The Egyptian Journal of Hospital Medicine (January 2023) Vol. 90 (2), Page 2574-2580

Inhibition, Characterization and Purification of α-AMY from Sera of Iraqi Breast Cancer Patients

Namariq A. Lafta*, Alaa Hussein J. Al-Qaisi
Department of Chemistry, College of Science, Al-Nahrain University, Jadriya, Baghdad, Iraq.
***Corresponding author:** Namariq A. Lafta, **Tel.:** +9647805725833, **Email:** namariqamer93@gmail.com

ABSTRACT

Background: α-Amylase enzyme is digestive enzyme that breaks down complex carbohydrates like starch into simpler sugars; it is being studied as a potential target for anti-cancer therapies.

Objective: The current study aimed to evaluate the general characterization, and inhibition of α-Amylase enzyme, which had been purified from the sera of patients diagnosed with Breast Carcinoma.

Materials and methods: A total of 100 blood serum samples were collected from patients with Breast cancer from Baghdad National Hope Hospital in Baghdad. α-Amylase enzyme was purified from serum clinical isolate in three stages; precipitation with 65% saturated ammonium sulphate, ion exchange chromatography utilizing a DEAE-cellulose column, and gel filtration chromatography with a sephadex G-200 column. Changing conditions in pH, temperature, inhibitor concentration, and kinetics all affect the activity of the α-Amylase enzyme.

Results: α-Amylase inhibition assay showed that *Vitis vinifera L.* was the most potent inhibitor (71.8%). During the kinetic investigation of the enzyme, it was discovered that the inhibitory mechanism that the extracts use is non-competitive. Vmax was 0.023, and 0.012 mmol min^{-1} at 75 μg/ml of *Vitis vinifera L.* , *Verbena Officinalis L* .extracts, respectively.

Conclusions: High specific activity for α-Amylase purified from sera of Iraqi breast cancer patients was obtained after three purification steps. Ethanolic plant extracts showed inhibitory effect on α-Amylase while *Vitis vinifera L.* was the most potent inhibitor.

Keywords: α-Amylase, Breast Cancer, Characterization, Inhibition, Purification, Al-Nahrain University, Iraq.

INTRODUCTION

α-Amylase (α-AMY) (E.C.3.2.1.1) is a hydrolase enzyme that catalyses the hydrolysis of starch's internal α-1, 4-glycosidic bonds to produce glucose and maltose. It is a calcium metalloenzyme, which means that its activity is dependent on the presence of a metal cofactor [(1)].

In addition to being found in significant concentrations in the saliva and pancreatic juice, α-AMY may be isolated from other tissues such the gonads, fallopian tubes, skeletal muscle, and adipose tissue. The impact of temperature, the circumstances of hydrolysis, and the origin of the enzyme greatly influence the hydrolysate composition obtained following starch hydrolysis [(2)].

In human, there are mainly two distinct genes, AMY1 and AMY2, which encode salivary (type 1) and pancreatic (type 2) α-AMY, respectively [(3)].

α-AMY may be isolated from humans, plants, animals, and microbes. Several purification procedures for isolating α-AMY from microbiological sources have also been developed [(4)]. Ultrafiltration, salt precipitation, dialysis, and column chromatography are the methods used. These methods produce varying yields and folds of enzyme purification [(5)].

In women, breast carcinoma (BC) is the most common form of cancer and the primary cause of cancer-related death. a-Amylase is utilized as a biomarker in the clinical diagnosis of diseases such as cancers and inflammation [(6,7)] has antibacterial properties [(8)], and has been found in the mammary gland [(9)], breast milk [(10)], and various other tissues [(11)].

In addition to that, α-AMY has been identified in cases of lung cancer [(12)], breast cancer, and ovarian cancer [(13)]. α-AMY inhibitors, commonly known as starch blockers, may offer possible mitigation by inactivating Amylase in saliva or pancreatic digesting juice, lowering blood glucose. Therapies, and they appeared to be a viable replacement for synthetic α-AMY inhibitors as well as for antioxidant effects [(14)].

Several in vivo and in vitro research on the plants have demonstrated that *Vitis vinifera L.* ,*Verbena officinalis L.*, and *Ammi visnaga L.* have several pharmacological and biological activities, such as anti-tumor, anti-diabetic [(15,16,17)]. As a result, in this investigation, α-AMY was isolated and characterized from the serum of breast cancer patients. This article also focuses on purification techniques and characterization of α-AMY in terms of temperature and pH stability, substrate selectivity, and plants as natural sources of inhibitors to α-AMY effects.

