Dhia Falih Al-Fekaiki et al /J. Pharm. Sci. & Res. Vol. 11(5), 2019, 1838-1841

ISSN:0975-1459
Journal of Pharmaceutical Sciences and Research
www.jpsr.pharmainfo.in

Preparation of an Amino Acid Alliin from the Iraqi Garlic and using it as a Substrate for Alliinase

***Dhia Falih Al-Fekaiki, **Iman Hadi Al-Taai**
**Palm Research Center, University of Basra, Basra, Iraq,*
***Branch of Basic Medical Sciences, College of Nursing, University of Basra, Basra, Iraq,*

Abstract

Alliin (S-allyl-L-cysteine sulphoxide) has been prepared from Iraqi garlic (*Allium sativum*) in various ways including microwave, MCW (12, 24, 48 h) and ultrasonic methods. Microwave method was given higher efficiency to prepare alliin compared with the other methods. The obtaining results showed identical of peaks for both standard and prepared alliin which analyzed by HPLC at retention time 4.167 min. on wavelength 240 nm. The concentration of prepared alliin 0.55 mg/ml was used in purification of alliinase with sodium potassium buffer of pH 6.5, then precipitation with ammonium sulfate at saturation of (30-70) %, finally collect the dissolving precipitating proteins, dialysis and freeze drying. The thermal stability of prepared alliin was examined with $MgCl_2$ and $FeCl_2$ at (20, 40, and 60) °C. The remaining activity% increasing gradually 73.75%, 100.53%, and 116.97% respectively by increasing temperature with $MgCl_2$, while decreasing with $FeCl_2$ in an aqueous solutions at the same temperature.

Keywords: Alliin; Alliinase; Freeze drying; HPLC; Iraqi garlic

INTRODUCTION

From the ancient ages, garlic *(Allium satiuum*) has been used widely not only as a herbal plant but also as a medicine, in ancient civilizations at 3000 B.C., the Babylonian, Egyptian, Greek, Indian, Roman, Viking, and Chinese, were used garlic for treatment of heart diseases, ulcers, headache, bites, wounds, and tumors [1-9].Garlic enzyme, alliinase (alliin alkyl-sulfenate-lyase EC 4.4.1.4) enzymatically formed allicin by the action of the garlic abundant sulfur compound, alliin (S-allyl-L-cysteine sulfoxide), allicin formation is rapidly happened when crushed or chewed the raw garlic due to its completely formed through 6 seconds [10,11]. The garlic potency is belong to the organosulphur compounds, especially cysteine sulfoxides and thiosulfinates, whereby alliin is derived from dipeptide c-glutamyl-S-allylcysteine which collects in high levels in the garlic [2, 12, 13]. There are several methods for alliin preparation, such as synthesized of alliin from S-allyl-L-cysteine, L-cysteine, ethanol, sodium hydroxide and allyl bromide at room temperature, then the product was recrystallized [14]. Alliin was synthesis by using tissue cultures of the callus tissue from serine and allylthiol [15]. The alliin was also extracted from dry garlic powder under inhibition condition of alliinase at room temperature using methanol–water (80:20, v/v) and formic acid [16, 17]. Extraction of alliin by chemical synthesis and extracted with deionized water from the garlic after the enzyme inhibition, then using HPLC for analysis the prepared alliin [18]. Also .Alliin was extracted from garlic laboratorial by the thermal treatment and the chemical components were analyzed by HPLC and LC-MS [19]. As well as a two-step process including aqueous two phase extraction (ATPE) with chromatography were developed for alliin extraction and purification from garlic powder [20]. The aim of this study was to preparation of alliin in simple method without any chemicals just water and in low cost with high yield can be used for the commercial production of alliin and in the pharmaceutical industries.

MATERIALS AND METHODS

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

Chemicals. Standard alliin was bought from Santa Cruz Biotech.Co., USA, pyridoxal 5-phosphate (PLP) from SDI, Samarra, Iraq and solvents from HAYMAN Co.

Apparatuses. High Performance Liquid Chromatography (HPLC), Shimadzu Co., Kyoto, Japan; UV-Vis spectrophotometer, Apel 303 U, England.

