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Purification and Characterization of Myrosinase from Local Broccoli (*Brassica oleracea var. Italica*)

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ABSTRACT

The study including purification and characterization of myrosinase (β-thioglucosidase glucohydrolase; E.C. 3.2.1.147) from the local broccoli. Myrosinase is a type of glycoproteins containing various thiol groups, disulfide, and salty bridges. The enzyme was extracted by using sodium phosphate buffer (pH 6.5), precipitation process was carried out by adding ammonium sulfate salt at saturation ratio (20-60)%. Myrosinase purified by gel filtration chromatography using ÄKTA Pure 25 apparatus with superdex 200 column, three peaks were obtained but one of them having enzymatic activity, and the purity of enzyme identified with the absence of SDS. The optimum pH for myrosinase activity and stability were 5, and (4-7), respectively, the optimum temperature for myrosinase activity and stability were 40°C, and (20-50) °C respectively. The molecular weight of pure myrosinase estimated by electrophoresis with the presence of SDS which found equal to 75.85 KDa. Myrosinase kept most of its activity regarding to magnesium chloride, potassium chloride, and sodium chloride, while loosing half of its activity regarding to zinc sulfate and copper chloride, then the enzyme gradually lost most of its activity regarding to nickel chloride and EDTA salt, whereas ferrous chloride and aluminum chloride having an inhibition role for myrosinase activity. Standard glucoraphanin using as substrate to determination kinetics of myrosinase which are represented by Mechalis-Menton constant Km and Vmax where equal to 0.37 mM and 606.25 µmol/ml/min., respectively by using different plots.

KEYWORDS: *Myrosinase; β-thioglucosidase glucohydrolase; broccoli; purificatian; glucoraphanin*

INTRODUCTION

Myrosinase (β-thioglucosidase glucohydrolase; E.C. 3.2.1.147) is an enzyme uniquely able to hydrolyze some S-linked glucosides, to produce different and wide bioactive compounds when the plant tissue is disrupted by crushing or chewing, in the presence of water, release glucose and an unstable intermediate compound (Angelino & Jeffery, 2014). Myrosinase is a type of glycoproteins containing various thiol groups, disulfide, and salty bridges, depending on its source, myrosinase has different molecular weights (135-480 KDa), a different number of subunits (2-12), a high content of carbohydrate (over than 22.5%) consist of hexoses sugars (Montaut & Rollin, 2016). Myrosinase catalyzes the hydrolysis of glucorapha- nin to liberate glucose and form an unstable aglycone that spontaneously rearranges to give a range of products, the most reactive form is the sulforaphane. Glucoraphanin occurs in all tissues of broccoli plants, and it is most abundant in the aerial portions, the developing florets, and the seeds (Yagishita *et al*., 2019). The reaction occurs through hydrolysis of glucoraphanin by the action of myr- sinase with the formation of an unstable intermediate compound (thiohydroxamate-O-sulfonate) releasing of an equimolar amount of glucose, followed by the spontaneous

conversion of thiohydroxamate-O-sulfonate into sulforaphane or sulforaphane nitrile, depending on the reaction conversion, the acidic pH with epithiospecifier protein (ESP) is suitable for formation of sulforaphane nitrile, where the neutral pH is suitable for the formation of sulforaphane (Mahn *et al*., 2020). Epithiospecifier protein (ESP) is a cofactor of myrosinase enzyme that assists the formation of epithionitriles and nitriles as the breakdown product of GSLs with a terminal double bond. ESP hinders the synthesis of ITCs but it has been observed that cooking at about 60 ºC denatures the ESP and help the formation of ITCs rather than epithionitriles (Bricker *et al*., 2014). Myrosinase was purified and characterized for the first time from broccoli by (Mahn *et al*., 2014), 3D structure of broccoli myrosinase monomer was designed by (Román *et al*., 2018). Myrosinase enzyme is extraction, purified, and characterized widely from different plant sources particularly Brassica family, also the enzyme purified from microbial sources including bacterial and fungal sources as well as from insects (Bhat & Vyas, 2019; Li *et al*., 2022). Myrosinase is an important enzyme found in broccoli, cabbage, and cauliflower, members in the *Brassica* family that have different nutritional importance due to its high content of bioactive compounds, particularly glucosinolates (Favela-González *et al*., 2020). The system of glucosinolate-myrosinase represented the entrance of defense mechanism in all *Brassica* vegetables, when the plant tissue is damage, myrosinase release and hydrolysis the glucosinolates to formation bioactive compounds such as isothiocyanates, which are displaying variety of healthy properties like anticancer activity (Wang *et al*., 2020; Pardini *et al*., 2021). Myrosinase extracted from broccoli has been widely studied in detail for its potential use in biotechnologyical applications, including the high production of sulforaphane for therapeutic properties (Wang *et al*., 2022). In Iraq, broccoli cultivation is still not widely known, and cultured with cauliflower (Al-Bayaty & Al-Sudani, 2021). The recent recommendations indicate that diet should be enriched with cruciferous vegetables to enhance phase II detoxification enzymes activity, and support its effect in prevention cancer diseases (Eagles *et al*., 2020; Mitra *et al*., 2022). Such a research on myrosinase purification didn't existent in Iraq which led us to carry out this search to studying characteristics and kinetics of myrosinase.