MATERIALS AND METHODS

Samples:

A total of 100 samples of blood serum were collected from patients with Breast cancer from Baghdad National Hope Hospital in Baghdad. during the periods from 15/3/2021 to 20/6/2021. Control group 50 samples were collected from healthy people from a local hospital in Baghdad Iraq. Using a disposable syringe, 2-4ml of blood was collected by

Received: 19/07/2022
Accepted: 21/09/2022

venipuncture in tubes containing a clot activator. The tubes were centrifuged and serum samples were separated. The samples were transported to the laboratory under refrigeration for analysis. Following that, all serum samples were collected for use in the following steps.

α-AMY activity assay:
Human Alpha-Amylase (Enzyme-Linked Immunosorbent Assay) ELIZA Kit (BT Lab, Shanghai, China) was used to evaluate α-AMY activity in samples, with the manufacturer's protocol modified for 96 well formats with suitable dilutions. The protein concentration was then measured using the Bradford technique.

Purification of α-AMY:
α-AMY was purified according to **Saleh *et al.*** [18] as follows:
Step 1: Ammonium sulfate precipitation (salting out).
The specific weight of solid ammonium sulfate was gradually added to 100ml of the crude enzyme to get saturation ratios of (10, 20, 30, 40, 50, 60, and 65% separately), then centrifuged at 6,000 rpm for 20min and the precipitate in each concentration was dissolved in suitable amounts of phosphate buffer. The activity of the enzyme was assayed to determine the best saturation ratio.

Step 2: DEAE-Cellulose Ion exchange chromatography.
Enzyme solution was applied to DEAE-cellulose column (2×20cm) which equilibrated with Tris-HCl buffer (0.05M; pH 8). The column was washed with an equivalent volume of the same buffer and eluted with NaCl gradient ranging from 0.1-1M). 3ml fractions were collected and fractions containing α-AMY were pooled and kept for the next step.

Step 3: Gel filtration on Sephadex G-200.
A purified α-AMY obtained from the ion exchange step was applied on to a Sephadex G-200 column (1.5×35cm) which was equilibrated with phosphate buffer. The elution was achieved at a flow rate of 3ml/fraction and the fractions containing α-AMY activity were used for the next steps.

α-AMY characterization
The characterization study was done according to [19]:

Determination of PH effects on α-AMY stability.
The influence of pH on α-AMY stabilization was used to investigate by a pre-incubating isolated enzyme in buffers ranging in pH from 5 to 10 for 30 minutes at 37 degrees Celsius, and the residual activity was assessed using the ELISA method.

Table 1. List of plants selected for the study of inhibitory potentials of their Ethanolic extracts

Plants	Species	Family
Vitis vinifera L. 	leaves	Vitaceae
Verbena officinalis L. 	leaves	Verbenaceae
Ammi visnaga L. 	seeds	Apiaceae

Determination of Temperature effects on α-AMY stability.
α-AMY stability was determined by pre-incubating of a purified enzyme at (30, 40, 50, 60, 70, and 80℃) and the remaining activity was measured using the ELISA technique.

Determination of α-AMY molecular weight (mol. wt.).
Protein molecular weight was measured by gel filtration chromatography. The pH 7 0.05M phosphate buffer was used to equilibrate and elute a 1.5 x 35 cm Sephadex G-200 column. Molecular weight standards were determined using crystalline proteins such as alcohol-dehydrogenase (mol. wt.= 150,000), albumin (mol. wt. = 66,000), carbonic anhydrase (mol. wt. = 29,000), and lysozyme (mol. wt.= 14300). Elution volumes of standard proteins were measured at 280 nm in a UV-Vis spectrophotometer, and void volumes were estimated with Blue Dextran at 600 nm. The mol.wt. of the α-AMY was evaluated by elution volume using the molecular weight of known standard proteins.

Plant Material and Ethanolic extraction
The leaves and seeds of plants (*Vitis vinifera, Ammi visnaga L., Verbena Officinalis L.*) were collected from Baghdad –Iraq through March–August, 2021 (Table 1). Ethanolic extract of each plant was prepared according to [20].

50 grams of each plant powder was extracted with 150ml of Ethanol at 65℃ for four hours using soxhlet apparatus. pressure in a rotary evaporator and collected in Petri dishes which were transferred to a hot-air oven (40℃) until drying completely. The resulting plant extracts were scraped from the surface of Petri dishes and stored until used.

Table 2. Purification steps of serum α-Amylase.