Preparation of substrate (Alliin)

1- Microwave method

Alliin was prepared by weighting 300 g of fresh Iraqi garlic which already peeled and cooled, put in microwave for 5 min. to stopping the activity of aliinase, then crashed well in a plastic mortar until getting a paste. Extract the paste with 1000 ml of distilled water and filtered by cheesecloth, then centrifuged at 5000 rpm for 10 min. the precipitate was discarded and collect 800 ml of the filtrate which was concentrated by freeze-drying until obtaining a powder (alliin) and finally the prepared alliin was diagnosed and estimated its concentration by HPLC from standard alliin.

2- The MCW method

Alliin has been prepared in accordance with the method described by [21] and mentioned by [22] with a little of modification, 50 ml of MCW solution (methanol, chloroform, Water) was prepared in the percent of 12:5:3 and used 40 g of garlic was put in this solution for 12 , 24 and 48 h by using 3 different samples of garlic, after the expiration of the installed time 4.5 ml of chloroform and 5.5 ml of distilled water were added to every 10 ml of the MCW solution and were left at a stable position in a separation funnel (250 ml) until obtaining a clear separation of an organic and aquatic layer , then the aqueous solution was left to evaporate the remaining solvent at the laboratory temperature, the final output was reduced to 100 ml with distilled water and stored at the temperature of 4°C.

3- Ultrasonic method

Alliin was prepared according to the method described by [22] with a little modulation by weighing 30 g of pre-chilled garlic cloves and flooded in 100 ml methanol for 30 minutes, then the mixture was put in the ultrasonic bath for an hour at temperature ranging 30-50 °C, followed by centrifuging for the extracted at 6000 rpm for 30 minutes, the precipitate was discarded and the filtrate was kept at the refrigerator temperature as a natural substrate.

HPLC. Prepared and standard alliin were filtered by millipore filter 0.22 µm and injected 1 µl of the extract in HPLC. The HPLC conditions were as following: an analytical column C18 (250 x 4.6 mm i.d, 5 um); column temperature 40 °C; UV detecting at 240 nm; column was equilibrated with distilled water and methanol (for HPLC) at ratio 50:50; flow rate 0.5 ml/min., run time 20 min.; chart speed 2 mm min-1.

Standard curve of alliin. 1 mg of standard alliin was dissolved in 10 ml of distilled water and making three concentrations (0.1, 0.5 and 1) mg/ml, then injected 1 µl from each concentration with three replicates in HPLC apparatus using a column C18 as a stationary phase with methanol and water (50:50) as a liquid phase. Concentration of prepared alliin was measured from standard alliin curve by drawing the relationship between the standard alliin concentration (mg/ml) and the area under the curve (%) as shown in the figure (1).

Extraction. Weighing 150 g of fresh Iraqi garlic were smashed in a plastic mortar and left for 30 min., then added a sodium potassium phosphate buffer 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% [23], then the mixture was filtered by some layers of cheesecloth, followed by centrifugation at 10000 rpm for 30 min. at temperature 4°C. Ammonium sulfate was added to the crude extract gradually with saturation of 30-70%, stirring continuously for 4 h, then collected the precipitate and dissolved in buffer contains sucrose 15% (w/v) and NaCl 1% [22]. Dialyzed for 24 h at 4°C and finally the product was freeze drying and kept at 4°C.

Enzyme assay. Estimated the enzyme activity spectrally by measuring the concentration of pyruvate (sodium pyruvate used as standard) according to the method of [24], the reaction mixture consists of 0.5 ml enzymatic extract, 0.5 ml standard alliin, 0.5 ml 2,4dinitr- phenyl hydrazine (0.0125% of DNPH in 2N of HCl), put in a water bath at 37°C for 15 min. after the incubation period added 2.5 ml of 0.6N NaOH. A UV-Vis spectrophotometer at 420 nm was used to measuring the pyruvate concentration.

Protein assay. Bovine serum albumin (BSA) was used as a standard to measuring the concentration of protein according to method of [25].