MATERIALS AND METHODS

Materials. Broccoli was brought from greenhouses/ Department of Horticulture /College of Agriculture/ University of Basra / Iraq. Cleaned, washed with distilled water and kept at 4 °C in polyethylene bags until use.

Chemicals. Standard glucoraphanin from Santa Cruz Biotech.Co., USA, and bovine serum albumin (BSA) from Sigma-Aldrich Co., USA. Protein Molecular Weight Marker from Bioneer Co., South Korea, solvents from HAYMAN Co.,UK and metal from BHD Co., England.

Apparatuses. UV-Vis Spectrophotometer, Apel 303 UV, England, ÄKTA Pure 25, GE Healthcare Life Sciences Co., Sweden and Slab electrophoresis, Biocom. Direct Co., UK.

Enzyme Extraction. Crude myrosinase extract was prepared according to (Mahn *et al*., 2014) with some modifications by using sodium phosphate buffer (pH 6.5) in a ratio of 1:3 (w/v), put about 50 g of broccoli, added 150 ml from the buffer solutions in the electrical blender to damage the plant tissue and releasing myrosinase, then leaving the mixture for 10 min. to complete enzyme reaction, then filtration with 4 layers of cheesecloth to get rid of the big parts of the plant, centrifuged at 5000 rpm/min. for 30 min., discard the sediment and keep the supernatant for determination of enzyme activity later. All steps were performed at 4 °C.

Enzyme Assay. Myrosinase activity was determined according to (Wu *et al*., 2022) that described by (Lim *et al*., 2014) with some modification, a reaction mixture consisting of 0.5 ml of broccoli extracted by 33 mM potassium phosphate buffer (pH 6.5) containing 0.5 mM ascorbic acid., 0.5 ml extracted glucoraphanin (from the best procedure), and 1ml of glucose reagent (kit GOD/POD ready to use), incubated in a water bath at a temperature of 37 °C for 15 minutes, the reaction was stopped at 95 ◦C for 5 min, enzyme activity was measured at 546 nm according to glucose kit procedure, the supernatants were used as a crude enzyme in the later purification steps.

Protein assay. A concentration of protein was measured using bovine serum albumin (BSA) as standard (Bradford, 1976).

Ammonium sulfate precipitation. Precipitation process was carried out by adding ammonium sulfate salt to the crude extract gradually with continuous stirring on the magnetic stirrer for 4 hours at a temperature of 4 °C until dissolution at a saturation rate ranging between 20%-60%, centrifugation performed at a speed of 5000 rpm. for 30 min. at a temperature of 4 °C, the sediment was collected and dissolved in potassium phosphate buffer pH 6.5, the specific activity and protein concentration in the filtrate and precipitate were estimated in each step, and then the dialysis against distilled

water using dialysis bags (previously activated) with a diameter of 28 µm was performed at a temperature of 4 °C for 24 hours, with changing of distilled water every 6 hours, the dialyzed solution was freeze-dried until concentration.

