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Full Length Research Paper

Immobilization of alliinase and its application: Flow-injection enzymatic analysis for alliin

Jianqin Zhou

School of Pharmaceutical Sciences, Medical College, Soochow University, Suzhou 215123, P.R., China.
E-mail: zkzhu@ustc.edu. Tel.: +86 512 65880025. Fax: +86 512 65880031.

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Alliinase was immobilized on chitosan microspheres by means of glutaraldehyde, and a flow-injection enzymatic analytical system was developed for determination of alliin based on the immobilized alliinase and an ammonia gas electrode. The factors affecting the activity of the immobilized enzyme, such as glutaraldehyde concentration, cross-linking time and the amount of alliinase, were investigated. Results showed that the maximum of enzyme activity could be obtained at glutaraldehyde concentration of 4%, cross-linking time of 2 h and the amount of alliinase 20.2 u. The properties of the immobilized alliinase were also studied in detail. For the immobilized alliinase, the highest activity was allowed at pH of 7.0 and temperature at 35°C. Besides, the immobilized enzyme showed good thermal and pH stabilities. The flow-injection enzymatic analytical system based on the immobilized alliinase and an ammonia gas electrode provided linearity in the 1×10^{-5} to 1×10^{-3} mol/L alliin concentration range and exhibited good repeatability and operational stability.

Key words: Alliinase, immobilization, enzyme properties, flow-injection analysis.

INTRODUCTION

Alliinase (alliin lyase EC 4.4.1.4) has been reported to occur in many plants of the genus *Allium* such as garlic (*Allium sativum*), onion (*Allium cepa*), leek (*Allium porrum*) (Hughes et al., 2005; Kuettner et al., 2002a; Nock and Mazelis, 1987). Its extensive characteristics are well documented. Alliinase is a PLP-dependent enzyme and a homodimeric glycoprotein with a total molecular mass of 103 kDa (Shimon et al., 2007). It belongs to the family of mannose-rich glycoproteins with an estimated carbohydrate content of about 5.5 - 6% (Smeets et al., 1997). Alliinase catalyzes the conversion of alliin (+S)-allyl-L-cysteine sulfoxide) to allicin, pyruvate and ammonia. Allicin is well known for its remarkable medicinal properties which includes antimicrobial, antihypertensive, antithrombotic, antiviral and anticancer activities (Elkayam et al., 2003; Hirsch et al., 2000; Larsen et al., 2006). Allicin also prevents the development of the atherosclerotic process, reduce serum cholesterol and normalize lipoprotein balance (Dausch and Nixon, 1990; Mansell and Reckless, 1991; Oron-Hermanet al., 2005). Alliin is the precursor of allicin, and alliin itself was reported to possess canceroprotective and antioxidant potential (Iberl et al., 1990). So the content of alliin in plants is the important determinants of plant quality.The aim of this study is to develop a convenient and accurate method to measure the content of alliin.

For this purpose, alliinase was immobilized, and a flow-injection enzymatic analytical system was developed based on the immobilized alliinase and an ammonia gas electrode. The enzymatically formed ammonia can be monitored using ammonia gas electrode and the amount of ammonia is proportional to the content of alliin. Therefore ammonia gas electrode can be used for an indirect quantification of alliin.

In the present study, alliinase was immobilized on chitosan microspheres by means of glutaraldehyde, and the assessments of activity and stability of the immobilized alliinase compared with free alliinase were present. Based on the properties of the immobilized enzyme, the working conditions of the flow-injection enzymatic analytical system were determined. Under these conditions, the calibration graph and operational stability of the system were investigated.

MATERIALS AND METHODS

Alliinase was isolated from garlic cloves according to the methods described by Dausch and Nixon (1990). Alliinase was stabilized with 10% glycerol, 0.17 M NaCl and 25 mM pyridoxal-5'-phosphate (PLP) dissolved in phosphate buffer (pH = 6.5, 20 mM). L-(+)-Alliin

was purchased from Fluka (Shanghai, China). Other chemicals were purchased from Sinopharm Chemical Reagent Co., Ltds (Shanghai, China).

