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RESEARCH ARTICLE

Separation and Purification of Alliinase and Alliin from Garlic (*Allium sativum*)

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Abstract

Two bioactive components, Alliinase and Alliin from garlic were successfully separated in the present investigation. Alliin was separated using microwave-assisted extraction followed by purification of garlic enzyme (Alliinase). This approach for extraction for the substrate (Alliin) illustrated higher activity when brought in contact with alliinase. The result obtained at optimum extraction conditions were analyzed by HPLC. The extraction efficiency of Alliin was 89.46±0.65%. Subsequently, Alliinase was extracted using different buffers at 4°C. The sodium phosphate buffer with the addition of EDTA, NaCl and Glycerol had highest stability and considerable activity. The total percentage of 25% (w/v) Polyethylene glycol 6000 (PEG 6000) was found to have higher activity with the addition of dual frequency 15% (w/v) followed by 10% (w/v). The freeze dried Alliinase was found to have recovery rate of >60% at optimum temperature of 31°C at optimum pH of 6.1 buffer system with natural substrate. The stability the garlic enzyme increased 10% by freeze drying. However, as the temperature increased its stability declined. The garlic bioactive compounds obtained in this study have a great potential in pharmaceutical industry when used in the form of tablets or capsules.

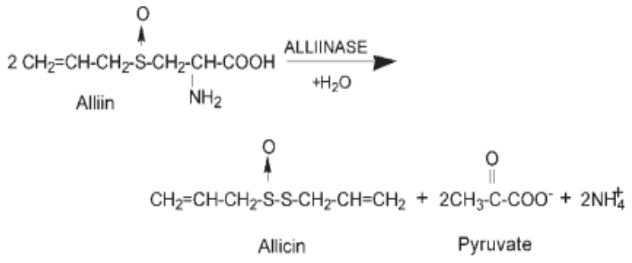
Keywords: *Allium sativum*, garlic, alliin, alliinase, polyethylene glycol, enzyme stability, bioactive compounds.

Introduction

Garlic (*Allium sativum*) is a widely distributed plant used throughout the world not only as a spice and a food, but also as a folk-medicine. Previous studies show that many Allium plants other than *A. Sativum* and *A. cepaare* of great importance due to their uses as flavoring agents, antioxidants, fragrance and therapeutics (Stajner *et al.,* 2006). The antioxidant activity of Allium species is due to a variety of sulphur-containing compounds and their precursors, but it is also related to other bioactive compounds such as polyphenols, dietary fiber and microelements (Nencini *et al.,* 2007). Other studies show that allicin prevents the development of the atherosclerotic process, reduces serum cholesterol, normalizes lipoprotein balance, decreases blood pressure and has antithrombotic and antiinflammatory activities (Sela *et al.,* 2004; Miron *et al.,* 2006). Studies indicated that garlic is effective in protecting against numerous diseases because of its cholesterol lowering, lipid lowering, antihypertensive, antidiabetic and antihyperhomocysteinemic characteristics. Other biological properties of garlic include antioxidant, antimicrobial, anticarcinogenic and antimutagenic activities (Reutar, 1995; Koch and Lawson, 1996; Kuettner *et al.*, 2002). The general composition of the fresh garlic bulbs is found to be 84.09% H_2O, 13.38% organic matter and 1.53% inorganic matter. Studies on the distribution of cysteine sulfoxides (CSOs) in Alliums show that they are found in a range of tissues, including leaf, bulb, root and flower scapes (Briggs *et al.,* 2002) as well as in cell culture and callus tissues (Lancaster and Collin, 1991). Alliinase is extremely abundant in garlic tissue, consisting of at least 10% of the total clove protein (Van Damme *et al.*, 1992; Ellmore and Feldberg, 1994). The enzyme from bulb tissues is a glycoprotein containing 6% carbohydrate and exists as a dimer of two subunits of MW 51.5 kDa each (Shimon *et al*., 2007). The reaction catalyzed by alliinase, which requires pyridoxal phosphate (PLP) as a cofactor, is categorized as a â-elimination-deamination reaction involving an aminoacryl intermediate bound to PLP (Tomofumi *et al.*, 1998).

