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RESEARCH ARTICLE

Purification and Characterization of Alliinase from Iraqi Garlic (*Allium sativum*)

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Abstract

The study including purification and characterization of alliinase (EC: 4.4.1.4, Cys sulfoxide lyase, alliin lyase) from Iraqi garlic. Enzyme extraction with sodium phosphate buffer, pH 6.5, the extract was precipitated by addition of an ammonium sulfate with saturation (30-70)%. Gel filtration chromatography was done to purification enzyme by ÄKTA Pure 25 apparatus using superdex 200 column, three peaks were obtained with a good enzyme activity and an enzyme purity were identified by the absence of denaturation substances for protein SDS. Characteristics of pure enzyme showed that optimum pH for an activity was 6 and optimum pH for the stability was between 6-8, while optimum temperature for an activity and the stability were 35°C, 30-45°C respectively. Effect of ions Na, K, Mg+2, Fe+2, Zn+2, and Mn+2 was found stimulate enzymatic reaction while Ca+2, Ni+2, Cu+2 and Hg+2 inhibit the enzymatic reaction. Standard alliin used to determine a value of Km and Vmax and found to be 0.35M and 121.5 µmol/ml/min. respectively. The molecular weight of pure enzyme by electrophoresis technique with the presence of SDS was equal to 49 KDa.

Keywords: *Alliinase, Alliin lyase, Iraqi garlic, Purification, ÄKTA Pure 25.*

Introduction

Alliinase (EC: 4.4.1.4, Cys sulfoxide lyase, alliin lyase, C-S lyase) is a glycoprotein with ideal di molecules belongs to the wrapped type I from enzymes that dependent on coenzyme pyridoxal-5-phosphate (PLP) from family aminotransferase which plays the role of a catalyst in stimulating the transformation of the non protein amino acid (+) S-allyl-L-cysteine sulphoxide to allicin (diallyl thiosulfinate), enzyme subunits consists of a series of 448 amino acid, there are 10 chains of amino acid cysteine (Cys) per monomer of the enzyme, eight of them in the form of four disulfide bridges, two forms are free thiol groups [1].

The first who described alliinase in garlic was the scientists Stoll and Sebeck in 1947, which responsible for editing the flavor compounds in Allium genus, the effective garlic compounds were studied well by many researchers [2-4]. Alliinase was founding in many plants of Allium genus like garlic (*Allium sativum*), onions (*Allium cepa*) and leek (*Allium porrum*) and others, alliinase stimulates transformation alliin to allicin, pyruvate and ammonia as byproducts and allicin is considered most important sulfuric compound which due to the beneficial properties of garlic, and because the enzyme inhibiting in gastric acid and loses its effectiveness, at present time many of packaging operations for enzyme to protect it from these conditions in capsules or tablets or powder these forms are available commercially in the name of food garlic supplements or enzymatic supplements [5].

The enzyme was isolated and purified from garlic by different researchers [6, 12]. Alliin is a non-protein amino acid derivative of the amino acid cysteine, owns four stereoisomers, however only one isomer found in garlic [13]. Alliin is a stable raw material and if cut or chop or smashing garlic cloves, the membrane rupture and turn alliin to allicin by enzyme alliinase, the process of turning into allicin compound very fast within seconds, studies have indicated the existence of alliin and

alliinase in compartments or separate parts in garlic [14]. Allicin treats many diseases, bacterial and viral infections such as acne, animal bites, arthritis, asthma, bedsores, blood pressure, cholesterol, anemia, colds, flu, cough, diabetes, diarrhea, arthritis, ear and eye inflammation, eczema, food poisoning, toothache and warts [15].

The unique flavor and health-promoting properties in garlic is generally attributed to the rich content of sulfur-containing compounds such as alliin and γ-glutamylcysteine and their derivatives, when smash or crush or cut up cloves of fresh garlic lead to the free enzyme alliinase from gaps and quickly convert alliin to allicin, which is a very unstable compound and turns immediately to a huge number of products containing sulfur and soluble in the oil including compounds diallyl disulfide (DADS), diallyl sulfide (DAS), diallyl trisulfide (DATS), diallyl tetrasulfide [16]. Such these studies on alliinase didn't existent in Iraq or the Arab Homeland that led us to carry out this search for studying characters and kinetics of enzyme.

