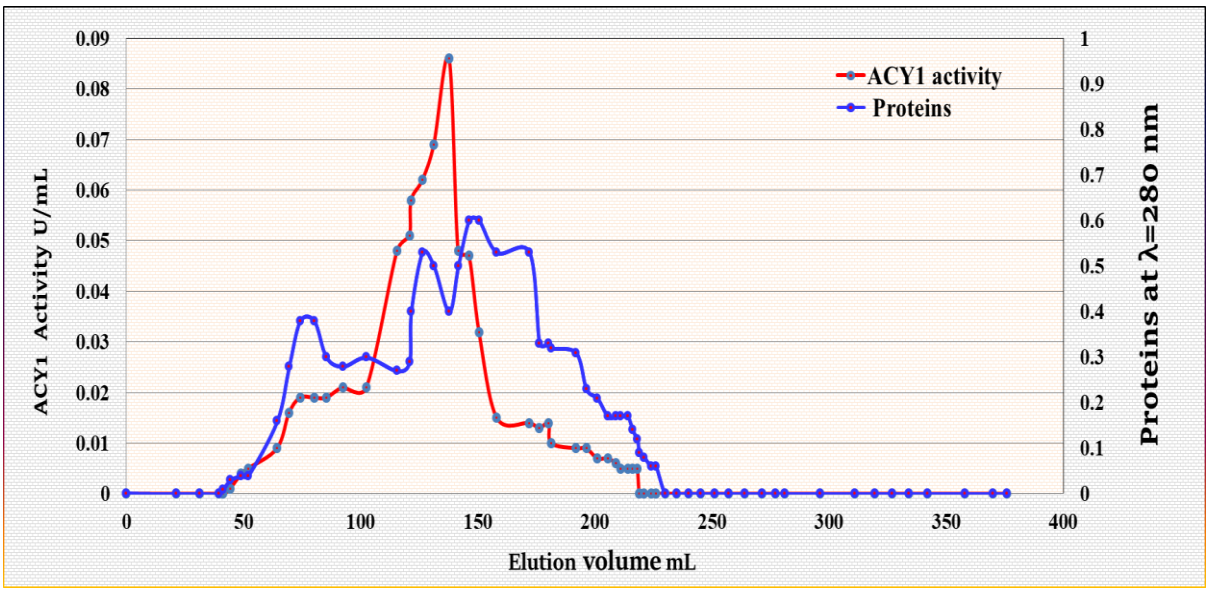

Figure (2) Ion exchange technique for purifying ACY1 enzyme from the serum of hepatocellular carcinoma patients

In the gel filtration step, the specific activity of purified ACY1 increased fourfold to (94.36 U/mg), while the fold increased three-quarters and a half times (3.54) and the yield became (98.43%) compared to the crude serum. In the gel filtration technique, a single distinct bundle was given at the highest peak (One isoenzyme) of ACY1 activity, note Figure (3).

European Science Methodical Journal
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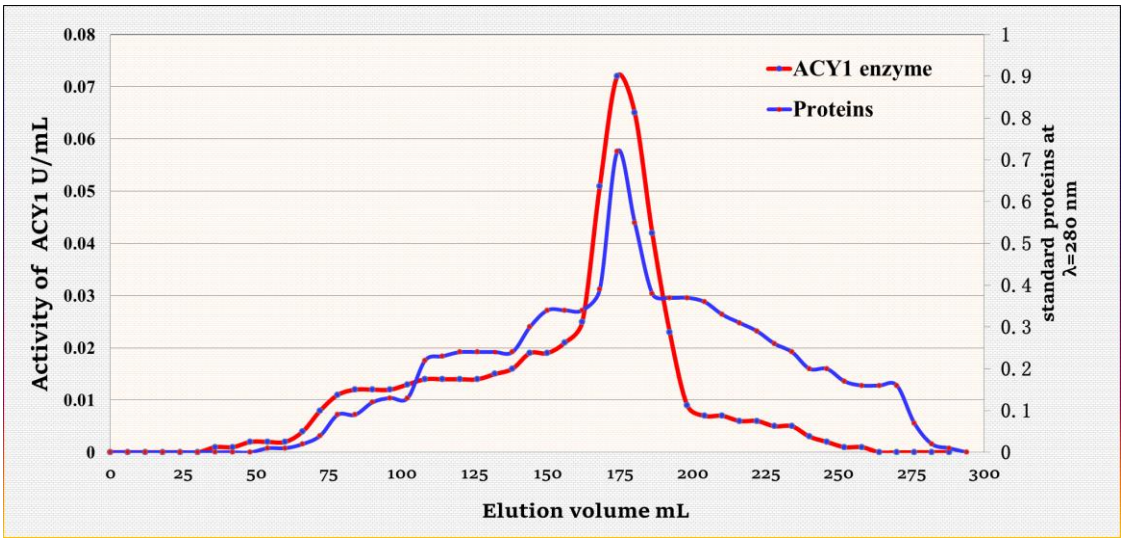