Steps of purification	Volume (ml)	α-AMY activity (U/mL)	Protein concentration (mg/ml)	Specific activity (U/mg)	Total activity (U)	Purification (folds)	Yield (%)
Crude α-AMY	75	0.758	0.4	1.895	56.850	1	100
Ammonium sulphate precipitation 65%	30	1.864	0.33	5.345	55.920	2.82	98.3
DEAE-cellulose	21	2.596	0.25	10.384	54.516	5.47	95.8
Sephadex G-200	16	3.108	0.1	31.380	50.240	16.55	88.3

In vitro inhibition of α-AMY

α-AMY was inhibited by three plant extracts according to [21]. A (0.005 g) of each inhibitor was dissolved in 10ml from ethanol and D.W. The extracts were concentrated under reduced and two concentrations (25, and 75 µg/ml) were prepared. The assay mixture was prepared by adding 40µl of different inhibitors (25, and 75 µg/ml) to 40 µl of purified enzyme (volume to volume). The mixture was incubated at 25℃ for 15min and then added to sample wells and α-AMY activity was measured using the ELISA technique.

Kinetic Parameters

Kinetic parameters of purified α-AMY were evaluated by varying substrate concentrations within a range of (0.6, 0.4, 0.22, and 0.125 mM). The ELISA method was used to determine the rate of product release. Lineweaver-Burk plots (19) were used to compute the Michaelis constant (Km) and the maximal velocity (Vmax). To investigate the manner of inhibition by ethanolic extracts of (*Vitis vinifera L., Verbena Officinalis L.*), α-AMY activity was evaluated in the absence or presence of one of the extracts at doses (25, and 75 µg /ml). After incubating the mixture at 25 °C for 15 minutes, the enzyme reaction was complete. Lineweaver-Burk plot analysis of data from enzyme tests comprising various dilutions of the substrate and inhibitors according to Michaelis-Menten kinetics was used to establish the manner of inhibition for these inhibitors [22,23].

Ethical approval:

The study was approved by Institutional Review Board (IRB) of the Al-Amal National Hospital for Cancer Management. Every patient signed an informed written consent for acceptance of participation in the study.

RESULTS

α-AMY purification:

Purification of α-AMY is essentil for developing a better understanding of the enzyme's function. It also necessitated property studies and a better understanding of the structure-function relationship. α-AMY was purified from sera of breast cancer patients under ideal conditions [18]. Purification was accomplished through a series of steps that included ammonium sulfate precipitation, ion-exchange chromatography, and gel filtration chromatography.

The sera for patients with the highest activity of α-AMY were collected and mixed to obtain a total volume of 60 ml. The mixture was subjected to ammonium sulfate with a 65% saturation ratio and it has been found that at this ratio, the obtained enzyme-specific activity was 5.345 U/mg which is a noticeable increase compared to 1.895 U/mg of crude enzyme-specific activity as shown in (Table 2).

After being purified using DEAE- cellulose chromatography, the enzyme's activity increased even more. (Figure 1) shows a graph of -Amylase enzyme and specific activity. In one step of washing, the first peak that appeared lacked -Amylase activity, so it was neglected. The second peak (eluted at 0.4 of NaCl) had α-AMY activity reached 2.596 U/ml. After finishing up the ion exchange chromatography, the active fractions that represent α-AMY were collected and pooled onto the Sephadex G-200 column (dimensions) which is previously equilibrated with phosphate buffer. The results illustrated in (Figure 2) showed just one absorption peak which represents α-AMY with maximum activity. The gel filtration technique purified the enzyme about (16.5) purification fold with a maximum activity of 3.108 U/mL and yielded 88.3%.

Wasan and Firas, revealed that the specific activity of α-AMY enzyme purified from the blood of diabetic patients was 21.8 U/mg and the degree of purification reached 16.1 with an enzymatic outcome of 108.2% using Ion exchange chromatography and Sephadex G-100 gel filtration column chromatography [24]. From the human pancreas and saliva, α-AMY was purified using solvent and salt fractionation and column chromatography to achieve specific activities of 63 and 279 kU/g, respectively [25].