Materials and Methods

Materials

Fresh Iraqi garlic was purchased from local markets in Basra, Iraq, then cleaning the plants, remove the outer parts, peels, washed with distilled water, and kept at the refrigerator temperature of 4°C in polyethylene bags until its use.

Chemicals

Alliin and allicin were bought from Santa Cruz Biotech. Co., USA, PLP from SDI, Samarra, Iraq, Protein Molecular Weight Marker from Bioneer Co., South Korea, and solvents from HAYMAN Co., UK and metal ions from BHD Co., England.

Apparatuses

Apparatus protein purification ÄKTA Pure 25, GE Healthcare Life Sciences Co., Sweden and Slab electrophoresis, Biocom. Direct Co., UK.

Enzyme Extraction

Peeled and chilled of garlic cloves (100 g) were crushed in a plastic mortar and leave for 30 minutes, then added a buffer sodium phosphate 20 mM, pH 6.5 at a ratio 0f 1:2 (w/v) contains EDTA 5 mM, NaCl 5%, PLP 20 µM and glycerol 10% and also left for 30 minutes, then the mixture was filtered by layers of cheesecloth, followed by centrifugation at 10000 rpm for 30 min. on temperature 4°C.

Enzyme concentration by adding ammonium sulfate to the crude extract gradually with saturation ranged 30-70%, stirring continuously for 4 hours, then the precipitate was collected and dissolved in buffer consists of sucrose 15% (w/v) and NaCl 1% [17] and dialyzed for 24 h at 4°C, the product was lyophilized and kept for studying enzyme characterization later.

Enzyme Assay

Enzyme activity was estimated spectrally by measuring pyruvate concentration (Standard curves was prepared by using sodium pyruvate) according to the method of [18], the reaction mixture consists of 0.5 ml enzymatic extract, 0.5 ml standard alliin, 0.5 ml 2,4dinitr-phenylhydrazine (0.0125% of DNPH in 2N of HCl), then put in a water bath at 37°C for 15 min. after the incubation period adding 2.5 ml of 0.6N NaOH, Pyruvate was measured using a UV-Vis spectrophotometer (Apel 303 UV, England) at 420 nm. The final concentration of pyruvate was measured as micromole/min.

Protein Assay

A concentration of protein was measured by using bovine serum albumin (BSA) as standard [19].

Gel Filtration

The process of gel filtration was done by using ÄKTA Pure 25 apparatus 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 of 0.5 ml/min) filled packaged with agarose and dextran and the column was calibrated with sodium phosphate buffer (5 liters) with pH 6.5. Injecting the concentrated sample (0.5ml) which was obtained from the precipitation of ammonium sulfate in the column gradually after filtered by Millipore filter 0.22 µm to get rid of the impurities and follow-up to the separated peaks along the wavelength of 280 nm through the diagram that appears on the computer screen, the recovered parts were collected as 2 ml/ part by the fractions collector (type F9-R, ÄKTA Pure 25, GE Healthcare Life Sciences Co., Sweden).The injection was done more than once and peaks collected with a size of 45 ml, then measurement of enzymatic activity (unit/ml) and specific activity (unit/mg) to the separated peaks.

Electrophoresis

The purification of an enzyme 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 polyacrylamide gel (10-12%) according to the method of [20] modified by [21]. Marker proteins (broadband from 6.5-116 kDa) were used to determine alliinase molecular weight by using ß-galactosidase, phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, lysozyme, and aprotinin.

Optimum pH for an Enzyme 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 an Enzyme Activity and Stability

Enzyme activity (unit/ml) estimated for optimum temperatures ranged between (20-80)°C, where the optimum temperature for stability was calculated as remaining activity(%) in the same ranges of temperatures.

An Effectiveness of Mineral Salts on the Enzyme Activity

Preparation of ionic solutions with two concentration(1,5 mM) including sodium chloride, potassium chloride, magnesium chloride, ferrous chloride, copper chloride, mercury chloride, calcium chloride, nickel chloride, manganese sulfate and zinc sulfate, 1ml of the enzyme was incubated with these solutions for 30 min. at 35 °C. The enzyme activity was calculated as the remaining activity (%).Kinetics of Enzyme. Different concentrations of alliin were prepared (0.1-1%) to estimate Michaelis-Menten (Km) and maximum velocity (Vmax) for enzyme using Line weaver - Burk reciprocal plot, Hanes - Woolf plot, Woolf - Augustinsson - Hofstee plot, Eadie-Scat chard plot.