Figure (3) Gel filtration technique for purifying ACY1 enzyme from the serum of hepatocellular carcinoma patients

Determination of the Molecular Weight of Aminoacylase-1

1- By Gel Filtration Chromatography

Standard proteins were used in gel filtration chromatography to determine the molecular weight of the purified ACY1 enzyme [23]. Table (2) shows the known molecular weights of the proteins.

Table (2) Standard proteins for extracting the molecular weight of the ACY1 enzyme purified from the serum of hepatocellular carcinoma patients

No.	standard proteins	Molecular Weight of Proteins	Elution Volume	Log M.wt
1	Blue dextrin	2000000	30.2	6.30
2	Bovine serum	67000	55.7	4.83
3	Amylase	58000	66.0	4.76
4	Egg albumin	45000	83.3	4.65
5	Pepsin	36000	103.2	4.56
6	Insulin	5750	123.3	3.76
7	Tryptophan	204	171.4	2.31
8	ACY1 Unknown	45593	144	4.65

The molecular weight of ACY1 is estimated by substituting the elution volume from Figure (4), for the highest peak of ACY1 activity in the straight-line equation (y = -0.0124x + 5.8951) obtained from plotting the calibration curve in Figure (5).

European Science Methodical Journal
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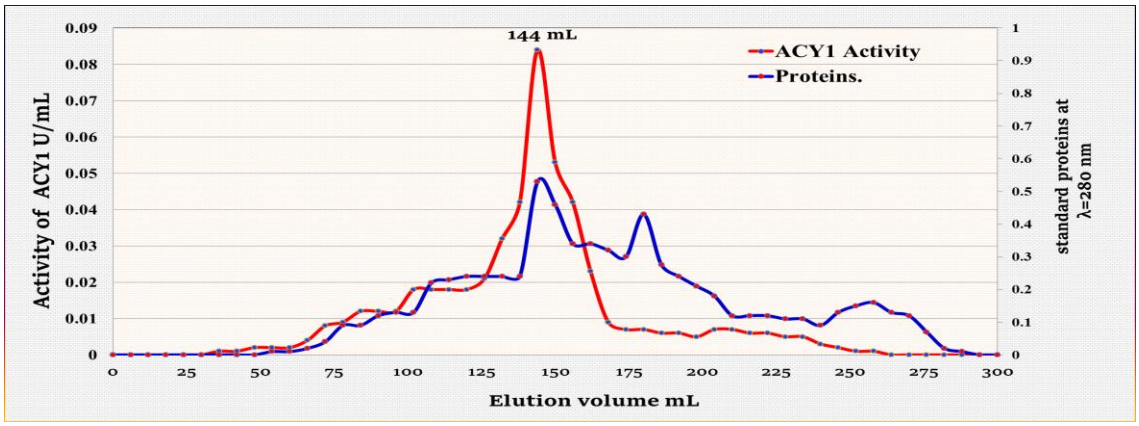

Figure (4) Gel filtration technique to determine the molecular weight of ACY1 enzyme purified from the serum of hepatocellular carcinoma patients

Substituting the elution volume (144 mL) for the value of (X) in y = -0.0124x + 5.8951, we obtain the value of (y) which represents (logMwt.). The approximate molecular weight of Aminoacylase-1 was (45593 Daltons).