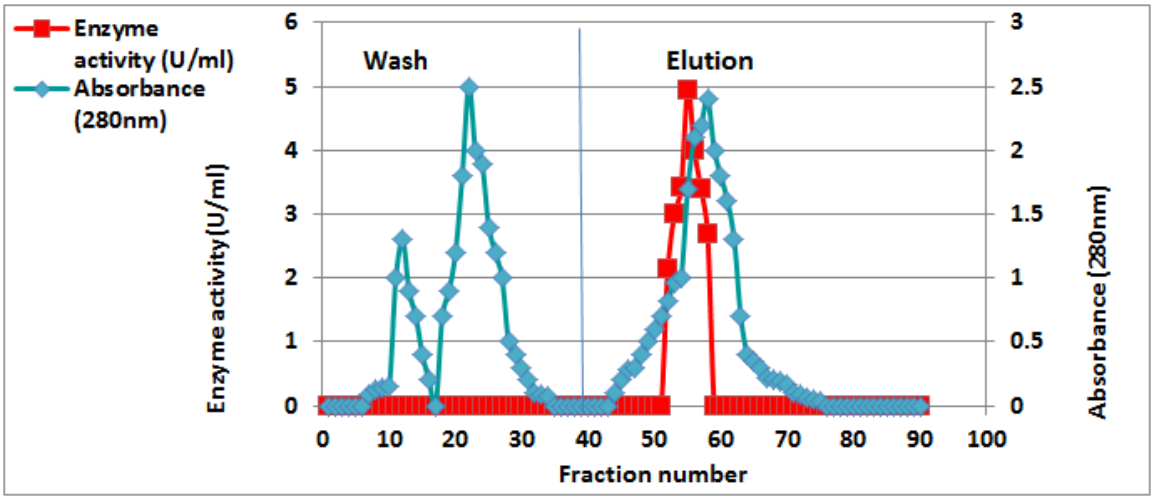

Figure 1. Ion exchange chromatography of serum α-AMY.

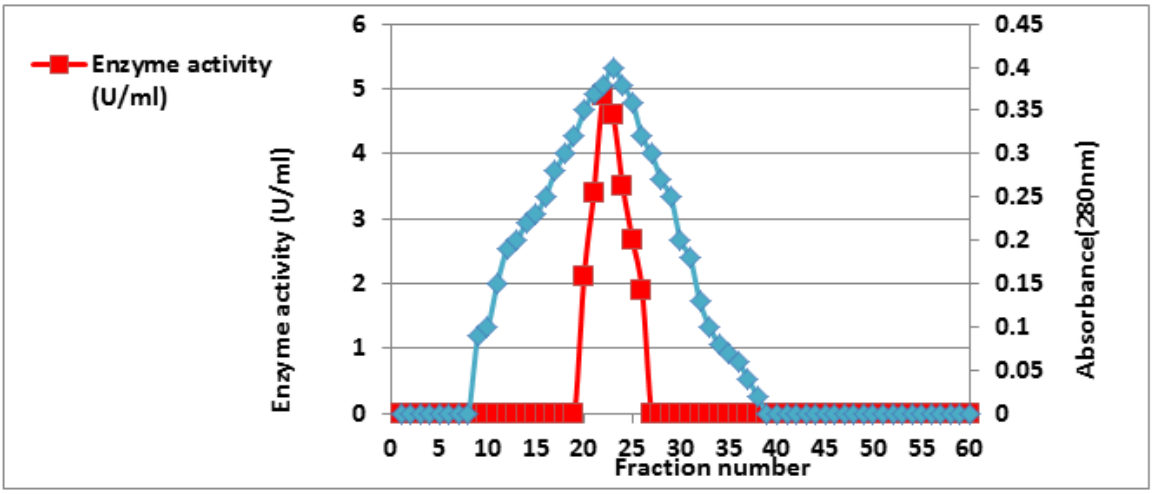

Figure 2. Gel filtration chromatography of serum α-Amylase.

Determination of α-AMY molecular weight

By using gel filtration chromatography, the mol.wt. of the purified α-AMY enzyme was determined. The 60 kDa mol.wt of α-AMY was interpolated from the molecular weights of known protein markers (**Figure 3**).

Effect of pH on α-AMY stability

α-AMY was stable in a wide range of pH (5- 9.0). The optimal pH for enzyme stability is between 6 and 7, with the highest stability at 7. And lower stability was found at pH 5 and 9 respectively (**Figure 4**). These results indicate that α-AMY tolerated an acidic and alkaline environment, but it prefers the neutral pH. pH affects an enzyme in multiple ways; changing enzyme conformation, the ionization groups in the enzyme's pocket, or the groups in the substrate [19].

Effect of Temperature on α-AMY stability

To evaluate how temperature affects the stability of α-AMY, the enzyme was incubated at a range of temperatures ranging from 30 to 80 °C, and the amount of activity that remained observable was determined using an enzyme activity assay [26]. The result presented in (Figure 5) shows that α-AMY was stable and maintained its activity 100% at (37-40) °C then when the temperature was increased the remaining activity began to decrease until reaches 20% at 80 °C.