Gel filtration. The technique of gel filtration was done according to the method of Al-Taai *et al*., (2019) with little modification by using ÄKTA Pure 25 device with Superdex 200 10/300 GL column (10 mm diameter, column size 23.562 ml, pressure 1.5 MPa, dimensions 10 x 300 mm, flow rate 0.5 ml/min) filled packaged with agarose and dextran, the column was calibrated with potassium phosphate buffer 0.01 M, NaCl 0.14 M, pH 7.4. Injecting the concentrated sample (0.5ml) which was obtained from the precipitation of ammonium sulfate in the column gradually after being filtered by Millipore filter 0.22 µm to get rid of the impurities, measuring the separated peaks at a wavelength of 280 nm through the diagram on the computer screen, the recovered parts were collected as 3 ml/part using the fractions collector (type F9-R, ÄKTA Pure 25, GE Healthcare Life Sciences Co., Sweden). The injection was performed more than once, and measurement of enzymatic activity (unit/ml) and specific activity (unit/mg) for the separated peaks, about 40 ml was collected from gel filtration, then concentrated by freeze-drying to determine the purity of the enzyme by electrophoresis.

Electrophoresis. The purification of myrosinase was estimated by using a polyacrylamide gel electrophoresis with an absence of the denaturation substances sodium dodecyl sulfate (SDS), either the molecular weight was assayed with a presence of SDS-PAGE in a polyacryla-mide gel (10-12%) according to the method of (Laemmli *et al*., 1970) modified by (Garrfin *et al*., 1990). Marker proteins (broad band from 6.5-116 KDa) were used to determine myrosinase molecular weight by using (β-galactosidase, phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, lysoz- yme, and aprotinin).

Optimum pH For Myrosinase Activity and Stability. The optimum pH for activity was calculated as enzymatic activity (unit/ml), while the optimum pH for stability was examined as remaining activity (%) using different buffer solutions with a concentration of 0.1M, by using acetate buffer of pH ranges between 3-5, phosphate buffer of pH ranges between 6-8, Tris-HCl buffer of pH ranges between 9-11.

Optimum Temperature for Myrosinase Activity and Stability. Enzymatic activity (unit/ml) estimated for optimum temperatures ranged between (20-90)°C, where the optimum temperature for stability was calculated as remaining activity(%) in the same ranges of temperatures .

Effectiveness of Mineral Salts on Myrosinase Activity. Prepared the salty solutions at concentrations of 1 and 5 mM by dissolving them in distilled water. Salts including NaCl, KCl, $CaCl_2$, $MgCl_2$, $NiCl_2$, $FeCl_2$, $AlCl_3$, $MnSO_4$, $ZnSO_4$, $CuCl_2.2H_2O$, EDTA. Incubating 1 ml of the purified enzyme with these solutions for 60 min. at 37 °C. The enzyme activity was calculated as the remaining activity (%).

Kinetics of Myrosinase. Different concentrations of glucoraphanin were prepared at range (0.1-1%) to estimate Michaelis-Menten (Km) and maximum velocity (Vmax) for enzyme using Lineweaver - Burk reciprocal plot, Hanes - Woolf plot, Woolf - Augustinsson - Hofstee plot, Eadie - Scatchard plot.

RESULTS AND DISCUSSION

Enzyme Purification. Showing **Table 1** steps of myrosinase purification, observing from sedimentation step for broccoli myrosinase by ammonium sulfate at saturation ratio (20%-60%) the enzymatic activity increasing gradually from 20% until the sedimentation process end completely at 60%, the optimal precipitation ratio observed at salty concentrations between (30%-50%) saturation, and at 40% the enzymatic activity reached at the highest value 461.224 (u/ml), specific activity 301.453 (u/mg), purification fold 2.77, and yield about 31.32% recovery. Using different concentrations of ammonium sulfate were compatible with optimal precipitation of salt at a saturation range (40%-60%) saturation achieved by (Mahn *et al*., 2014), and with results of Cai *et al*. (2019) of using ammonium sulfate at 50-60% saturation to precipitation of myrosinase, as well as a saturation of ammonium sulfate 50% was optimal to purify broccoli myrosinase (Román *et al*., 2018; Mahn *et al*., 2020), also Cao *et al*. (2021) precipitate broccoli myrosinase at 50% of ammonium sulfate for improvement sulforaphane content. Ammonium sulfate is highly effective in precipitating proteins because to its strong salting out effect which allows for the selective precipitation of proteins based on their solubility, which decreases as the ionic strength of the solution increases (Duong-Ly & Gabelli, 2014). By reducing the solubility of the proteins, ammonium sulfate prevents denaturation and degradation, which is important for maintaining the enzyme activity (Wingfield, 2016). The results showed in the **Figure 1** represented the gel filtration chromatogram by using Superdex 200 Increase 10/300 for the freeze-dried extract that results from sedimentation step, the results