Results and Discussion

Enzyme Purification

Showing Table 1 steps of enzyme purification, observing there was a good specific enzyme activity127.058 (unit/mg protein) by using sodium phosphate buffer, where found that the addition of glycerol 10% maintained the stability of the enzyme completely, it was noted that the enzyme is kept its effectiveness at a temperature of 10 °C after a month, and adding sodium chloride to the crude extract prevent particles of extract from gathering and keep them in a high degree of homogeneity for a long time, while the addition of PLP stimulated the enzymatic reaction especially when the coenzyme in a high degree of purity [17-22].

Ammonium sulfate (30-70%) was used extensively in precipitation processes for their availability, high solubility, and low cost compared with other organic solvents. Sulfates works on precipitation enzymes by equilibrium the charges on the protein surface and disrupting the surrounding water layer around it, so can pull the water molecules which reduces protein solubility and then precipitate it [23,24], the specific activity from this step was 165.517 (unit/mg protein).

Table 1: Purification of an alliinase from the Iraqi garlic

Steps of purification	Protein (mg/ml)	Activity (unit/ml)	Specific activity (unit/mg)	Total activity (unit)	Purification	Recovery%
Crude extract	1.36	172.8	127.058	51840	1	100
Precipitation of $(NH_4)_2SO_4$ (30-70)%	1.16	192	165.517	19200	1.30	37.04
Gel filtration Superdex 200	0.61	155.4	254.754	6993	2.01	13.48

The results showed in Figure 1 explain the presence of three peaks of enzyme extracted by gel filtration technique (ÄKTA Pure 25, superdex 200), the second peak was giving a high enzymatic activity reached to 139.2 (unit/ml), while the first and third peaks were giving low activity, so the second peak was chosen to making electrophoresis for enzyme. A presence of more than one peak for an enzyme activity may belong to the isomers of alliinase, the specific activity which obtained was 254.754 (unit/mg protein).Two separated peaks for the purified enzyme in the garlic was found by using gel filtration technology with Sephadex G-200 column [22], as well as [25] was able to obtain three peaks of the pure

enzyme using fast protein liquid chromatography (FPLC), while [9] purified alliinase by using a superdex 200 column in a chromatography of gel filtration and have got two peaks, the highest peak was attributed to alliinase which having little subunits or may refer to the complex forming between alliinase and lectin, while the high activity peak may be returning to the pure alliinase that having two subunits. The variety of results may attribute to the different devices used in the purification, type, a number of columns were used, temperature, pressure, and type of buffers.

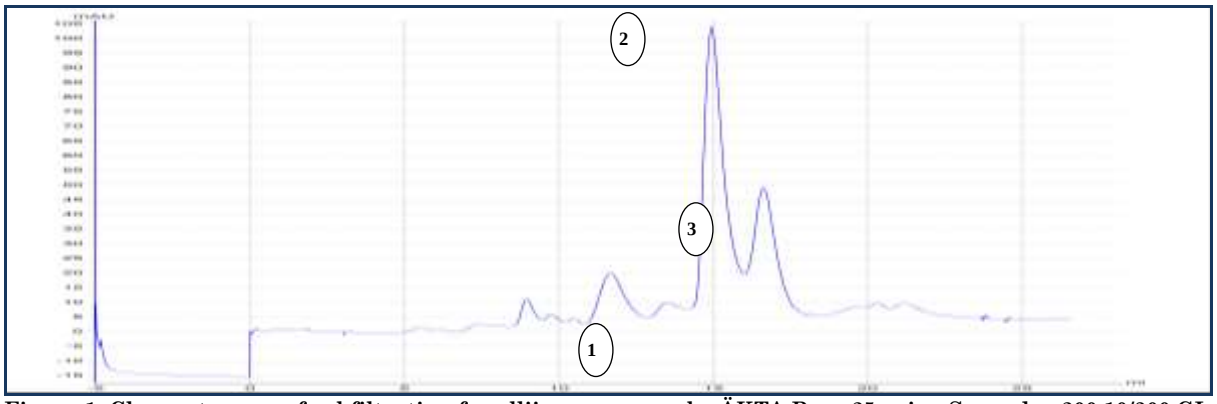

Figure 1: Chromatogram of gel filtration for alliinase enzyme by ÄKTA Pure 25 using Superdex 200 10/300 GL in the flow rate of 0.5 ml/min