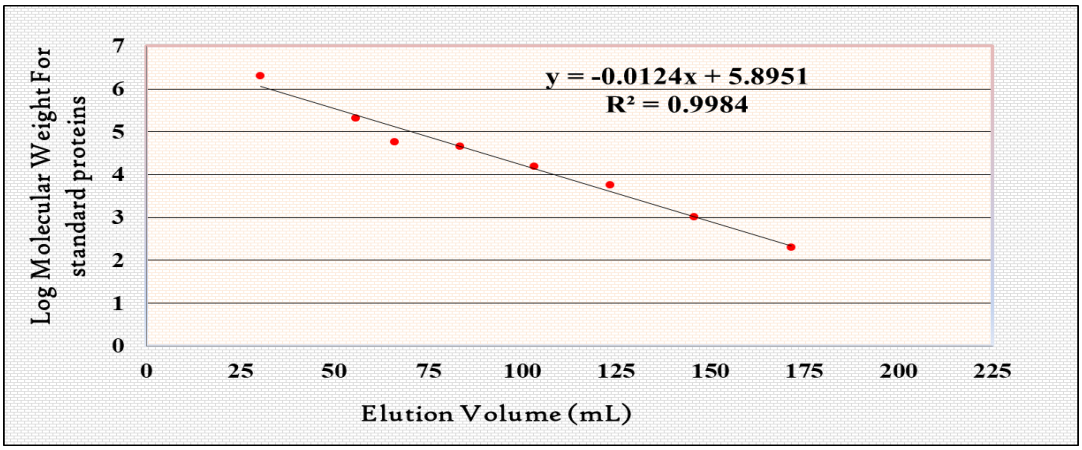

Figure (5) plots the calibration curve for standard proteins in gel filtration to find the straight-line equation

2- Electrophoresis Method

The enzyme minoacylase-1 was also isolated by electrophoresis, as shown in Figure (6). Its mechanism of action is based on molecular weight, not shape or charge. An electric current is passed after placing ACY1 in the gel, and it moves towards the positive electrode. The molecular weight of ACY1 was approximately (450,000 Daltons) [24].

This study is consistent with several studies in mammals where the molecular weight of ACY1 ranged (43000-47000 Daltons). In the study by Somar et al., the molecular weight of ACY1 was (46000 Daltons) [25]. In a study by Chung et al., the molecular weight of ACY1 in human blood was found to be (47000 Dalton) [26]. While the molecular weight of ACY1 in the rest of

the mammals was: in the kidneys of mice (43000 Dalton) [26,27] and in the kidneys of pigs (45260 Dalton), and it was also identical in the liver of pigs [10].