Determination the thermal stability of alliin. The thermal stability of alliin was examined at different temperature (20, 40 and 60) °C. Alliin was prepared with concentrate of 0.5 mg/ml, incubated with $MgCl_2$ (0.001M) and $FeCl_2$ (0.001M) in a water bath at the temperature that mentioned above for 1h. The enzymatic activity has been estimated depended on the remaining activity%.

RESULTS AND DISCUSSION

Preparation of alliin. Explain the figure 1 the standard curve of prepared alliin that its concentration equal to o.55 (mg/ml) from the equation under area (%) which means high yield with a low cost by using distilled water only for extraction alliin from garlic without any chemicals which may because toxicity. [26] was diagnosed and measured alliin by HPLC with UV at wavelength 210 nm in different types of the garlic (Iraqi, Iranian, Lebanese, French, and Chinese) by using different solvents (methanol, ethyl acetate, and water) and found that the aqueous extract of Iraqi garlic has the highest level and concentrations of alliin compared with the other types of garlic (17.4 ppm, 0.9%) which agree with our findings about containing Iraqi garlic a high concentration of alliin, while the Chinese garlic has the lowest level and concentrations of alliin (4.3 ppm, 0.22%). The difference of alliin amount and concentration belongs to the change of the environmental and normal conditions for garlic's growth and the content of sulfur in the plant [27]. Sulfur is also an important for improvement the quality of sulfur compounds like alliin and other sulphurous derivatives compounds. The content of alliin increased by increasing the level of sulfur in plant [16].

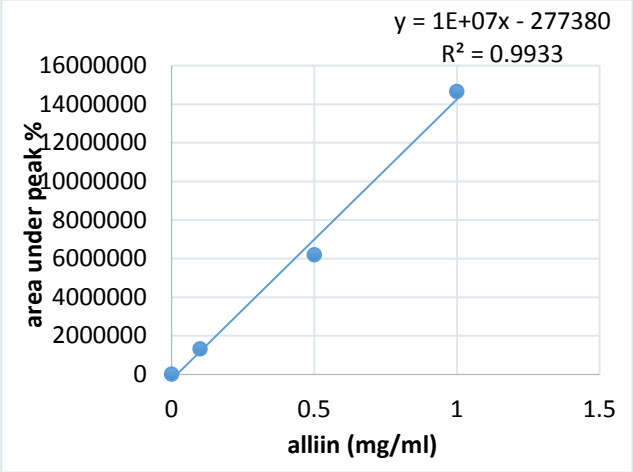

Figure 1. Standard curve of alliin

The figure 2 showing the results of alliin preparation where the microwave method gave the highest enzymatic activity 189.2 (unit/ml) and MCW method for the periods (12, 24 and 48 h) was gave an enzymatic activity 145, 166 and 150.1 (unit/ml) respectively, while the ultrasonic method was gave an enzymatic activity about 122.5 (unit/ml). .The methods above making on inhibition of alliinase activity through the temperatures and solvents that used, so the alliin can be isolated easily from garlic just with water and with high efficiency. The previous methods of preparation, especially the microwave method considers one of the encouraging methods of preparing the pharmaceutical manufacturing for the garlic tablets or capsules, this method is characterized of its little toxicity when it was taken by humans because of using water only for alliin extraction, as well as the methods of MCW and ultrasonic are consider good preparation methods for extract alliin because of the relatively low toxicity [22].

Both standard and prepared alliin was diagnosed by HPLC, the separated peak of prepared alliin was detected at a retention time 4.450 minutes which matching of the resulting peak of standard alliin at a retention time 4.167 minutes (figure 3), that means water was the best solvent for alliin extraction because of the important alliin's properties which is high solubility in water [28].