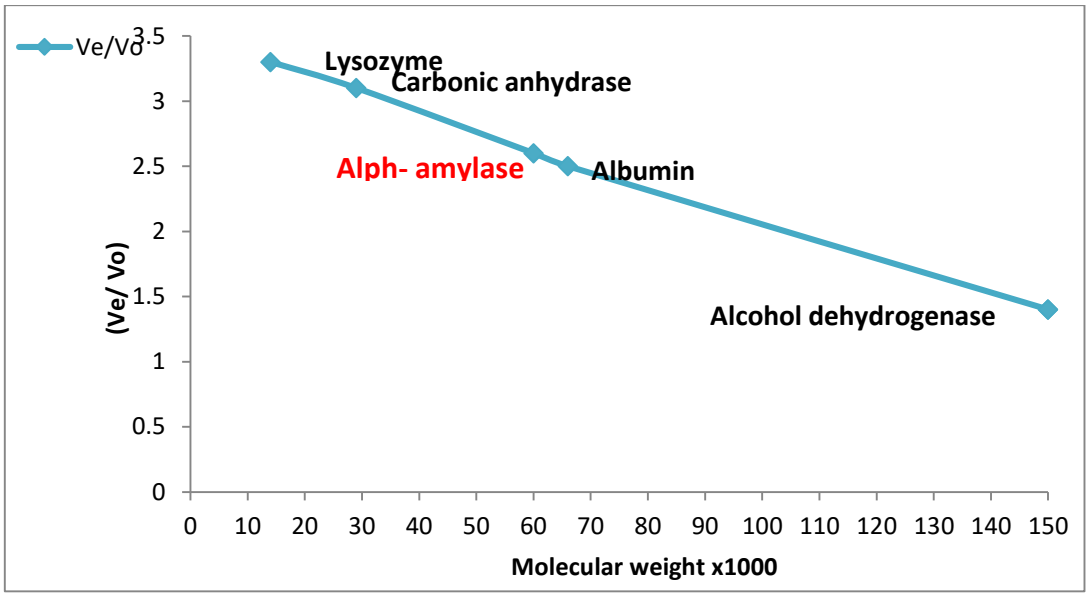

Figure 3. Determination of molecular weight for purified α-AMY.

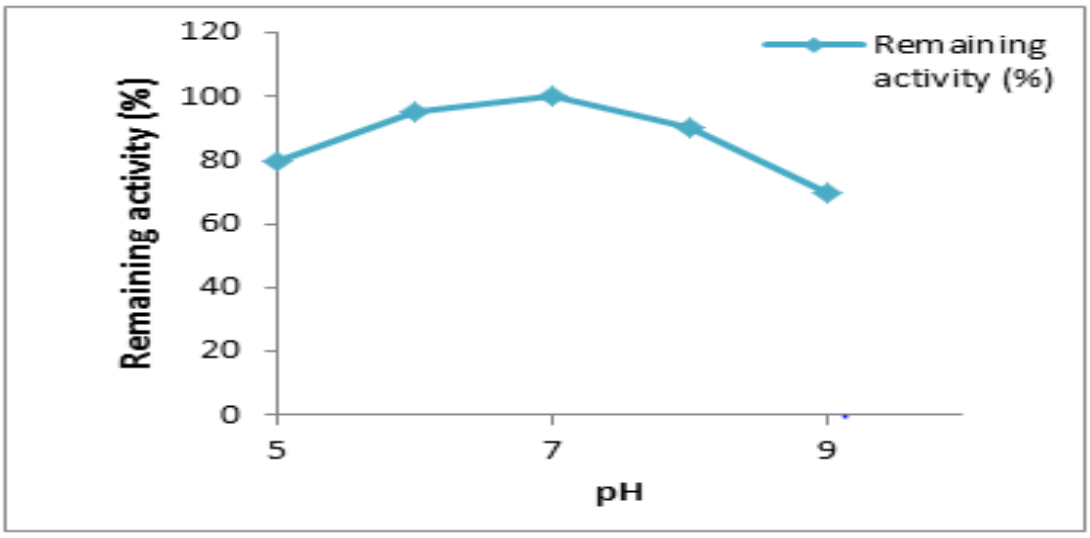

Figure 4. Effect of pH on α-AMY stability.