Alliinase is the enzyme that initiates the conversion of the alkyl cysteine sulphoxide flavour (ACSO) precursor (alliin) to allicin and its derivatives. Alliinase is located in the vacuoles of vascular bundle sheath cells which are located around the phloem (Ellmore and Feldberg, 1994), rather than the abundant storage mesophyll cells that contain the CSOs (Koch and Lawson, 1996) which may be linked to the dynamic re-mobilization of CSOs during development. This contrasts with onion where both alliinase and CSOs are present in all cells, respectively in the cell vacuoles and cytoplasmic vesicles (Lancaster and Shaw, 2000). Garlic alliinase more rapidly hydrolyzes (+)-alliin, a naturally occurring substrate for the enzymatic synthesis of allicin, than (-)-alliin (Stoll and Seebeck, 1951; Lancaster and Collin, 1981; Kuettner *et al.*, 2002; Shimon *et al*., 2007).

Alliinase (Cys sulfoxide lyase, alliin lyase, C-S lyase; EC 4.4.1.4) from garlic (*Allium sativum*) is an enzyme that uses pyridoxal-5-phosphate (PLP) as a cofactor to catalyze the conversion of a non-protein amino acid alliin (þ) (Sallylcysteine sulfoxide) to allicin (diallyl thiosulfinate), pyruvate and ammonia as shown in the following scheme:



Alliin and alliinase precursors must remain separated prior to consumption to preserve the allicin potential. The S-alk (en) yl-L-cysteine sulfoxides (ACSO) substrates are located in the cytoplasm, while alliinase is compartmentalized in the vacuole until cell rupture (Lancaster and Collin, 1981). The production of allicin from alliin and alliinase is hampered by the instability of both enzyme and allicin and their tendency to aggregate (Miron *et al*., 2006). Most of the supplements are either garlic powder or the allicin derived from garlic or garlic extracts. However, allicin is not suitable during the intake due to its high instability. But for the pharmaceutical utilization, deriving of pure garlic compounds could be of higher aptitude. The objective of this study was to isolate two bioactive compounds primarily for pharmaceutical purpose. Alliin was isolated using various methods from the fresh garlic. The method with higher activity and lesser toxicity was considered as the potential substrate in enzyme purification procedure. Additionally, the process of protein sedimentation using PEG 6000 was studied for the enhancing refinement of alliinase followed by dialysis and freeze drying.

Materials and methods

Chemicals: Alliin standard (Shanxi Ciyuan Biotech), Pyridoxine 5'-phosphate (PLP), Sodium Pyruvate, Bovine serum albumin (Standard, Ourpharm, Shanghai), Ethyl acetate, Methanol and EDTA. All the chemicals and solvents unless mentioned were bought from Sino Pharm chemical reagent and were of analytical grade.

Plant material: Garlic bulbs were purchased from local markets in Wuxi, Jiangsu, China. The bulbs were stored in the refrigerator at 4°C before use.

Equipments: WD750B Microwave oven, UV-Vis3000 spectrophotometer, Vortex, KJ-300 Ultrasonic bath, and Waters 2695 HPLC system connected to Waters UV-Vis detector and (Nacalai Tesque Inc., Japan), R-205 Rotary evaporator.

Alliin preparation

Microwave-assisted method: Alliin was prepared according to the method described by Li *et al*. (2011) with slight modifications. About 50 g of freshly peeled garlic were taken and were microwaved for 90 sec, 750 W for permanent deactivation of the enzyme. In addition of 80 mL ethyl acetate for the removal of fat soluble components, they were blended for 5 min with the lab blender. The so formed homogenate was left still for an hour and then was centrifuged 8000 rpm for 5 min to separate the layers of organic solvent mixed along with the crude garlic oil. As the supernatant was discarded, the precipitate is then taken for extraction. Water was used as extracting solvent. Vacuum filtering is necessary to get the final product.

Chemical method (MCW): Alliin was prepared according to the method described by Yanhui *et al*. (2008) with slight modifications. The composition of 50 mL MCW (Methanol, chloroform and water) was prepared in the ratio of 12:5:3. About 20 g of garlic was immersed in the MCW solution for 12, 24, and 48 h respectively for preparation of 3 different samples. With the addition of 4.5 mL chloroform and 5.5 mL water per 10 mL of MCW solution, the samples were left at stationery position until the sharp demarcation was seen in separator funnel. The water solution was extracted and moved to rotary evaporator. The final solution was then diluted to 100 mL.