display appearing three peaks starting from fraction No.9 to fraction No.13, and when measuring the enzymatic activity for each peak was founding that only one peak having an enzymatic activity at fraction No.9-No.10 while there aren't any enzymatic activity at fraction No.11 and fraction No.12-No.13. Gel filtration technique was carried out for many times to collect about 40 ml from the active peak, determined the enzymatic activity which equal to 432.108 (u/ml) and the specific activity 583.929 (u/mg) with purification fold 5.36 and recovery 14.67%. The results of the specific activity were less than the results of Ye *et al*., (2022) which equal to 6951 (u/mg) when using Superdex 200 Increase 10/300 column in gel filtration chromatography to purify myrosinase from bacteria *Shewanella baltica* Myr-37. In contrast, the results of the specific activity were higher than the results that obtained by Huang *et al*., (2023) which equal to 267.00 (u/mg) for the purified myrosinase from marine bacteria *Pseudomonas oleovorans* when using gel filtration chromatography and with the same Superdex 200 Increase 10/300 column. One of the major benefits of gel filtration technique is that it operates under mild, non-denaturing conditions so that enzymes can be purified without losing their biological activity, which is crucial for functional studies and industrial applications where enzyme activity is necessary (Ó'Fágáin *et al*., 2016).

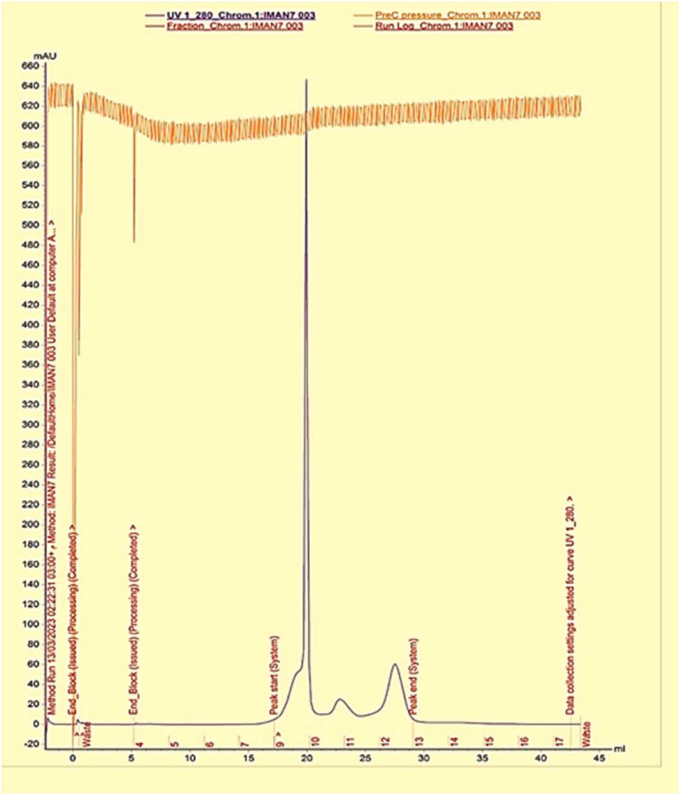

Figure 1. Gel filtration chromatogram for dialyzed solution from ammonium sulfate precipitation by using ÄKTA Pure 25 apparatus, Superdex 200 Increase 0/300 GL column (10 mm diameter, column size 23.562 ml, pressure 1.5 MPa, dimensions 10 x 300 mm, flow rate 0.5 ml/min), potassium phosphate buffer at pH 7.4.