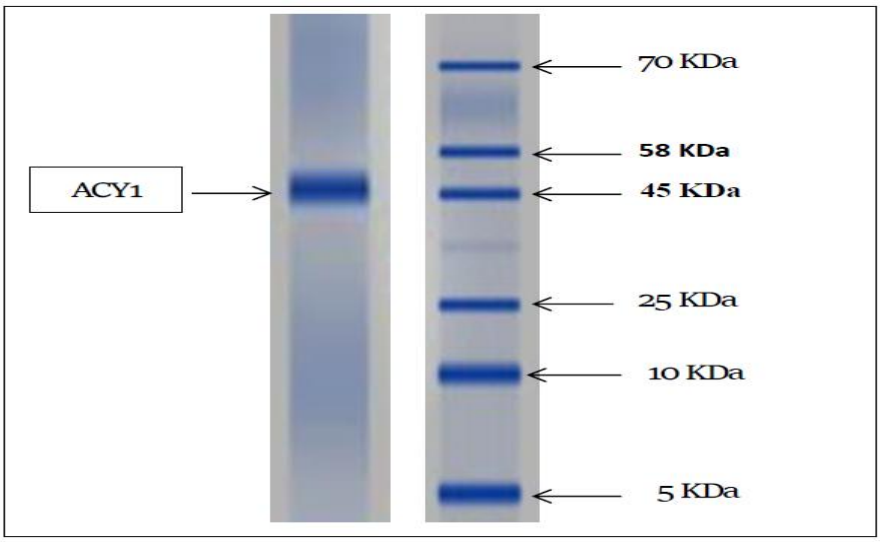

Figure (6) Electrophoresis of purified ACY1 enzyme via PAGE-SDS gel Kinetic study, optimal conditions and factors affecting purified Aminoacylase-1 enzyme 1- Studying the effect of the substrate (L-Nacetyl-L-methionine) on the activity of purified ACY1 enzyme.

Multiple concentrations of the substrate (L-Nacetyl-L-methionine) were prepared, ranging from (0.5-8.5 mM). An increase in ACY1 activity was observed as the substrate concentration increased, as shown in Figure (7). The enzyme activity continued to rise until the enzyme was saturated with the substrate and reached its maximum velocity (Vmax), at which point the velocity remained constant regardless of the substrate concentration. The optimal substrate concentration (7.5 mM) was at the highest activity of ACY1.

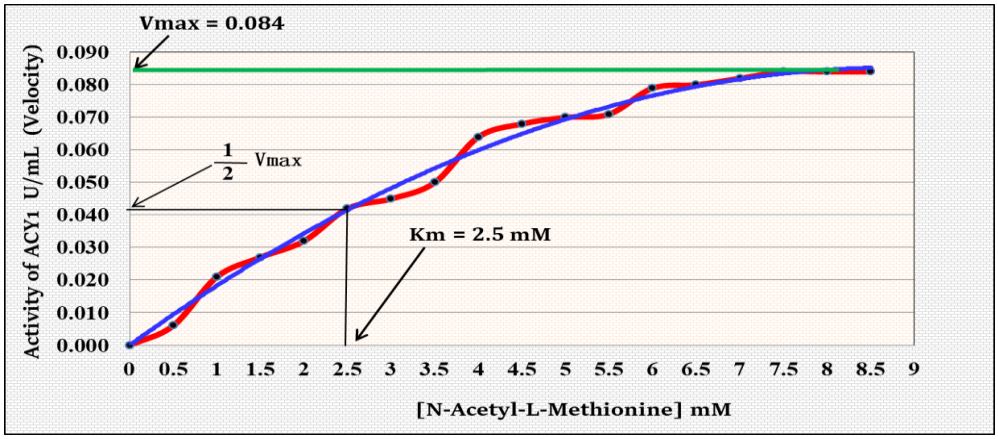

Figure (7) Impact of the substrate L-Nacetyl-L-methionine on the activity of purified ACY1

European Science Methodical Journal
ISSN (E): 2938-3641
Volume 4, Issue 5, May- 2026

To find the values of the Michaelis-Menten constant (Km) and the maximum speed (Vmax), it is necessary to draw a Lainover-Berck diagram between the x-axis (1/[S]) and the y-axis (1/V), as in Figure (8).