Hot water method: Alliin was prepared with slight modification as mentioned by Wei and Luo (2012). Pre-cooled garlic cloves were used and were boiled for 15 min. The crushed garlic was then filtered through four layers of cheese cloths. Acetone was added with the ratio of 1:4 to the initial volume. The homogenate was centrifuged at the speed of 8000 rpm for 15 min. The precipitate was discarded and the supernatant obtained was concentrated to a certain volume.

Sonication: The preparation of this natural substrate was done using 20 g of pre-cooled garlic cloves. They were immersed in 100 mL of methanol for 30 min. The slurry was then prepared by multiple sonications and centrifuged at 8000 rpm for 15 min. The supernatant obtained was used as natural substrate.

Preparation of substrate (Alliin): About 50 g of freshly peeled garlic were taken and microwaved for 90 sec, 750 W for permanent deactivation of the enzyme. To remove fat soluble components, 80 mL ethyl acetate was added and blended for 5 min with the lab blender. The so formed homogenate was left still for an hour and then was centrifuged at 8000 rpm for 5 min to separate the layers of organic solvent mixed along with the crude garlic oil. As the supernatant was discarded, the precipitate is then taken for extraction. The extracting solvent is water in the solid-liquid ratio1:5, at 35°C for 60 min.

The constant stirring is required. Vacuum filtering is necessary to get the final product. Alliin was stored at 4°C for further use with exception of freeze dried which was stored at room temperature.

$$Alliin\ obtained = \frac{Total\ amount\ of\ Alliin\ in\ extracting\ solvent}{Total\ amount\ of\ Alliin\ in\ garlic} \times 100\%$$

Quantification of Alliin by HPLC: The concentration of alliin was determined using HPLC system C18 ODS (4.6 mm X 250 mm, 5 µm particle size). The sample volume of 20 µL was injected and monitored at 195 nm. Alliin was eluted in mobile phase of 5% methanol and 95% phosphate buffer (pH 5) at the flow rate of 1.0 mL/min. The column temperature was maintained at 25°C. The quantification of alliin was done by comparing the peak area produced by freshly prepared alliin with that of standard alliin (99%).

Enzyme (Alliinase) extraction and purification: Alliinase was purified from fresh garlic bulbs obtained from the local market in Wuxi, Jiangsu. The isolation procedure was followed as described by Stoll and Seebeck (1951), Rabinkov *et al*. (1995), Miron *et al*. (1998) and Kuettner *et al.* (2002). All purification and column steps were done at 4°C except for peeling. Peeled and refrigerated bulbs (100 g typically) was homogenized in the ratio of 1:1.5 (w/v) of Buffer A (pH 6.5, 20 mM of Sodium phosphate buffer, 5 mM (EDTA) 5% (w/v) NaCl, 10% (v/v) glycerol and 20 µM pyridoxal-5-phosphate (Mendel and Laurie, 1968; Nock and Mazelis, 1986). The inclusion of PLP and glycerol in all sample buffers is recommended to stabilize the enzyme during purification (Krest and Keusgen, 1999; He, 2010). The homogenate was squeezed through four layers of cheese cloth followed by centrifugation at 10,000 rpm for 30 min in polyethylene centrifuge tubes. The protein precipitating between 20%-25% PEG 6000 was collected and dissolved in Buffer B (15% (w/v) Sucrose, 1% NaCl). The supernatant was obtained through centrifugation (10,000 rpm for 30 min) and dialyzed (18 h at 4°C) using the Buffer C (1% NaCl, 15% Sucrose) (Krest and Keusgen, 1999). The obtained product was freeze dried to obtain the partially purified enzyme.

Protein assay: Protein concentration was measured according to the method of Bradford using bovine serum albumin as standard (Bradford, 1976).

Enzyme assay (Pyruvic acid content): Each reaction test tube contained 1 mL of diluted filtrate, 1 mL of distilled H_2O and 1 mL of 2,4-dinitrophenylhydrazine (0.0125% DNPH in 2 N HCl) with natural L-(+)-alliin. A blank was prepared with NaOH, DNPH, and substrate. All reaction tubes were vortexed and placed in a water bath (37°C) for 10 min. The enzymatic reaction was terminated using Tricholroacetic acid.