Steps of purification	Volume (ml)	Enzymatic activity (u/ml)	Protein (mg/ml)	Specific activity (u/mg)	Total activity (unit)	Purification fold	Recovery %
Crude extract	300	392.652	3.61	108.767	117795.6	1	100

Precipitation of $(NH_4)_2SO_4$ (20-60)%	**80**	**461.224**	**1.53**	**301.453**	**36897.92**	**2.77**	**31.32**
Gel filtration Superdex 200	**40**	**432.108**	**0.74**	**583.929**	**17284.32**	**5.36**	**14.67**

Table 1. Purification steps of myrosinase from broccoli

Electrophoresis. Determined the purity of the myrosinase by electrophoresis technique in the absence of SDS (**Figure 2**) which refers to the polyacrylamide gel electrophoresis (native-PAGE) of purified myrosinase without SDS, noting there were appearing six protein bands in the crude extract, two protein bands from precipitated extract by ammonium sulfate (20-60%) after dialysis, and one clear band from gel filtration step which indicating to the high purity of enzyme. The results don't agree with the results of Mahn *et al*., (2014) on the purity of broccoli myrosinase by using polyacrylamide gel electrophoresis, which obtained two bands of protein, as well as Galádová *et al*., (2022) obtained two bands of protein from gel electrophoresis in the presence of SDS when purify myrosinase from *Lepidium sativum* seeds, while the results are compatible with the results of Ye *et al*., (2022) for obtaining one clear protein band that appearing in native-PAGE of gel filtration gel filtration step in the purification of myrosinase from bacteria *Shewanella baltica* Myr-37.

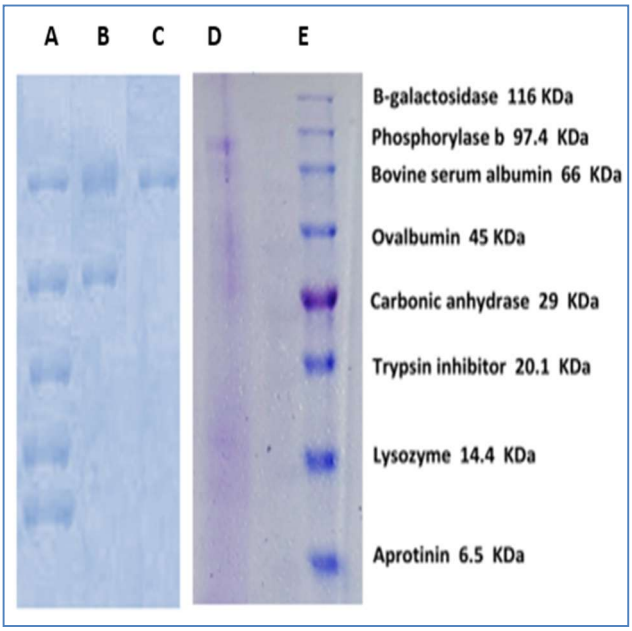

Figure 2. Determination of myrosinase purity using electrophoresis and molecular weight by SDS-PAGE where; (A): crude extract, (B): precipitated extract by ammonium sulfate (20-60%), lane (C): pure enzyme from gel filtration using Superdex 200 column, (D): pure myrosinase, (E): Marker proteins.

Polyacrylamide gel is easy to prepare and effective material compare to its cost, making it accessible for routine use in laboratories, also allows for the repeated use of gel, which is beneficial for multiple rounds of analysis or purification without affect the resolution (Gaytán *et al.,* 2013). Myrosinase molecular weight found equal to 75.85 KDa which is agreeing with the results of Okunade *et al*., (2015) of the molecular weight of the purified myrosinase from ripe seeds

that is equal to 71.7 KDa. Determining an enzyme's molecular weight can provide insights into its stability, particularly under various environmental conditions. Enzymes with higher molecular weights may exhibit greater stability, which is often essential for industrial applications where enzymes are exposed to extreme conditions (Ebrahimi *et al*., 2011). (SDS-PAGE) is a common method for determining enzyme molecular weight because it also provides information on the purity and homogeneity of enzyme preparations, which is essential for both research and industrial applications (Rachmania *et al*., 2017).