Electrophoresis

Determined the purity of the enzyme by electrophoresis technique in the absence of denaturation materials of protein SDS (Figure 2) explained the existence of 6 protein bands in the crude extracted in polyacrylamide gel and 3 protein bands in the saturated extract with ammonium sulfate (30-70%) after dialysis, while one band was found from gel filtration step. The appearing of one band indicates arrival the enzyme to an advanced stage of purification and can be considered as high purity enzyme, as necessary to get pure enzyme at a degree of homogeneity to can studying the truth properties of this enzyme[26].The molecular weight of alliinase determined by SDS-PAGE through drawing the inverse relationship between relative motion of standard proteins and logarithm the molecular weight of these proteins and by measuring the relative motion of alliinase in the gel, its molecular weight was found that equals 49 KDa , the studies show similar values for alliinase molecular weight in garlic, [27] found alliinase molecular weight was 42 KDa and the molecular weight of enzyme in Chinese garlic equals 48±2 KDa [28], while determined the molecular weight of enzyme to be 53 KDa [6,9].Indicated both of [27] that the enzyme purification with more than technique produced three major peaks but it disappeared when they stained and only one band was existent by using electrophoresis technique, that evidenced to the presence of only one type of enzyme subunits in the garlic and existence of more than one band in the different techniques as a result of their interaction with the materials of the columns and the buffer solutions.

Figure 2: Determination of alliinase purity and molecular weight by electrophoresis without SDS and SDS-PAGE using (10 -12)% polyacrylamide gel, A: crude; B: dialysis extract; C: pure enzyme after gel filtration step; D: standard proteins marker (broadband)

Optimum PH

An enzyme activity increased gradually from acidic pH numbers until higher activity of the enzyme at optimum pH 6 with 159.84 (unit/ml), and then decreased gradually in the alkaline pH numbers as shown in the Figure 3A, the result of optimum pH agrees with [29] and nearby to the results of [17,30,31]. The enzymes are often influenced by the effectiveness of the hydrogen-ion concentration in the interaction, so enzyme activity was determined by the limited extent of the acidity, which depends on the ionic strength, temperature, nature of the buffers, a concentration of stimulants and inhibitors and concentration of the enzyme and substrate [26].