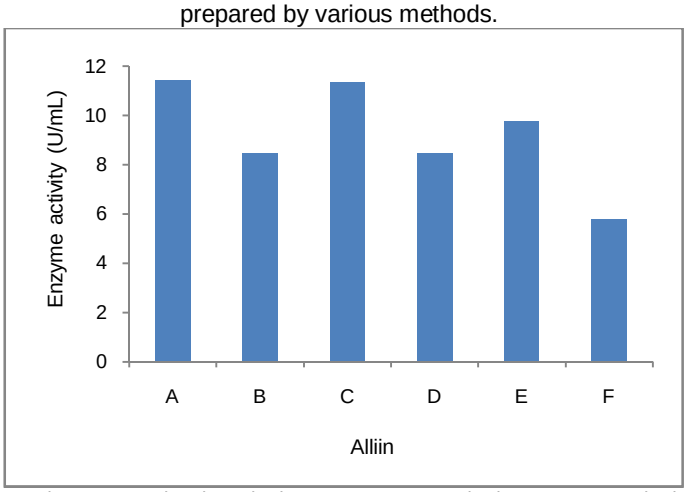
After the incubation period, 5 mL of 1 N NaOH was added and test tubes were vortexed and let stand for 5 min. Pyruvate was measured using a UV-Vis spectrophotometer at 445 nm for both control and sample filtrates. Standard curves were prepared with sodium pyruvate. Final pyruvate concentration per bulb (micromoles per gram fresh weight) was calculated from the difference between pyruvate levels in the sample and control wedges. One unit of alliinase activity is defined as the amount of enzyme which produces 1 mol pyruvate per min.

Statistical analysis: The obtained data were expressed as the means ± standard deviation (SD) of triplicate determinations.

Results and discussion

Figure 1 reports higher enzyme activity with the chemical method where a certain ratio of organic solvents is used to deactivate alliinase for alliin extraction. The second highest activity was from microwave-assisted alliin. However, the toxicity of chemical method remains of highest concern for human body on the intake. Therefore, the data resulted in an encouraged use of microwave assisted extraction of alliin as this procedure ensures the edibility in particular amount as tablets or capsules. The only chemical used for extraction is ethyl acetate, which was evaporated if remained after centrifuged at 8000 rpm for 10 min along with the withdrawal of garlic oil. Other than these two methods, the enzyme activity was found to be lower when combined with alliinase. The sonication method and hot water method could also be optimized for better results as its toxicity is relatively low. However, for further experiment microwave-assisted alliin was optimized and used as a natural substrate. The standard alliin (purity >99%) was used for analysis. The retention time was at 3.862 min. Figure 2 illustrates the retention time of alliin obtained by microwave-assisted method at 3.833 min. This shows the uniformity of alliin with the standard alliin. The extraction efficiency of alliin was confirmed to be 89.46±0.65%. The purity of this intensity can be used for pharmaceutical purposes. It was plainly seen that there was not a considerable difference between buffer systems F, G, H and I (Fig. 3). All of these systems can be used for further experiments. However, it was found that the homogeneity of the alliinase was stable for longer duration with the addition of NaCl. NaCl prevents the aggregation of crude enzyme for longer time period than $CaCl_2$. Kuettner *et al*. (2002) also reported homogeneity in presence of NaCl. Therefore, buffer system F was used for further experiments. Mendel and Laurie (1968) mentioned that the enzyme was unstable to storage at -10°C, particularly in dilute concentrations, but the addition of glycerol (final concentration 10%, v/v) stabilized the activity completely for at least 30 d. The addition of PLP stimulated the reaction rate and the stimulation became more marked when the purity increases.

Fig. 1. Enzyme activity with natural substrate alliin prepared by various methods.



A: Microwave-assisted method, B: Hot water method, C: MCW method (12 h), D: MCW (24 h), E: MCW (48 h), F: Sonication method. The activity was calculated with the same concentration of alliinase while using the alliin from different sources with a general concentration of 1 mg/mL.

Fig. 2. HPLC chromatogram of alliin obtained by microwave-assisted method.