Optimum pH. The results shown in

Figure 3 display myrosinase enzymatic activity increasing gradually from acidity values reaching to high enzymatic activity at pH 5 which is equal to 270.571 (u/ml), then the enzymatic activity was decreasing regarding to neutral and basic values, enzymatic activity reaching to 152.176 (u/ml) at pH 11. The results agree with the results of (Oliviero *et al*., 2014; Román *et al*., 2018; Zhang *et al*., 2020) which indicate to the optimum pH for broccoli myrosinase activity which is equal to 5, while disagree with the results of the optimum pH 7 for bacterial myrosinase from *Citrobacter* (Albaser *et al*., 2016). as well as disagree with the results of (Jiménez *et al*., 2022; Curiqueo *et al*., 2022) about findings that pH 3 is the optimum pH for myrosinase activity from *Escherichia coli* and *Saccharomyces cerevisiae* respectively. The optimum pH of an enzyme is the pH at which the enzyme's active site is found in the most favorable conformation for substrate binding and catalysis, so variation in this optimum pH can lead to changes in the ionization state of amino acids in the active site, which in turn can alter the enzyme's structure and reduce its activity (Talley & Alexov, 2010). pH considers as one of the most important characteristics that affect the function and enzymes stability, and enzymes can work in non-native pH conditions, that enhancing their activity and stability especially in the industrial applications, so enzymes have adapted to work in extreme pH conditions by altering their amino acid composition to maintain stability and activity (Kumar *et al*., 2020).

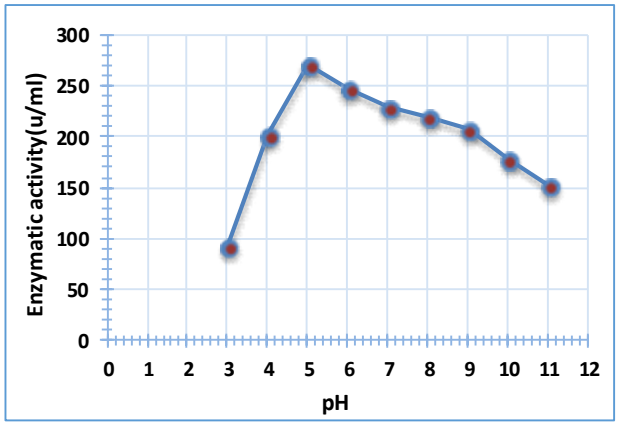

Figure 3. Optimum pH for myrosinase activity

While **(Figure 4)** indicates to the optimum pH for myrosinase stability ranging between (4-7), the remaining activity ranges between (94.15%-93.6%), where the enzyme loses most of its activity in extreme acidic and basic conditions. At very low acidity at pH 3 remaining activity is equal to 35.76%, and in the basic condition at pH 11, the remaining activity is equal to 40.67%. The results are compatible with the results of (Mahn *et al*., 2014; Oliviero *et al*., 2014) which show the stability of broccoli myrosinase at pH 4-8, and pH 5-7 respectively, whereas disagree with the results of Navarro-Rico *et al*., (2014) found the best temperature stability of broccoli myrosinase is under acidic conditions, especially at pH 4.5-5.0. The formation of additional hydrogen bonds and salt bridges within the enzyme's structure can significantly stabilize the enzyme across varying pH levels by maintaining the integrity of the active site and preventing denaturation (Sáez-Jiménez *et al*., 2015). Adjusting the solvent environment and ionic strength can help stabilize enzymes by minimizing unfavorable interactions between charged groups, which is critical for maintaining activity at extreme pH levels. (Idrees *et al*., 2016).