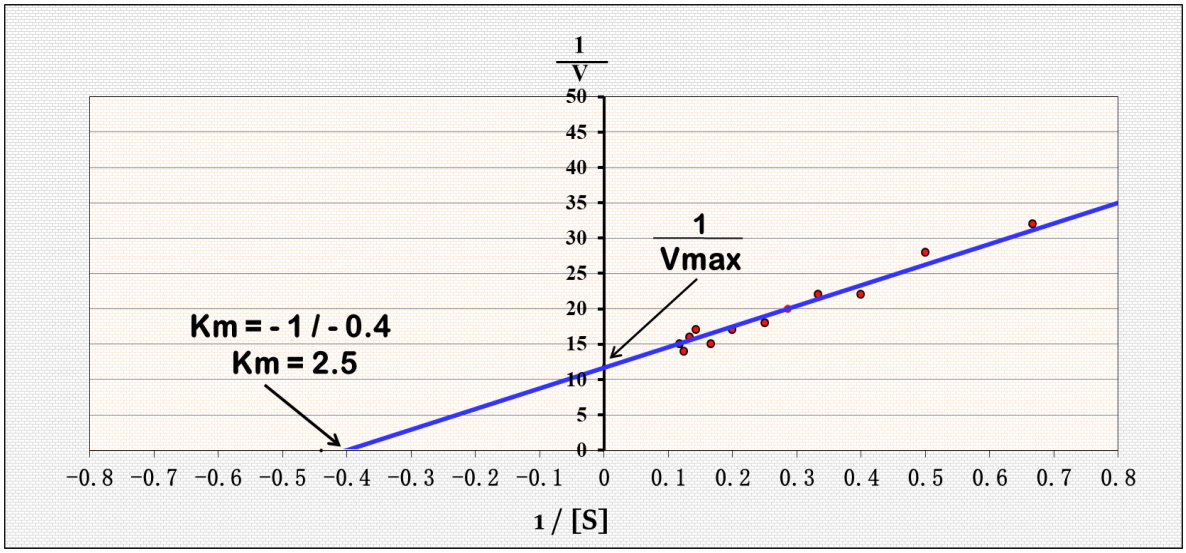

Figure (8) Lineweaver-Berke plot for finding the values of the Michaelis-Menten constant (Km) and the maximum velocity (Vmax) of purified ACY1 activity.

Figures (5, 6) show that the value of the Michaelis-Menten constant is (Km = 2.5 mM). In Figure (5), the Km value is the substrate concentration when the highest activity of the enzyme ACY1 is half the maximum speed (1/2 Vmax). The maximum velocity value is (Vmax = 0.084 U/mL).

2- Studying the effect of temperature on the activity of the purified Aminoacylase-1 enzyme

The activity of ACY1 was measured at different Celsius temperatures ranging from (30-44 C°), as shown in Figure (9). It was observed that the highest activity of ACY1 was at the optimum temperature (37°C).

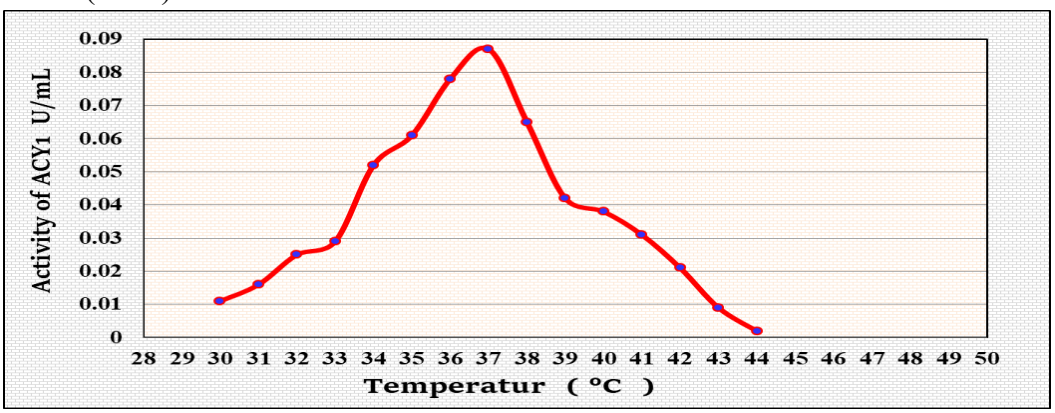

Figure (9) Optimal temperature affecting the activity of purified ACY1

European Science Methodical Journal
ISSN (E): 2938-3641
Volume 4, Issue 5, May- 2026

3- Studying the effect of pH on the enzymatic reaction rate of purified ACY1 enzyme

In Figure (10), solutions of varying pH levels (3-10) were prepared. The activity of ACY1 was highest at the optimum (pH=7.5).