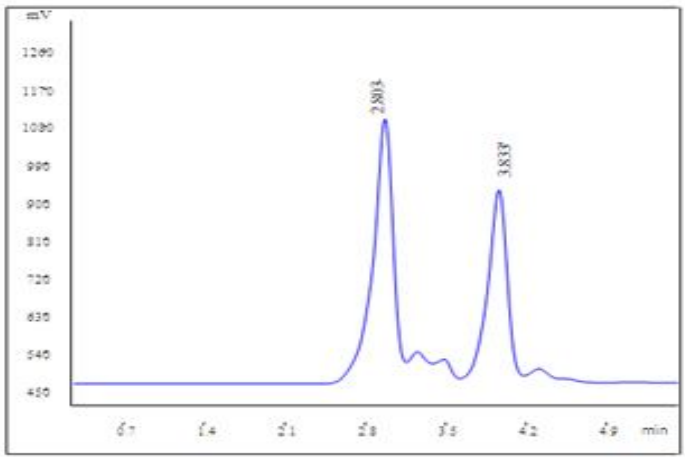
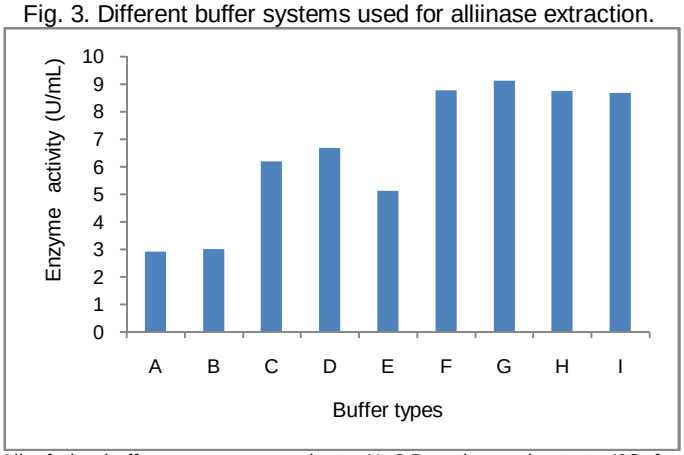


Fig. 3. Different buffer systems used for alliinase extraction.



All of the buffers were prepared at pH 6.5 and was kept at 4°C for 12 h before use. There was also an addition of PLP in each of them. A: Na^+ Phosphate buffer: Na^+/K^+ Phosphate buffer: [A+$CaCl_2$], D: [A+NaCl], E: [A+Sucrose], F: [D+EDTA+Glycerol], G: [C+EDTA+Glycerol], H: [B+EDTA+ Glycerol+NaCl], I: [B+EDTA+Glycerol+$CaCl_2$].

Fig. 4. PEG 6000 precipitation (10-70%) chart of crude alliinase.

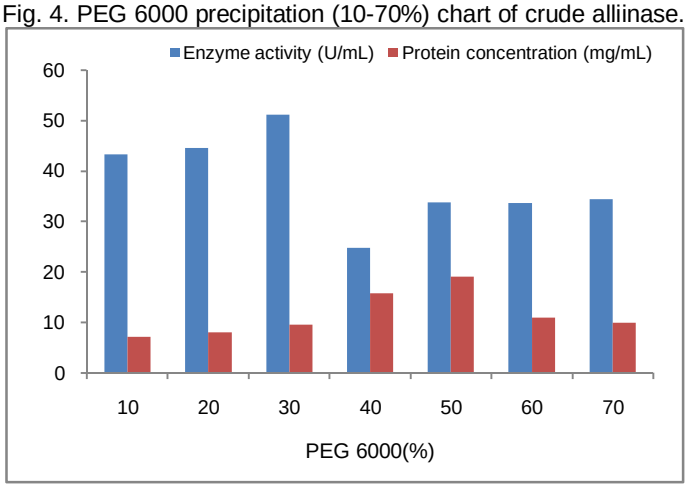
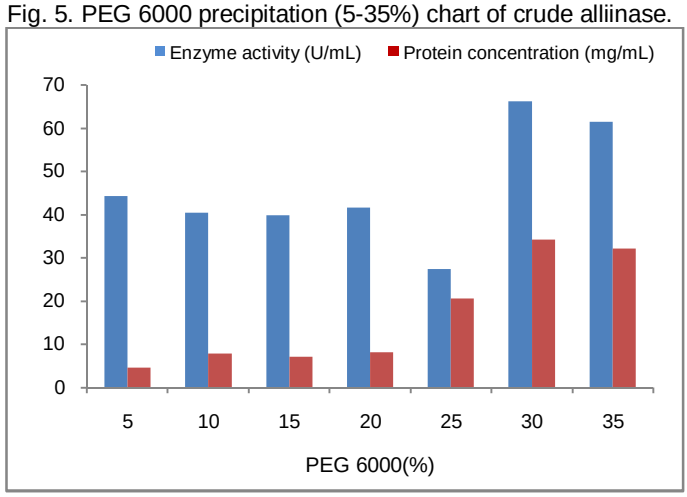