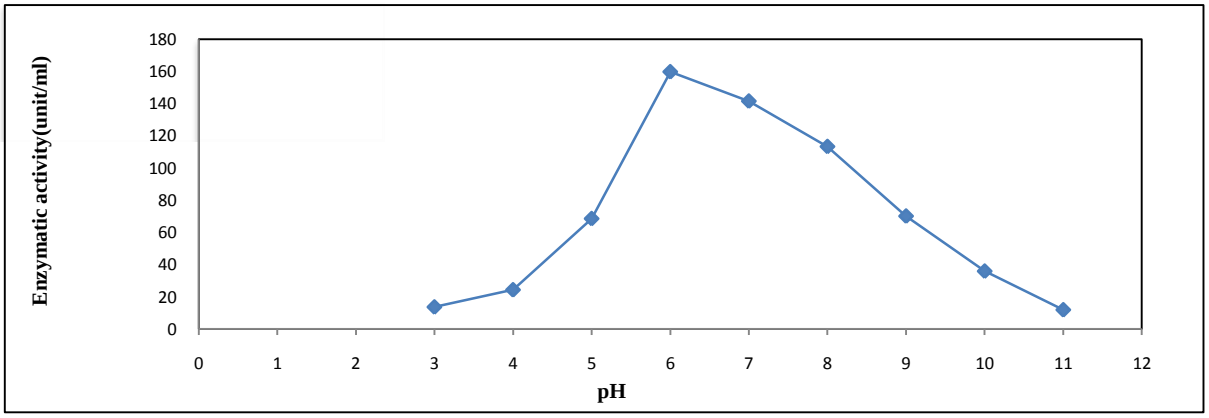

Figure 3A: Optimum pH for alliinase activity

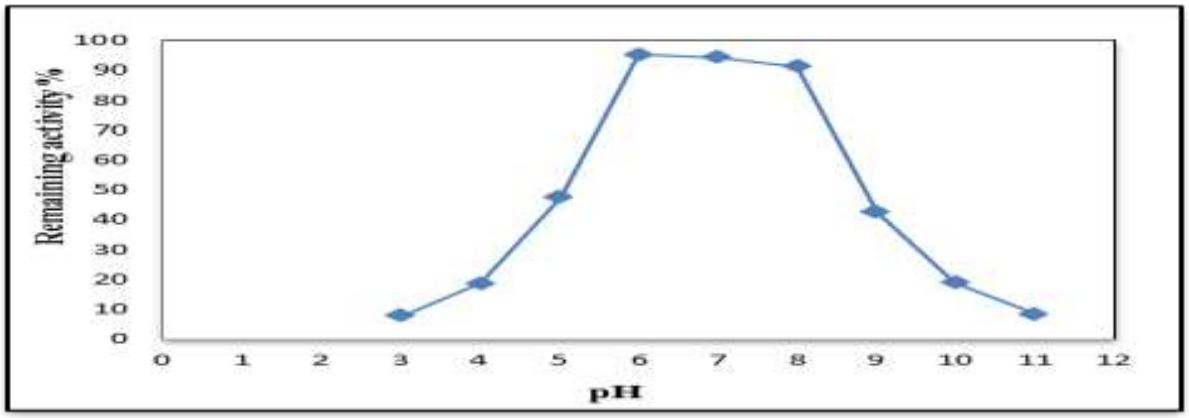

Figure 3B: Optimum pH for alliinase stability

Either the optimum pH for enzyme stability was at a range between 6-8 and the enzyme kept with 95.13% of its activity at that values (Figure 3B) while the enzyme began to losing its activity at the extreme acidic and alkaline values of pH, this value was matching with the result of [6].

Optimum Temperature

An alliinase activity reached to its maximum value when rising the temperatures gradually. The optimum temperature for an enzyme activity was at 35°C where the activity reached 137.4 (unit/ml), then the activity reduction to 54.6 (unit/ml) at a temperature of 80°C (Figure 4A), the result deals with the results of optimum temperature for enzyme activity who reaches to it [11,29]. The reason for increasing speed of enzymatic reaction by rising temperature to a limited extent, lead to an increase in the kinetic energy for enzyme molecules and substrate, leading to increased clashes between them and thus increase the speed of interaction, but the height of temperature more than this limitation leads to quickly lower in enzymatic activity due to the impact of heat on the secondary and tertiary structure of enzyme, which leads to denaturation of enzyme and decreases the activity [26]. while observing from Figure 4B the optimum temperature for enzyme stability which ranges between (30-45)°C, the remaining activity ranged between 96.82%-97.85% which shows the stability of the enzyme within this range of temperatures, and then enzyme activity began to decline at high temperature where the remaining activity reached 23.82% at a temperature of 70°C. The enzyme was found stable at a temperature less than 50°C [32].

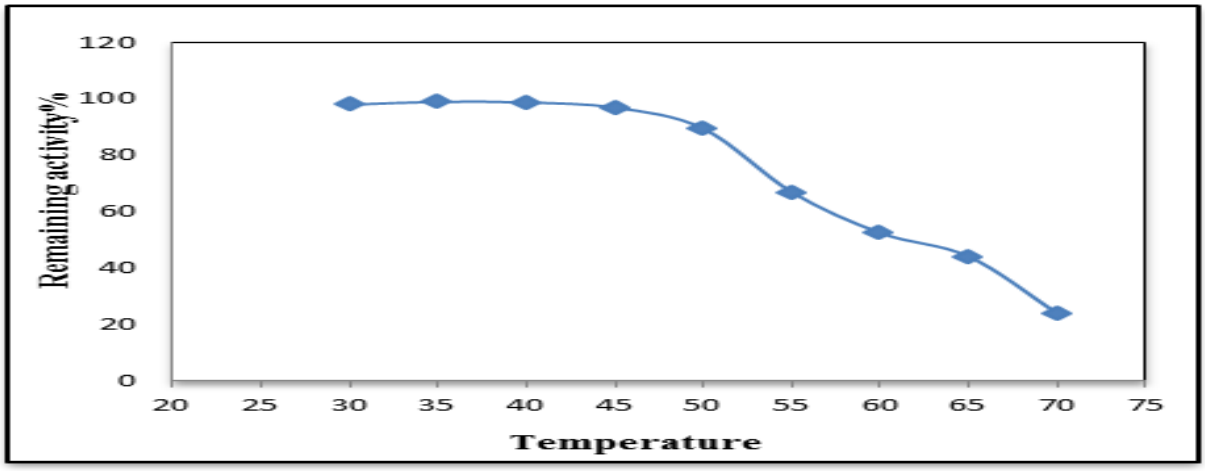

Figure 4A:

The optimum temperature for alliinase stability

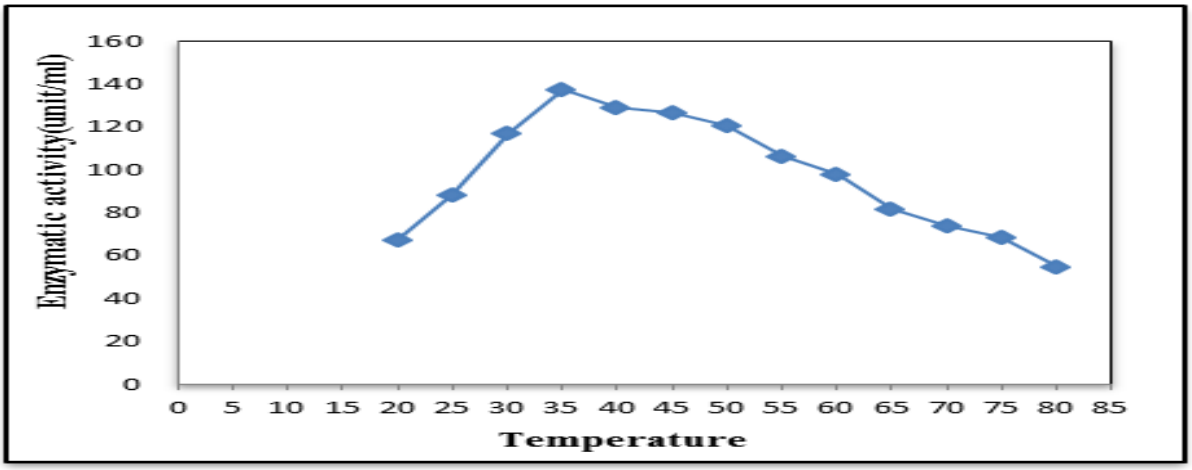

Figure 4B: The optimum temperature for alliinase activity

The lowering activity by increasing temperature due to the sensitivity of enzymes towards the temperature which affects its stability that depends on several factors like sulfur linkage, pH, ionic strength, and a presence of coenzymes [33].

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. An influence of different mineral salts were observing on alliinase activity, the most effective salts were magnesium chloride which showed an increasing of remaining activity to 105.02%, while the remaining activity with sodium and potassium chloride equal to 92.82% and 91.75% respectively, either when incubation iron chloride with alliinase led to slightly lowering in activity, the remaining activity reached to 88.94%, then gradually decrease enzyme activity with zinc and manganese sulphate which reached to 72.74% and 68.38% respectively, but addition of nickel, calcium, copper and mercury salts inhibition enzyme activity in a range between (14.64-37.04)% . Explained [29] the addition of bivalent ions Zn^{+2}, Ni^{+2}, Ca^{+2}, Mg^{+2}, Co^{+2}, and Mn^{+2} didn't effect on enzyme activity, while [34] was found the addition of Cu^{+2} and Hg^{+2} inhibition the alliinase activity.

Kinetics of the Enzyme

A first whom calculated Michaelis-Menton constant (K_m) and the maximum velocity (V_{max}) of alliinase reaction in garlic was [35] using different types of substrate can the enzyme working on it, the speed of reaction increase depends on the increase of a substrate concentration until the speed become fixed and not increase even if a substrate concentration increasing because the enzyme was saturated of substrate and velocity of reaction be limited [26]. The values of K_m and V_{max} were calculated from four types of plots with using alliin as a substrate for the enzyme (Figure 5), the average of K_m was 0.35M and V_{max} 121.5 µmol/ml/min. Michaelis-Menton constant estimated by [22] in the garlic using S-methyl-L-cysteine sulfoxide, S-ethyl-L-cysteine sulfoxide, S-propyl-L-cysteine sulfoxide and S-butyl-L-cysteine sulfoxide as substrates, as

well as many researchers, could assay the values of K_m and V_{max} in garlic, onion, leek and other plants or microorganisms, so there is no ideal value of this kinetics due to the differences in the substrate, pH, temperature and other conditions of experiments.