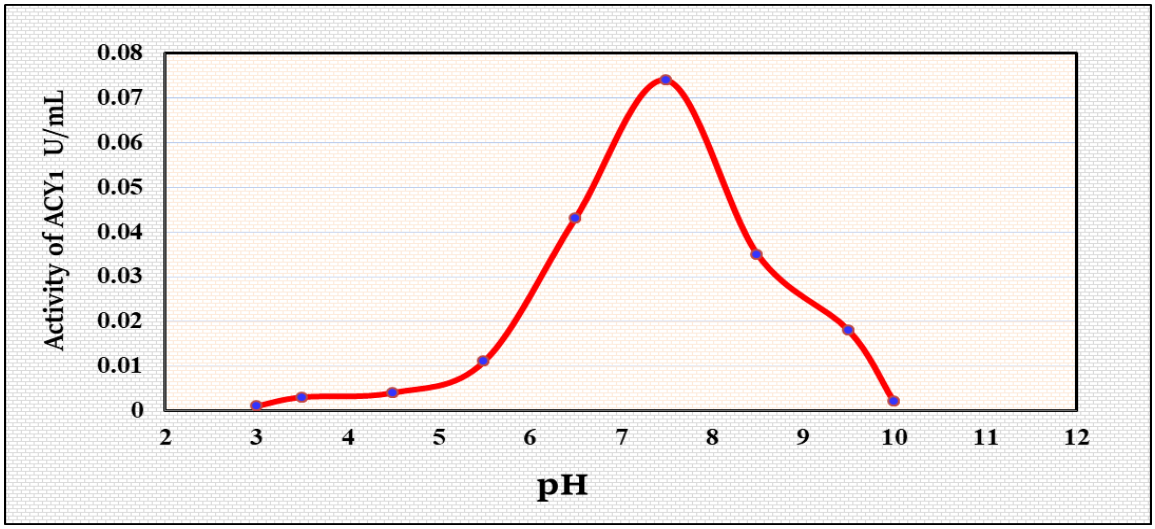

Figure (10) Impact of optimum pH on purified ACY1 activity

4- Studying the Impact of time period on the activity of purified ACY1 enzyme

The optimal incubation time period for the highest activity of purified ACY1 enzyme was determined to be (25 minutes), as shown in Figure (11).

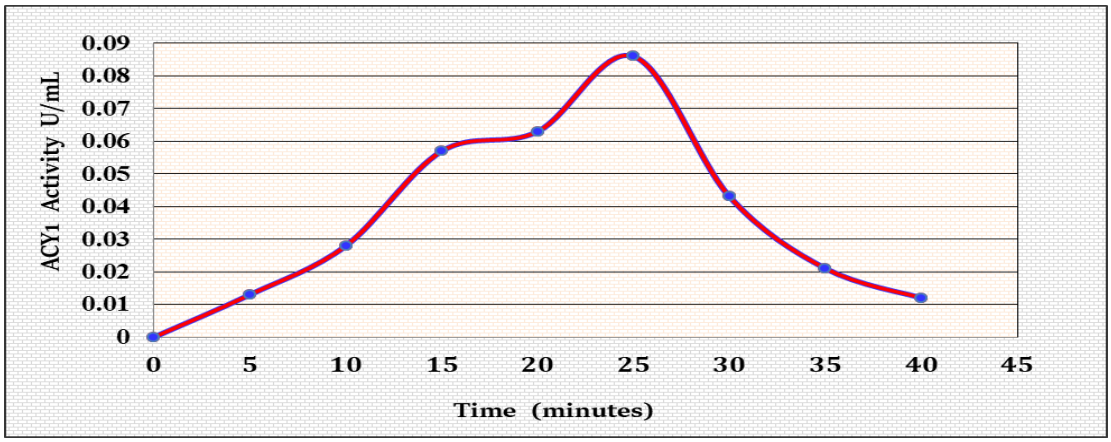

Figure (11) Impact of incubation time on purified ACY1 activity

Table (3) Optimal conditions for maximum activity of ACY1 enzyme purified from the serum of hepatocellular carcinoma patients

[S] mM	Tempertur (Celsius)	pH	Time (minutes)	Vmax U/mL	Km (mM)
7.5	37C°	7.5	25	0.084	2.5