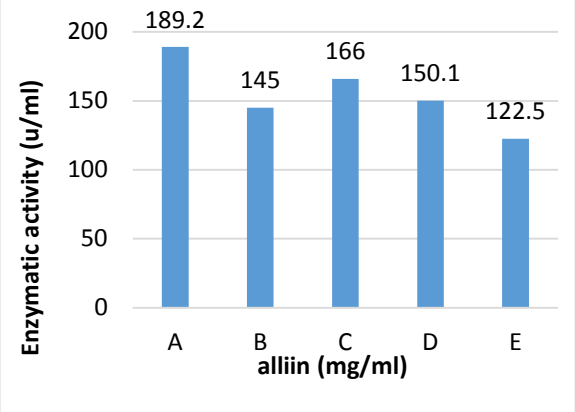

Figure 2. Methods of preparation alliin A: simple microwave; B: MCW (12 h); C: MCW (24 h); D: MCW (48 h); E: ultrasonic

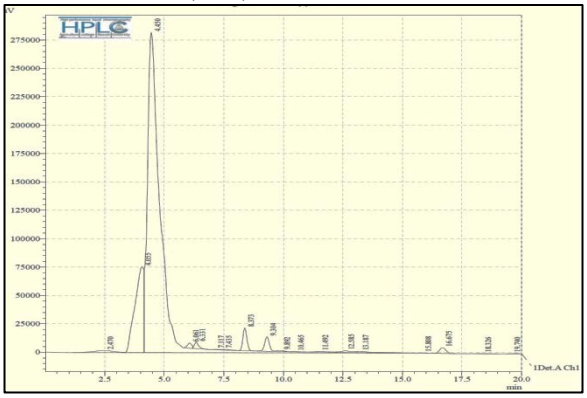

Figure 3. Chromatogram of prepared alliin diagnosed by HPLC

Enzyme purification.

Showing in table 3 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 [22,23]. Ammonium sulfate (30-70%) is 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 [29,30], the specific activity from this step was 165.517 (unit/mg protein).

The thermal stability of alliin.

Explain figure 4 the thermal treatment of prepared alliin with $MgCl_2$ and $FeCl_2$ at different temperature and effect on the stability of alliin. Notice that increasing the remaining activity% gradually 73.75%, 100.53%, and 116.97% respectively by increasing temperature from (20- 60) °C when using $MgCl_2$, while decreasing the remaining activity% of alliin and $FeCl_2$ in an aqueous solutions at the same temperature from (73.81-64.55) %. Showed [23] that Mg^{+2} and Fe^{+2} when added as chloride or sulphate to the garlic extract were stimulated the enzymatic reaction, while found [31] the alliin content decreased during the thermal processing in the first 2 days, stabilized at 4 to 7 days, then increased gradually as heating time increasing in the black garlic. It was suggested that the heating process decrease alliin content the garlic sample (sample was processed at 100 °C for 20 and 40 min) is that alliin converted into SAC, S-allylmercapto-cysteine, arginine and other compounds when using the thermal processing [19]. In fact the mechanism of reaction between alliin and these ions which enhancement the stability was not clearly understood, may be these ions were forming a complex compound more stable than the reactive compounds, that needs more studies and researches to know and clarify alliin stability at thermal processing.

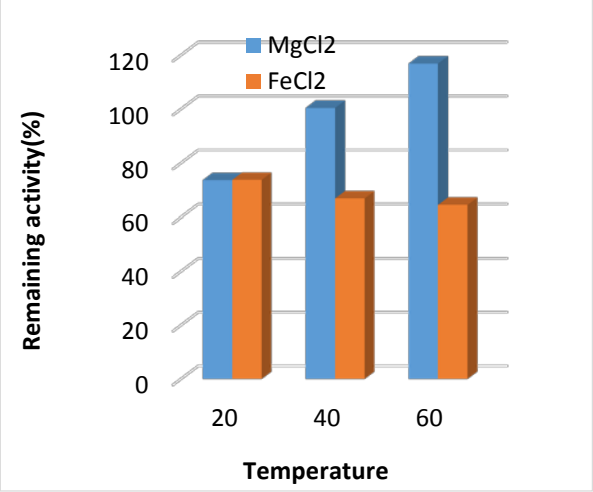

Figure 4. Thermal stability of prepared alliin

Table 1. Purification of an alliinase from Iraqi garlic

Steps of purification	Protein(mg/ml)	Activity(unit/ml)	Specific activity(unit/mg)	Total activity(unit)	Purification fold	Recovery%
Crude extract	1.30	259.2	199.38	25920	1	100
Precipitation of $(NH_4)_2SO_4$(30-70)%	1.27	220	173.22	17600	0.86	67.90
Dialysis	1.22	192.8	158.03	10604	0.79	40.91

CONCLUSION.