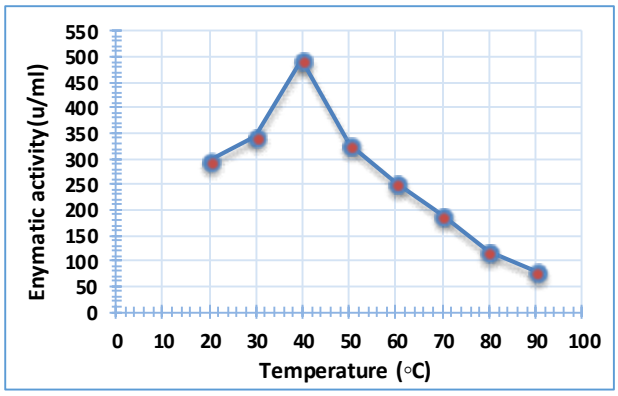

Figure 4. Optimum pH for myrosinase stability

Optimum Temperature. The optimum temperature for myrosinase activity increased gradually reaching to high enzymatic activity at a temperature of 40 °C, where found equal to 494.149 (u/ml), then the enzymatic activity decreased regarding a high temperature of 90 °C which is equal to 79.863 (u/ml) **(Figure 5)**. The results are agreeing with the results of (Mahn *et al*., 2014; Jin *et al*., 2014; Oliviero *et al*., 2014; Okunade *et al*., 2020) about the optimum temperature for broccoli myrosinase activity that equal to 40°C, and agree with the results of Wang *et al*., (2022) of the optimal activity at 40°C for novel myrosinase from *Rahnella inusitata*, while disagree with the results of Dosz & Jeffery (2013) about findings the optimum temperature for broccoli myrosinase activity equal to 66°C , as well as disagree with the results of Guo *et al*., (2014) which showing the optimum temperature for broccoli myrosinase activity equal to 25°C, also disagreement with the results of Pérez *et al*., (2014) for the optimum temperature which is equal to 57 °C for broccoli myrosinase activity. The role of entropy in enzyme function is significant, as temperature increases, the entropy of the enzyme and the surrounding environment also increases, which can enhance catalytic activity up to an optimum point, while excessive entropy can lead to structural instability and loss of enzyme function (Siddiqui, 2016).

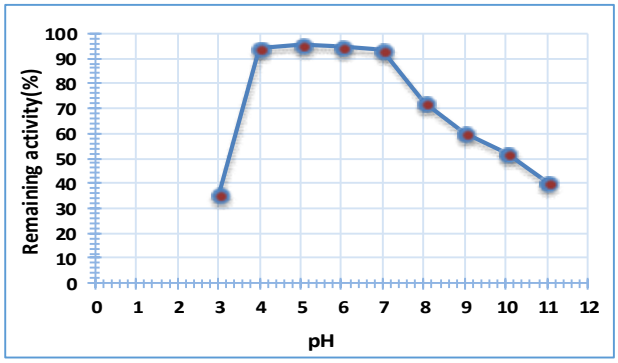

Figure 5. Optimum temperature for myrosinase activity

while observing from **Figure 6** myrosinase was kept most of its activity at temperature (20-50) °C, the remaining activity ranging (95.36%- 92.74%), then the enzyme lost most of its activity gradually regarding high temperature above 60°C, until the remaining activity reach to 18.64% at 90°C. The results are compatible with the results of (Jin *et al*., 2014; Román *et al*., 2018) about broccoli myrosinase temperature range of stability (25-60)°C, and agreeing with the results of Curiqueo *et al*., (2022) about the temperature range of stability (20-50)°C for myrosinase from *E. coli*, and range (20-60)°C for myrosinase from *Saccharomyces cerevisiae*, also with the results of Jiménez *et al*., (2022) about recombinant

myrosinase from *E. coli* temperature range of stability (30-50)°C, whereas disagree with the results of (Pérez *et al*., 2014; Oliviero *et al*., 2014) about range (40-70)°C, as well as the results of Guo *et al*., (2014) for range (25-30)°C of broccoli myrosinase .

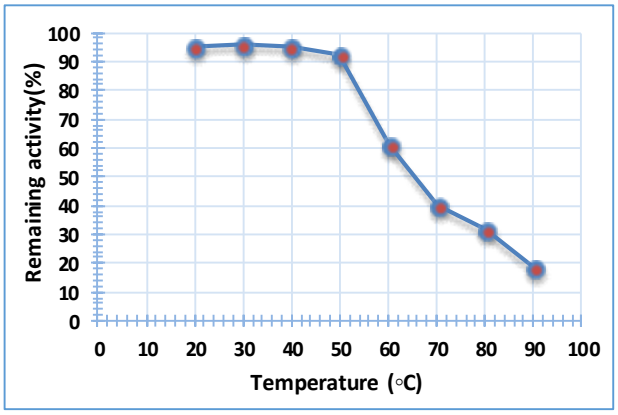

Figure 6. Optimum temperature for myrosinase stability

Molecular mechanisms such as changes in amino acid composition, enhanced hydrogen bonding, and the formation of salty bridges play crucial roles in the adaptation of enzymes to different temperatures, these adaptations help maintain the enzyme's structure and function under varying thermal conditions (Pinney *et al*., 2021).