Immobilization of alliinase

Chitosan solution (2.5% w/v) was prepared by dissolving a given amount of chitosan into 1% (v/v) acetic acid solution. The solution was then extruded through a needle into a solution of NaOH (20% w/v) and CH_3OH (30% v/v) to form microspheres. After 2 h of hardening, the obtained microspheres were washed with distilled water to neutrality and then stored at 4℃ for use.

The chitosan microspheres were put into glutaraldehyde solution and incubated at 4℃ for several hours, followed by being rinsed with distilled water to get rid of glutaraldehyde. The cross-linked chitosan microspheres were immersed in enzyme solution for 2 h. Then the microspheres were again washed with distilled water to remove excessive alliinase and stored at 4℃.

Enzyme activity assay

Alliinase was assayed by determining the production of pyruvate from alliin. The reaction mixture consists of the selected amount of enzyme and 2 ml of 20 mmol/L alliin containing 20 µmol/L PLP. The mixture was incubated at 25℃ for 5 min and then was filtered to quench the reaction. 2.5 ml aliquots of filtrate were taken to determine the production of pyruvate. One unit of alliinase activity is defined as the amount of enzyme which produces 1 µmol pyruvate per min. As to the activity of free alliinase, trichloroacetic acid was used to stop reaction.

Measurement procedure

A schematic diagram of the flow-injection enzymatic analytical system is shown in Figure 1. The flow-injection enzymatic analytical system mainly consisted of a constant flow pump (DHL-B, Shanghai Qingpu Huxi Instrument Factory), a column (13 mm × 30 mm), an ammonia gas electrode (pNH3-1, Shanghai Precision and Scientific Instrument Co. LTD), a pH-meter (pHS-2F, Shanghai Precision and Scientific Instrument Co. LTD). The immobilized alliinase was filled in the column. Samples for analysis were dissolved in phosphate buffer (0.01 M, pH 7.0) and injected into the column. After 5 min enzymatic reaction, the buffer solution was pumped into the column and the resulting solution in the column was driven to the electrode cell. The pH meter was used to measure the output voltages (△E). For constructing a calibration curve, the experiment was repeated with different alliin solutions of known concentrations.

RESULTS AND DISCUSSION

Optimal conditions for immobilizing alliinase

Chitosan was cross-linked by means of glutaraldehyde to bind the alliinase through amino groups. The immobilization was performed with excessive pyridoxal -5'-phosphate to protect lys251 from reacting with the activated carrier (Miron et al., 2006). Moreover, addition of pyridoxal-5'-phosphate in immobilization medium is helpful to reserve enzyme activity because the excessive pyridoxal-5'-phosphate could occupy the unfilled PLP-sites on alliinase (Kuettner et al., 2002b; Lancaster et al., 1998).

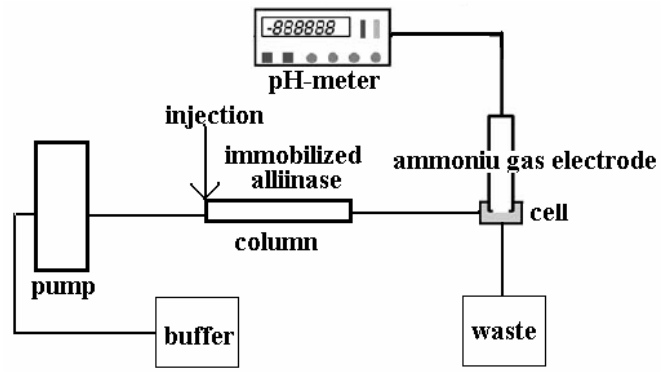

Figure 1. Schematic diagram of the the flow-injection enzymatic analytical system.