Fig. 5. PEG 6000 precipitation (5-35%) chart of crude alliinase.



The addition of EDTA or Mg^{2+}, Mn^{2+}, Co^{2+} or Fe^{2+} stimulated the reaction rate. Other bivalent cations either had no effect or gave a strong inhibition. In the presence of EDTA, no further increase of activity was observed with added Mg^{2+} (Jansen *et al.*, 1989). So far, these buffer systems aimed at maintaining the enzyme activity and homogeneity throughout the purification procedure. Figure 4 and 5 shows the enzyme activity and protein concentration of partially purified alliinase with natural substrate alliin. As the percentage of PEG in the supernatant is increased, the protein concentration increased as well. Figure 4 concluded that the specific activity of the enzyme is not directly proportional to the percentage of PEG. With the increment of PEG 6000 till 30% (w/v) there is progression of activity and protein concentration. As it's above 40% (w/v), with the sudden increase of protein concentration, the enzyme activity is highly reduced. The increasing concentration of PEG causes the solution to be extremely viscous which is not convenient for dialysis or freeze drying. Besides, the activity also remains almost unmoved with lower purification fold. When PEG 6000 was added from 10% (w/v) to 30% (w/v) both the activity and specific activity amplified.

Table1. Polyethylene glycol addition in various ways and its purification fold.

PEG addition methods	Enzyme activity loss (%)	Protein residue (%)	Specific activity (U/mg)	Purification fold
Homogenate (after centrifuging)	0	100	1.4	1
PEG 400 (20%)	47.54±0.01	66.2±0.015	2.02	1.46
PEG 6000 (20%)	15±0.032	57.63±0.041	3.37	2.6
PEG 6000 (25%)	35.74±0.021	61.33±0.008	2.67	1.9
PEG 6000 (10%+10%)	37.16±0.019	31.7±0.021	5.06	3.6
PEG 6000 (15%+10%)	23.5±0.066	45.91±0.126	4.25	3.03
PEG 6000 (20%+10%)	21.86±0.058	73.83±0.013	2.7	1.92

This table is calculated by the total volume of 15 mL of each before addition of different percentages of PEG. Values were means±SD of at least three duplicate, independent measurements.

Table 2. General extraction procedure of alliinase.

Fraction	Total protein (mg)	Total enzyme activity (U)	Specific activity (U/mg)	Purification fold	Recovery (%)
Homogenate (after centrifuging)	1405.53±0.02	1920.07±0.06	1.37	1	100
PEG 6000 (15%+10%)	534.5±0.06	1322.58±0.02	2.5	1.8	69.0
Dialysis	515.4±0.03	1291.7±0.04	2.51	1.83	67.27
Freeze drying	-	1278.5±0.01	-	-	66.58