Conclusion

This study concludes that hepatocellular carcinoma is a malignant disease. Early diagnosis of HCC is difficult because symptoms that confirm the presence of cancer are not present until its advanced stages. HCC is influenced by modifiable underlying risk factors. It has rare but serious biliary and arterial complications. The enzyme Aminoacylase-1 is closely related to HCC due to its active role in regulating metabolism and its effect on the growth and spread of cancer cells. ACY1 gene expression levels decrease in HCC, causing metabolic reprogramming dysfunction. ACY1 functions as a potential therapeutic target and as an inhibitor that prevents the growth and spread of cancerous tumors in the liver, and as a biomarker for detecting tumors in cancerous tissues.

We conclude that ion exchange and gel filtration techniques gave an excellent result for the single-band (one isotope) of ACY1. The molecular weight of the enzyme was estimated by gel filtration and electrophoresis and was approximately (450,000 Daltons). The optimal conditions for the enzyme ACY1 were at a substrate concentration of (7.5 mM), a value of (Km=2.5 mM), a maximum velocity of (Vmax=0.084 U/mL), a pH value of (pH=7.5), an incubation time of (25 minutes), and a temperature of (37°C).

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Consent for publication

The researchers have no objection to publication.

Data availability

Study data will be available upon request to the corresponding

Funding Statement

No funding

Conflicts of Interest

The author declare that there are no conflicts of interest regarding the publication of this manuscript.

References

1- Li, P., Ding, Z., Feng, Y., Ren, X., Wei, Y., Xia, C., ... & Cao, G. (2026). Global, regional, and national burden of hepatocellular carcinoma and contribution of nine modifiable risk factors across 185 countries/territories in 2022. *Science Bulletin*, *71*(4), 838-849.

2- Pham, G. A., Le, V. K. A., Pham, T. H. Y., Nguyen, S. L., Nguyen, L. H., Quach, C. N., ... & Luu, H. N. (2026). Risk Factors for Hepatocellular Carcinoma: Findings from a Case-Control Study in Vietnam.

3- Wan, Y. X., Lin, Z. Y., Chen, L. T., Wu, R. Q., Zhang, Y., & Du, Z. Q. (2026). Arterial and biliary complications after transarterial chemoembolization for hepatocellular carcinoma. *World Journal of Clinical Oncology*, *17*(1), 113618.

4- Zhang, X., Zhao, L., Ngo, L. H., Dillon, S. T., Gu, X., Lai, M., ... & Zhang, X. (2024). Prediagnostic plasma proteomics profile for hepatocellular carcinoma. *JNCI: Journal of the National Cancer Institute*, *116*(8), 1343-1355.

5- Malik, A. K., Geh, D., Jeffry Evans, T. R., Chow, P. K., Mann, D. A., & White, S. A. (2026). Improving surgical treatments for hepatocellular carcinoma. *Nature Reviews Gastroenterology & Hepatology*, *23*(3), 208-226.

6- Wei, X., Li, J., Xie, H., Ling, Q., Wang, J., Lu, D., ... & Zheng, S. (2014). Proteomics-based identification of the tumor suppressor role of aminoacylase 1 in hepatocellular carcinoma. *Cancer letters*, *351*(1), 117-125.

7- Chen, Q., Xia, F., Guo, B., Liu, Z., Liu, X., Shu, C., ... & Zhu, P. (2026). Development and Internal Multicenter Validation of a Deep Learning Model for Predicting Post-Hepatectomy Liver Failure in Patients with Hepatocellular Carcinoma: A Multicenter Study. *Cancers*, *18*(6), 926.

8- Smolka, V., Friedecky, D., Kolarova, J., Tkacik, O., Foltenova, H., Bekarek, V., ... & Srovnal, J. (2024). Aminoacylase 1 deficiency: case report on three affected siblings. *AME Case Report*, *8*, 18.

9- Mohammadi, M. F., Dehghani, A., Zarabadi, K., Kahani, S. M., Sayyad, S., Ashrafi, M. R., ... & Tavasoli, A. R. (2024). Persistent basal ganglia involvement in aminoacylase-1 deficiency: expanding imaging findings and review of literature. *Irish Journal of Medical Science (1971-)*, *193*(1), 449-456.