Alliin obtained from garlic extract by microwave method showed a high efficiency and potential for using in the pharmaceutical industries due to the low cost of preparation, good concentration levels, and high an enzymatic activity. Also, the thermal treatment enhancement alliin stability in water that need more studies to understand the mechanism between alliin and magnesium ion or others ions when increasing temperature that making alliin more stable at heating for further studies in the future.

REFERENCES

[1] Ariga, T.; Oshiba, S.; Tamada, T. Platelet aggregation inhibitor in garlic. *Lancet.* 1981, 150-151.

[2] Block, E. The chemistry of garlic and onions. Sci. Am. 1985, 252, 114-119.

[3] Block, E. Antithrombotic agents from garlic: a lesson from 5000 years of folk medicine. In Folk Medicine: The Art and the Science; Steiner, R. P., Ed.; *American Chemical Society*: Washington, DC, 1986; pp 125-137.

[4] Block, E. Biologically active organosulfur compounds from garlic and onions: the search for new drugs. *In Sulfur-Centered Reactive Intermediates in Chemistry and Biology*; Chatgilialoglu, C., Asmus, D., Eds.; Plenum Press: New York, 1991; pp 282-289 (NATO Conference Series).

[5] Block, E. The organosulfur chemistry of the genus Allium implications for organic sulfur chemistry. *Angew. Chem., Int. Ed. Engl*. 1992, 31, 1135-1178.

[6] Block, E.; Ahmad, S.; Catalfamo, J. L.; Jain, M. K.; Apitz- Castro, R. Antithrombotic organosulfur compounds from garlic: structural, mechanistic, and synthetic studies. J. Am. Chem. SOC. 1986,108, 7045-7055.

[7] Block, E.; Iyer, R.; Saha, C.; Grisoni, S.; Belman, S.; Lossing, F. Lipoxygenase inhibitors from the essential oil of garlic. Markovnikov addition of the allyldithio radical to olefins. *J. Am. Chem. SOC.* 1988,110, 7813-7827.

[8] Block, E.; Naganathan, S.; Putman, D.; Zhao, S.-H. *Allium* chemistry: HPLC analysis of thiosulfinates from onion, garlic, wild garlic (ramsons), leek, scallion, shallot, elephant (great head) garlic, chive, and Chinese chive. Uniquely high allyl to methyl ratios in some garlic samples. *J. Agric. Food Chem*. 1992, 40, 2418-2430.

[9] Freeman, F. & Kodera, Y. Garlic Chemistry: Stability of S(2-Propenyl) 2-Propene-1 sulfinothioate (Allicin) in Blood, Solvents, and Simulated Physiological Fluids. *J. Agric. Food Chem.* 1995, 43, 2332-2338.

[10] Lawson, L. D. & Hughes, B. G. Characterization of the formation of allicin and other thiosulfinates from garlic. *Planta Med.* 1992, 58, 345-350.

[11] Lawson, L. D. & Wang, Z. J.. Low Allicin Release from Garlic Supplements: a Major Problem Due to the Sensitivities of Alliinase Activity. *J. Agric. Food Chem.* 2001, 49, 2592-2599.

[12] Block, E., Naganathan, S., Putman, D., Zhao, S.H. Organosulphur chemistry of garlic and onion (*Allium cepa* L.) cultured in situ: comparison between sugar feeding and light induction. *Ann. Bot.* 1993, 69, 551–555.

[13] Nasim, S.A.; Dhir, B.; Samar, F.; Rashmi, K.; Mahmooduzzafar; Mujib, A. Sulphur treatment alters the therapeutic potency of alliin obtained from garlic leaf extract. *Food and Chemical Toxicology*. 2009, (47) 888–892.