An Effectiveness of Mineral Salts. The presence of salts changing the case of a reaction by stimulate or inhibition enzyme activity through changing the equilibrium state of reaction. Myrosinase activity increased when using manganese sulfate at concentrations of (1 mM, and 5 mM), where the remaining activity reaching (103.96% and 105.75%) respectively, while the myrosinase kept most of its activity regarding to magnesium chloride the remaining activity equal to (88.47%, and 85.32%) respectively, potassium chloride the remaining activity equal to (86.76%, and 83.54%) respectively, and sodium chloride the remaining activity equal to (85.12%, and 84.26%) respectively at the same concentrations. The enzyme began to loosing half of its activity regarding to zinc sulfate and copper chloride, then gradually the enzyme lost most of its activity regarding to nickel chloride and EDTA salt, and in the other hand the ferrous chloride and aluminum chloride having an inhibition role for myrosinase activity where the remaining activity equal to (13.70%, and 11.05%) respectively for ferrous chloride, and the remaining activity equal to (12.63%, and 10.47%) respectively for aluminum chloride.

Kinetics of the Enzyme. The results in **Figure 7** explain the kinetics of myrosinase regarding to glucoraphanin as substrate using plots of Lineweaver-Burk, Hanes-Woolf, Woolf-Augustinsson-Hofstee, and Eadie-Scatchard. The kinetics of myrosinase represented by Mechalis-Menton constant (Km) which is equal to 0.37 (mM), and maximum velocity (Vmax) which is equal to 606.25 (µmol/ml/min.). Most researchers using Michaelis-Menten model always to describe enzyme kinetics, according to this equation, the low value of Km indicates to a higher affinity between the enzyme and substrate concentration, so as substrate concentration increases, the velocity of reaction increases too until reaches to its maximum velocity (Vmax) where the enzyme is saturated with substrate and working at all capacity (Pinto *et al*., 2015).

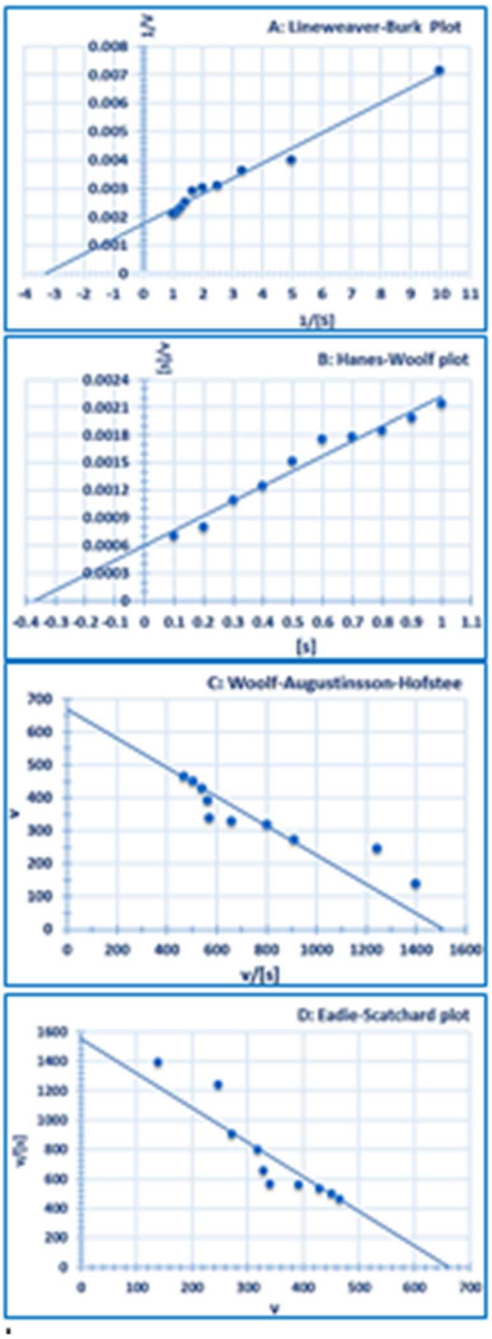

Figure 7. Plots of myrosinase kinitics using glucoraphanin as substrate at concentrations (0.1-1)% where; (A): Lineweaver - Burk reciprocal plot ; (B): Hanes - Woolf plot ; (C): Woolf-Augustinsson-Hofstee plot ; (D): Eadie - Scatchard plot

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