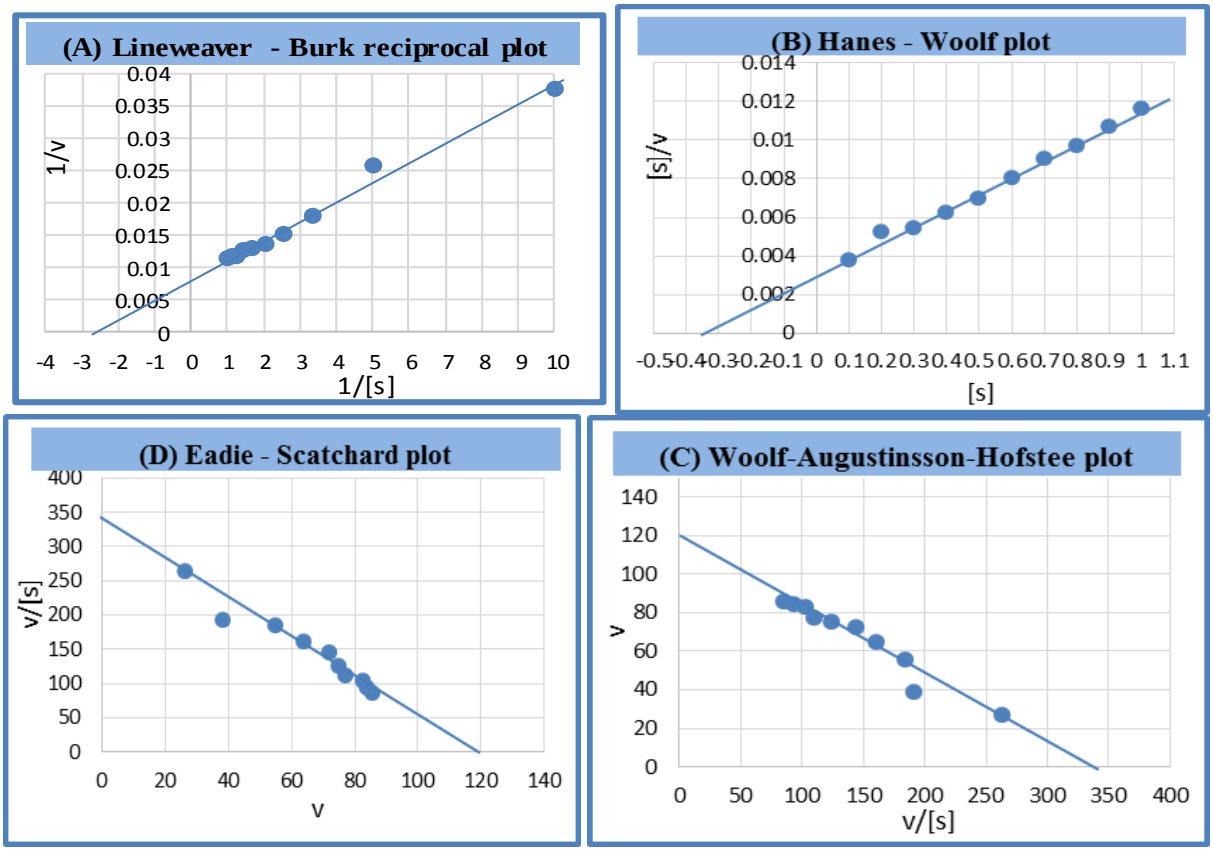

Figure 5: Plots of alliinase kinetics using alliin (0.1-1)% where (A) Line weaver - Burk reciprocal plot ; (B) Hanes - Woolf plot ; (C) Woolf-Augustinsson-Hofstee plot ; (D) Eadie – Scat chard plot

Conclusion

In this study, we found that Iraqi garlic was a good source for alliinase extraction and purification with a high enzymatic activity where there was not any research about garlic enzyme already in Iraq or Arab homeland. Alliinase has a very well stability at a good range of pH and temperature so it can be used in the pharmaceutical industries as a therapeutic enzyme in the future for its high activity, stability and high content of sulfur compounds.

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