Likewise, regarding the general use of 25% (w/v) used by Rabinkov *et al*. (1994) and Linda *et al*. (2002), the further experiment was carried out by addition of PEG 6000 from 5% (w/v) to 35% (w/v). Figure 5 shows a gradual rise in protein concentration coinciding with Fig. 4, but the enzyme activity is quite assorted. In addition of 5% (w/v), the enzyme activity is elevated whereas the existence of the minimal amount of protein shows the inadequate precipitation. Whereas, concurring with Fig. 4, the enzyme activity is found to be highest at 30% (w/v). The purification fold is reduced due to over-precipitation of non-active protein in garlic mixture. At 25% (w/v), it can be reckoned as the fine demarcation where inactive proteins get precipitated reducing the enzyme activity with the sudden increase of protein concentration. The best percentage determined to be 20% (w/v) of PEG 6000. Lower the residue of protein remnant in the mixture is the better purification fold. From Fig. 5, 25% (w/v) was considered to be the best percentage. Table 1 shows the frequency in addition of PEG 6000 to ensure better purification-fold and lessen the loss of enzyme activity. The purification fold was the highest at 10%+10% as the specific activity is the highest, however, the enzyme loss was found to be the lowest at 15%+10%. This low percentage of PEG 6000 dissolves readily in the garlic mixture which minimizes the higher possibility of enzyme degradation with reduced time. Therefore, taking the time factor into consideration which is highly important in industrial level, the addition of PEG 6000 in dual frequency in total of 25% (w/v) is selected which is 1.6 times in specific activity when added once at 25% (w/v). Zhang *et al*. (2012) has mentioned enhancement of activity 5.42 times in addition of PEG 8000 in dual frequency. The protein residue remaining is the least which helps in the advancement of the further purification steps and analysis steps (Zhang *et al.*, 2012).

After freeze drying, the partially purified alliinase was much more stable (Table 2). The loss of enzyme activity during the freeze drying process reduced to 10%, which is found to be higher in the study done by Krest and Keusgen (1999) which is about 15%. After freeze drying, it was found to be stable for 30 d, with degradation of enzyme activity less than 15% when stored at 4°C. Liquid system of alliinase is less stable in which within 30 d about 35% of activity was reduced. Despite being partially purified, the garlic enzyme is suitable for pharmaceutical use. As it is freeze dried, it can be used for pharmaceutical use with alliin powder to make the tablets or capsules. Higher purification is recommended, but as the purification steps proceed higher, less the stability was ascertained (Krest and Keusgen, 1999).

Stability of partially purified enzyme: The dialyzed enzyme was stored in the phosphate-sucrose buffer; it showed a higher loss of activity than glycerol. However, this buffer was suitable when the partially purified enzyme was freeze dried. However, Mendel and Laurie (1968) suggested the storage of concentrated enzyme rather than in dilution. This concentrated enzyme had more than 50% of its original activity at –10°C even after 57 d. Therefore, freeze drying was used as a process to maintain the concentration for longer stability. This experiment was conducted by using dialyzed enzyme and freeze dried enzyme in the same conditions. It was stored at 4°C for 35 d. There was gradual depreciation of activity in both the samples (Fig. 6). With the freeze dried sample, the declination was less than 10% after 35 d whilst it was more than 25% for the other one of the original activity. This result differs from that of Mendel and Laurie (1968) due to varying temperature condition and absence of glycerol in the buffer system. The absence of glycerol and substitution with sucrose was found to be quite cooperative for freeze drying.

Fig. 6. Remaining enzyme activity at 4°C.

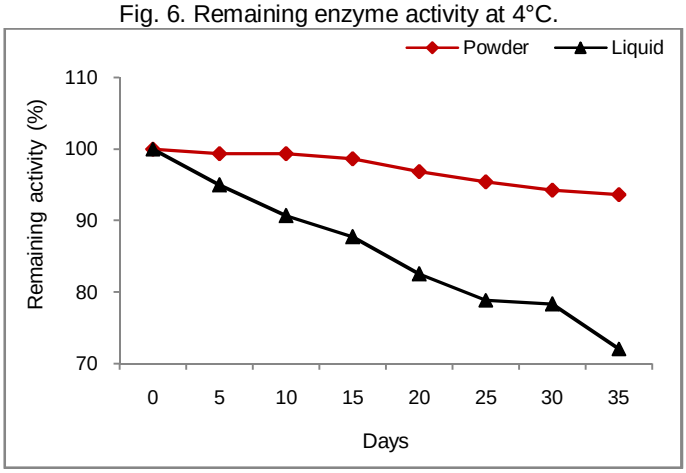


Fig. 7. Remaining enzyme activity in different reaction temperatures.