[14] Iberl, B.; Winkler, G.; Muller, B.; Knobloch, K. Quantitative determination of allicin and alliin from garlic by HPLC. *Planta Med*. 1990, 58, 320-326.

[15] Hughes, J., Tregova, A., Tomsett, A. B., Jones, M. G., Cosstick, R., Collin, H. A. Synthesis of the flavour precursor, alliin, in garlic tissue cultures. *Phytochemistry*. 2005, 66, 187–194.

[16] Arnault, J.P., Christides, N., Mandon, T., Haffner, R., Kahane, J., Auger, J. High performance ion-pair chromatography method for simultaneous analysis of alliin, deoxyalliin, allicin and dipeptide precursors in garlic products using multiple mass spectrometry and UV detection. *J. Chromatogr. A.* 2003, 991, 69–75.

[17] Nasim, S.A.; Mujib, A.; Rashmi, K.; Samar, F.; Junaid, A.; Mahmooduzzafar. Improved alliin yield in somatic embryos of *Allium sativum* L. (CV. YAMUNA SAFED) as analyzed by HPTLC. *Acta Biologica Hungarica.* 2009, 60 (4):441–454.

[18] Dethier, B.; Laloux, M.; Hanon, E.; Nott, K.; Heuskin, S.; Wathelet, J.P. Analysis of the diastereoisomers of alliin by HPLC. *Talanta.* 2012, 101: 447–452.

[19] Zhang, M.; Lei, N.; Zhu, T.; Zhang, Z. Thermal processing effects on the chemical constituent and antioxidant activity of s-alk(en)ylcysteine s-oxides (alliin) extract. *LWT-Food Sci. Technol*. 2013, (51): 309–313.

[20] Jiang, X.; Lu, Y.; Tan, C.; Liang, Y.; Cui, B. Combination of aqueous two-phase extraction and cation-exchange chromatography: New strategies for separation and purification of alliin from garlic powder. *Journal of Chromatography.* 2014, B 957: 60–67.

[21] Yan-hui, G.; Zhao, J.; Min, D.; Feng, X. Study on kinetic characteristics of alliinase. *Agr. Sci. Technol*. 2008, 9 (1), 139-142.

[22] Mallika, T.; Omer, E.; Lianfu, Z. Separation and purification of alliinase and alliin from garlic (*Allium sativum*). *Journal of Academia and Industrial Research*.2014, 2(11), 599–605.

[23] Mazelis, M. & Crews, L. Purification of the alliin lyase of garlic, (*Allium sativum* L.). *Biochem. J.* 1968, 108: 725-730.

[24] Schwimmer, S. & Weston, W.J. Onion flavor, and odor: Enzymatic development of pyruvic acid in onion as a measure of pungency. *Agricultural and Food Chemistry*.1961, 9(4), 301-304.

[25] Bradford, M. M. A Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*. 1976, 72,248-254.

[26] Abdul Ghani, M.J. Determination of Alliin and Allicin in different types Garlic using High Performance Liquid Chromatography. *J. of University of Anbar for Pure Science.* 2010, 4(2):1-8.

[27] Bloem, E.; Haneklaus, S.; Schnug, E. Influence of nitrogen and sulphur fertilization on the alliin content of onions and garlic. *J. Plant Nutr*. 2004, 27, 1827–1839.

[28] Stoll, A. & Seebeck, E. Chemical investigation of alliin, the specific principle of garlic. *AdV. Enzymol.* 1951, 11,377–400.

[29] White, A.; Handler, P.; Smith, E. Principles of biochemistry. *Mc Graw-Hill Book company*. Alba Kiston publication, New York.1973.

[30] Englard, E. M. & Seifter, S. Precipitation techniques. In *Methods in Enzymology*. National Science and Mathematical.1990, 21(2), 199-208.

[31] Zhang, M.; Lei, N.; Liu, R.; Gao, Y.; Xu, M.; Zhang, M. Evaluation of alliin, saccharide contents and antioxidant activities of black garlic during thermal processing. *Journal of Food Biochemistry.* 2014, 1–9.