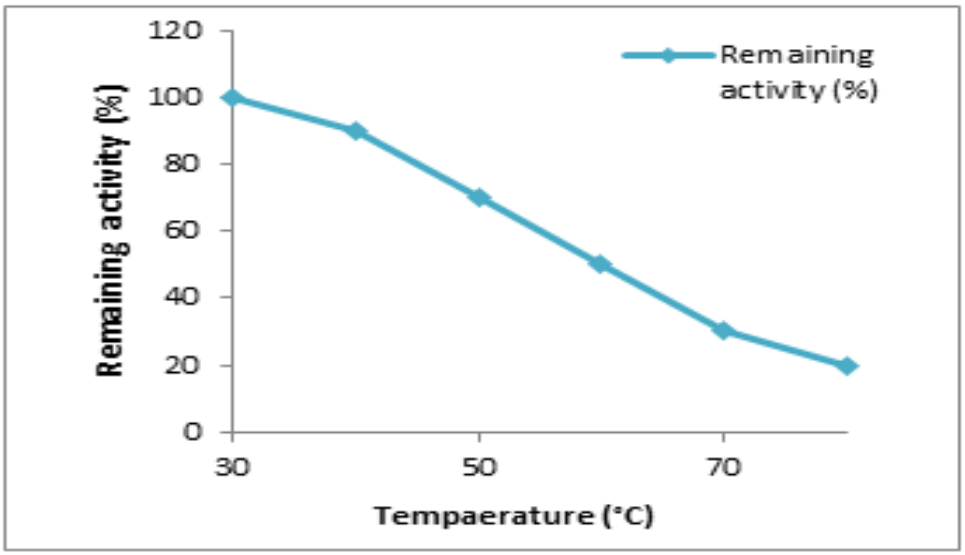

Figure 5. Effect of Temperature on α-AMY stability.

α-AMY inhibition

This study is being conducted in order to identify medicinal herbs that may be useful in the prevention or treatment of diseases [27].

The principal bioactive components of herbal plants have been utilised to treat cancer for a long time due to their anti-tumor and antioxidant effects [28]. These -Amylase inhibitors, also known as starch blockers, restrict the hydrolysis of α-1,4-glycosidic bonds in starch and other oligosaccharides into maltose, maltotriose, and other simple sugars, hence inhibiting or slowing the absorption of starch into the body [29]. We present the first evidence that ethanolic plant extracts of (Vitis vinifera L., Verbena Officinalis L., and Ammi visnaga L.) inhibit -Amylase. Inhibition of -amylase activity has been shown to be associated with some substances. These include tannins, phenols, flavonoids, and antioxidants [30].

Results displayed that, extracts plants showed inhibitory activity on α-AMY at all concentrations but in different percentages. The highest inhibitory activity was observed at 75 µg ml-1. strong inhibition was observed for Ethanol extracts of *Vitis vinifera L* (71.8%), and *Verbena Officinalis L.* (65.5 %) at 75 µg ml-1 , whereas low inhibition was noted in the case of *Ammi visnaga L.* (41.6%) as show in Table (3). This indicates that *Vitis vinifera L.* is extremely effective at inhibiting *α*-Amylase. This might explain the presence of several extract constituents (phenols, flavonoids, saponins, steroids, alkaloids, and terpenoids) that are effective *α*-Amylase inhibitors [31].

Table 3. *α*- Amylase inhibitory activity of three plant extracts at (25, 75 µg/ml).

Ethanol Extract	Inhibition % *α*-Amylase	
	75 (µg/ml)	25 (µg/ml)
Vitis vinifera L	71.8%	68.7%
Verbena officinalis	65.5%	52.4%
Ammi visnga L.	41.6%	36.2%

Vitis vinifera L. leaves have been shown to have a variety of biological activities, including hepatoprotective, spasmolytic, hypoglycemic, and vasorelaxant effects, according to the findings of several of in vivo and in vitro studies on the plant material [32].

3.6 Kinetic parameters of α-AMY.

The Lineweaver-Burk plot is a method that is utilized quite frequently in the process of determining the mechanism of enzyme inhibition that is most likely to occur. Here, Lineweaver—Burk plots were used to investigate the inhibition mode of active plants (*Vitis vinifera* , and *Verbena Officinalis*) against α-AMY. The mechanisms of *α*-Amylase inhibition of extracts were noncompetitive, according to double-reciprocal plots of enzyme kinetics (**Figures 6 and 7**).

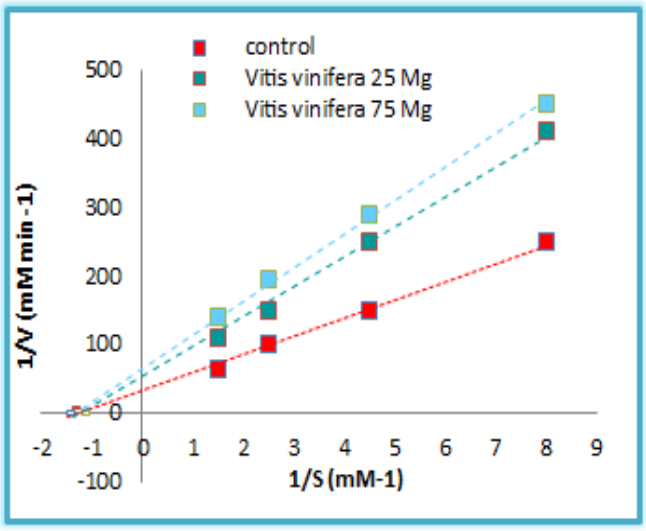

Figure 6. This kinetic analysis, displayed as a Lineweaver-Burk plot, of α-AMY inhibition by *Vitis vinifera L.*