10- Anders, M. W., & Dekant, W. (1994). Aminoacylases. *Advances in Pharmacology*, *27*, 431-448.

11- Procházková, D., Borská, R., Chrastina, J., Fajkusová, L., Konečná, P., Pešková, K., ... & Honzík, T. (2023). Aminoacylase 1 deficiency: the first Czech patient.

12- Guan, Y., Jonas, Z., Wang, A., Tendoh, F. G., Manish, M., Shah, R., ... & Benson, M. (2025). Aminoacylase-1 Regulates Hepatic Mitochondrial Respiration and Whole-Body Metabolism. *Circulation*, *152*(Suppl_3), A4357898-A4357898.

13- Chen, Z., Gao, J., Sun, J., Wu, Z., & Wang, B. (2021). Aminoacylase 1 (ACY-1) mediates the proliferation and migration of neuroblastoma cells in humans through the ERK/transforming growth Factor β (TGF-β) signaling pathways. *Medical Science Monitor: International Medical Journal of Experimental and Clinical Research*, *27*, e928813-1.

14- Li, Y., Li, L., Hou, Y., Peng, X., & Li, H. (2025). Research advances on amino acid starvation interventions for hepatocellular carcinoma. *Biochimica et Biophysica Acta (BBA)-Reviews on Cancer*, 189400.

15- Morine, Y., Utsunomiya, T., Yamanaka-Okumura, H., Saito, Y., Yamada, S., Ikemoto, T., ... & Shimada, M. (2022). Essential amino acids as diagnostic biomarkers of hepatocellular carcinoma based on metabolic analysis. *Oncotarget*, *13*, 1286.

16- Peterson, G. L. (1983). [12] Determination of total protein. In *Methods in enzymology* (Vol. 91, pp. 95-119). Academic Press.
17- Rosen, H. (1957). A modified ninhydrin colorimetric analysis for amino acids. *Archives of biochemistry and biophysics*, *67*(1), 10-15.
18- Lowry, O., Rosebrough, N., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *J biol Chem*, *193*(1), 265-275.
19- Wingfield, P. T. (Ed.). (2016). Protein precipitation using ammonium sulfate. *Current protocols in protein science*, *84*(1), A-3F.
20- Robyt, J. F., & White, B. J. (1987). Biochemical techniques: theory and practice. *(No Title)*.
21- Harisha, S. (2005). An introduction to practical biotechnology. Firewall Media.
22- Ó'Fágáin, C., Cummins, P. M., & O'Connor, B. F. (2016). Gel-filtration chromatography. In *Protein Chromatography: Methods and Protocols* (pp. 15-25). New York, NY: Springer New York.
23- Debnath, S., Das, M., Mondal, S., Sarkar, B. K., & Babu, G. (2025). Advances in chromatography: contemporary techniques and applications. *Essential Chem*, *2*(1), 1-27.
24- Qavi, H., & Kit, S. (1980). Electrophoretic patterns of aminoacylase-1 (ACY-1) isozymes in vertebrate cells and histochemical procedure for detecting ACY-1 activity. *Biochemical Genetics*, *18*(7), 669-679.
25- Sommer, A., Christensen, E., Schwenger, S., Seul, R., Haas, D., Olbrich, H., ... & Sass, J. O. (2011). The molecular basis of aminoacylase 1 deficiency. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease*, *1812*(6), 685-690.
26- Zhong, Y., Onuki, J., Yamasaki, T., Ogawa, O., Akatsuka, S., & Toyokuni, S. (2009). Genome-wide analysis identifies a tumor suppressor role for aminoacylase 1 in iron-induced rat renal cell carcinoma. *Carcinogenesis*, *30*(1), 158-164.
27- Giardina, T., Perrier, J., & Puigserver, A. (2000). The rat kidney acylase I, characterization and molecular cloning: Differences with other acylases I. *European journal of biochemistry*, *267*(20), 6249-6255.