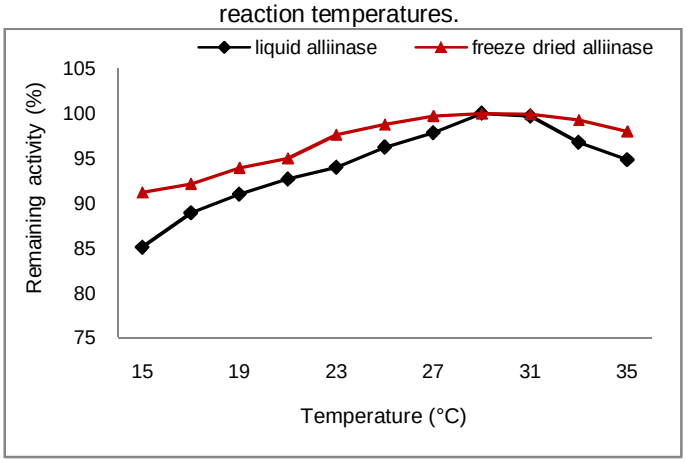
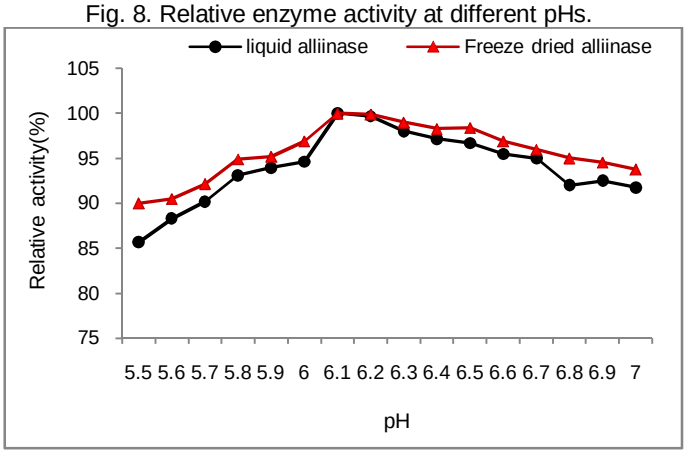


Fig. 8. Relative enzyme activity at different pHs.



The enzyme activity was found to be higher with the buffer system with glycerol. The dialyzed enzyme is more stable at -18°C, where the loss was less than 10% of the original activity. However, each time with freezing and thawing, there is a certain loss of activity (Krest and Keusgen, 1999). Freeze dried enzyme can be recommended for the utilization for a longer time period. To confirm the best reaction temperature and pH of the freeze dried alliinase, it was compared with the partially purified alliinase before freeze drying.

Figure 7 and 8 showed the uniformity in the reaction temperature and pH of both of them. The best reaction temperature is concluded as 31°C at pH 6.1. These optimum conditions are both lower than the ones mentioned in other studies where temperature is approximately 35°C. On the other hand, it is higher than that established by Yanhui *et al.* (2008), where the optimum reaction temperature is 29°C. Similar pattern was followed in pH where highest was recorded to be 6.5 and lowest at 6.1 (Krest and Keusgen, 1999; Linda *et al.,* 2002; Miron *et al.,* 2006; Yanhui *et al.,* 2008; Jing *et al.*, 2011). The substrate alliin obtained by microwave-assisted process can considered to be the main reason behind followed by the differences in buffer systems.

Conclusion

Alliinase is the enzyme that initiates the conversion of the precursor (alliin) to allicin and its derivatives. The results portrayed the efficiency of microwave-assisted alliin with alliinase in comparison to procedures aided with organic solvents and water for extraction. The alliinase enzyme activity seemed to improve with the addition of PEG 6000 in dual frequency with the total concentration of 25% (w/v). Freeze drying method is highly encouraged than to leave the partially purified enzyme in its dialyzed form as the depreciation of activity was reduced two-fold. The aggregation of alliinase which degrades not only the enzyme activity but also the stability can be prevented completely by freeze drying. The partially purified enzyme has optimum enzyme activity at 31°C and pH 6.1 buffer system with the use of microwave-assisted alliin. Furthermore, the enzyme activity can be highly improved using this procedure. The enzyme activity not only depends upon the purity and purification procedure, but also in the selection of source of garlic and peculiarly on the substrate used. This procedure of extraction can be used for pharmaceutical use for making tablets and capsules when freeze dried.

Acknowledgements

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