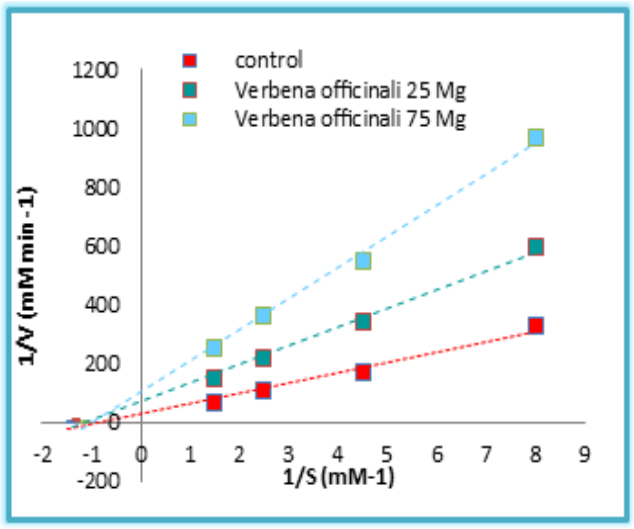

Figure 7. This kinetic analysis, displayed as a Lineweaver-Burk plot, of α-AMY inhibition by *Verbena officinalis L.*

According to the data, the average values of Km and Vmax of α-AMY were 0.714 mM and 0.047 mM/min, respectively. The inhibitors were no effect on Km, while Vmax in presence of 75 µg/ml of each of the extracts was decreased to 0.023, and 0.012 mmol min^{-1}, respectively [25].

CONCLUSIONS

In the current study, α-AMY was purified from the sera of breast cancer patients The purification factor was (16.55-folds) with a high specific activity. The molecular mass was calculated to be approximately 60.5 kDa. With pH, the enzyme was active. and temperature optima at moderate degrees. On the other hand, *Vitis vinifera* was the most potent noncompetitive inhibitor, and a kinetic study was used as a preliminary step for the in vitro α-AMY inhibition assay.

Declaration of conflict of interest: There was no disclosure of any possible conflicts of interest related to the research.

Funding: No funding

REFERENCES

1. **Shilpa S, Smruti S (2020)**: Review on microbial α-Amylase, types & their industrial application. Kala Sarovar, 23(4):105-112.
2. **Peter J, Frederick J, Peter R (2011)**: Human a-Amylase and starch digestion: An interesting Marriage. Starch, 63(11):395-405.
3. **Zainulabdeen J (2014)**: Is serum Amylase normal in women with polycystic ovarian syndrome? Baghdad Science Journal, 11(8):1583-1591.
4. **Ajita S, Thirupathihalli P, Krishna M (2014):** α-Amylase Production and Applications. A Review. Journal of Applied & Environmental Microbiology, 2(4):166-175.
5. **Sky-Peck H (1977):** Human pancreatic alpha-Amylase. I. Purification and characterization. Ann Clin Lab Sci., 7(4):298-309.
6. **Tanahashi C, Yasuki S, Akamine N *et al.* (2007)**: Pure acinic cell carcinoma of the breast in an 80-year-old Japanese woman. Pathol Int., 57(4):43-46.
7. **Attia M, Najlaa S (2016):** Progress of pancreatitis disease biomarker alpha Amylase enzyme by new nano optical sensor. Biosensors and Bioelectronics**,** 86(7):413-419.
8. **Abdel Ghany M, Hassan M, Ismail A *et al.* (2022):** Breast Asymmetry and Value of Contrast-Enhanced Spectral Mammography. The Egyptian Journal of Hospital Medicine, 89(1):4762-4766.
9. **Chaudhuri B, Rojek J, Vickerman M *et al.* (2007)**: Interaction of salivary alpha-Amylase and Amylase-binding-protein A(AbpA) of Streptococcus gordonii with glucosyltransferase of S. gordonii and Streptococcus mutans. BMC Microbiology, 60(7):1-12.
10. **Heitlinger L, Lee P, Dillon W *et al.* (1983)**: Mammary Amylase: a possible alternate pathway of carbohydrate digestion in infancy. Pediatr Res., 17(5):15-18.
11. **Andrea C, Marita M, Alessandro L *et al.* (2016):** Paraneoplastic lipase and Amylase production in a patient with small-cell lung cancer. Case Report BMC Cancer, 118(16):1-4.
12. **Minami S, Ryu J, Suguru Y *et al.* (2014)**: Amylase-Producing Lung Cancer with a Positive Epidermal Growth Factor Receptor Mutation Treated With Gefitinib. A Case Report, World J Oncol., 5(1):41-46.
13. **Gowhar R, Gulzar A, Aaliya S *et al.* (2022)**: Serum Amylase Levels with Respect to Different Clinical Disorders: A Hospital-based Observational Study from Kashmir. Acta Scientific Clinical Case Reports, 3(1):65-68.
14. **He L, Haochun Z, Jian Z *et al.* (2021)**. Proteinaceous α-Amylase inhibitors: purification, detection methods, types and mechanisms, International journal of food properties, 24(1):277-290.
15. **Didem O, Nilufer O, Berrin O *et al.* (2009)**: Biological activities of *Vitis vinifera* L. Leaves. Turk J Biol., 33(9):341-348.
16. **Ezz El Din M, Salah R, Kamel T, *et al.* (2022):** Evaluation of the Effect of Combination Chemotherapy with Five-day Infusion of Fluorouracil plus Vinorelbine in Pretreated Metastatic Breast Cancer Patients. The Egyptian Journal of Hospital Medicine, 88(1):2554-2560.
17. **Alam S, Anjum N, Akhtar J *et al.* (2018):** Pharmacological investigation on Khella (Ammi vinaga L.), World Journal of Pharmaceutical Research, 7(13):212-224.
18. **Saleh A, Yaaser Q, Youssri M *et al.* (2014)**: Purification and characterization of α-Amylase from Miswak Salvadora persica. BMC Complementary and Alternative Medicine, 119(14):1-10.
19. **Rehab J (2010):** Kinetics of α-Amylase enzyme in human serum. Journal of Kerbala University, 8 (3):237-244.
20. **Altemimi A, Lakhssassi N, Baharlouei A *et al.* (2017):** Phytochemicals: Extraction, isolation, and identification of bioactive compounds from plant extracts. Plants, 6(4):34-42.
21. **Khadayat K, Bishnu P, Hira G *et al.* (2020)**: Evaluation of the alpha-Amylase inhibitory activity of Nepalese medicinal plants used in the treatment of diabetes mellitus, Clinical Phytoscience, 6(34):1-8.
22. **Diana P, Luis F, Diana M (2015)**: Inhibitory effects of an extract of fruits of Physalis peruviana on some intestinal carbohydrases. Rev Colomb Cienc Quim Farm., 44(1):72-89.
23. **Ihcen K, Amar D & Mohamed Y (2014)**: Twenty Traditional Algerian Plants Used in Diabetes Therapy as Strong Inhibitors of *α*-Amylase Activity, International Journal of Carbohydrate Chemistry, 22(12):1-12.
24. **Wasan A, Firas T (2020)**: Isolation and partial Purification and for Amylase from diabetic patients and some kinetic properties. Tikrit Journal of Pure Science, 25(6):54-58.
25. **Eric J, Patricia H, Douglas M, et al. (1981):** Characterization and lntermethod Relationships of Materials Containing Purified Human Pancreatic and Salivary Amylase. Clinical Chemistry, 27(5):714-720.
26. **Prakarn R & Jarunee K (2012)**: Kinetic Inhibition of Human Salivary alpha-Amylase by A Novel Cellobiose-Containing Tetrasaccharide. J Med Assoc Thai., 95(1):102-108.
27. **Soheila M, Elham P, Mahmoodreza M *et al.* (2017):** Evaluation of Antioxidant Potentials and *α*-Amylase Inhibition of Different Fractions of Labiatae Plants Extracts: As a Model of Antidiabetic Compounds Properties. Biomed Research International, 14(2):1-8.
28. **Kunyanga C, Imungi J, Okoth M *et al.* (2012)**: Total phenolic content, antioxidant and antidiabetic Properties of methanolic extract of raw and traditionally processed Kenyan indigenous food ingredients. LWT Food Science and Technology, 45(2):269-276.
29. **De Sales P, de Souza P, Simeoni L *et al.* (2012)**: *α*-Amylase inhibitors: a review of raw material and isolated compounds from plant source. Journal of Pharmacy and Pharmaceutical Sciences, 15(1):141-183.
30. **Mohsina P, Faheem P, Amine M *et al.* (2022)**: Assessment of Antioxidative and Alpha-Amylase Potential of Polyherbal Extract. Evidence-Based Complementary and Alternative Medicine, 1:10.
31. **Yaseen K, Shujaul M, Ihsan u *et al.* (2021)**: Antioxidant potential in the leaves of grape varieties(*Vitis vinifera L.*) Grown in different soil Compositions. Arabian Journal of Chemistry, 14(2):1-10.
32. **Mirela L, Rahela C, Ionel F *et al.* (2020)**: Phytochemical Profile and Biological Activities of Tendrils and Leaves Extracts from a Variety of Vitis vinifera L. Antioxidants, 373(9):1-20.