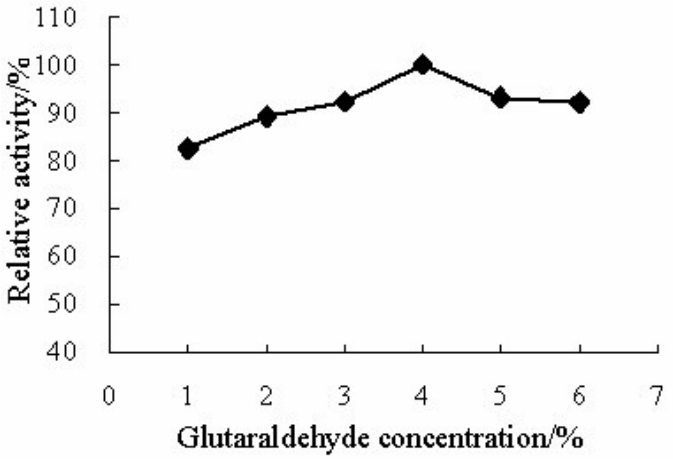

Figure 2. Effect of glutaraldehyde concentration on immobilization of alliinase. Immobilization conditions: amount of alliinase 6.7u; glutaraldehyde concentration 1-6%; cross-linking time 3 h. Relative activities were calculated by using the highest activity of immobilized alliinase as 100%.

Effect of glutaraldehyde concentration on immobilization

The effect of glutaraldehyde concentration on the activity of immobilized enzyme was shown in Figure 2. The relative activity of immobilized alliinase increased by increasing the concentration of glutaraldehyde from 1 to 4% and then decreased. At low concentration of glutaraldehyde, few bonds involving the carrier and the enzyme molecules were formed, thus not sufficient to give a high immobilization yield. The increase in the concentration of glutaraldehyde allowed the immobilization of maximum enzyme. However, since glutaraldehyde is toxic to the enzyme, a high concentration of glutaraldehyde above 4% also led to enzyme denaturation and decreased enzyme activity. Thus 4% of glutaraldehyde concentration was accepted as the optimal concentration for enzyme immobilization.

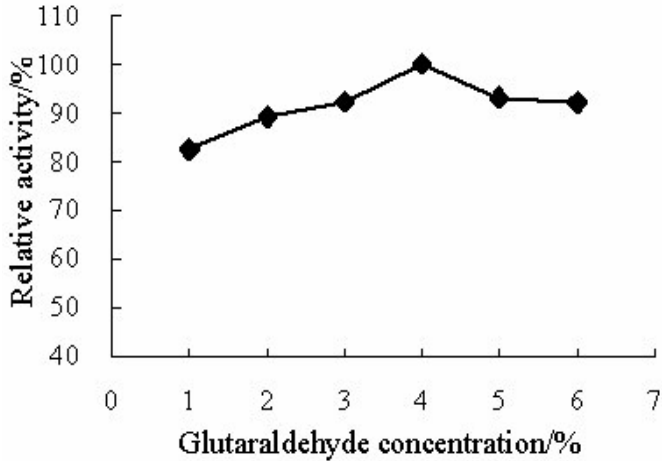

Figure 3. Effect of cross-linking time on immobilization. Immobilization conditions: amount of alliinase 6.7u; glutaraldehyde concentration 4% cross-linking time 0.5-3 h. Relative activities were calculated by using the highest activity of immobilized alliinase as 100%.

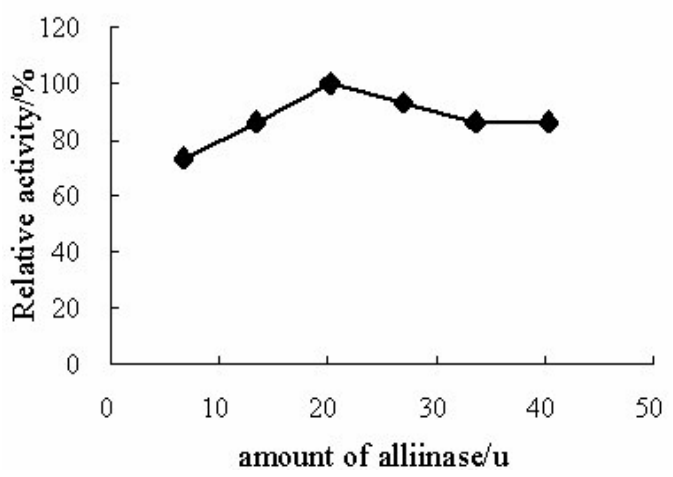

Figure 4. Effect of the amount of alliinase on activity of immobilized enzyme. Immobilization conditions: amount of alliinase between 6.7-40.3u; glutaraldehyde concentration 4%; cross-linking time 2 h. Relative activities were calculated by using the highest activity of immobilized alliinase as 100%.

Effect of cross-linking time on immobilization

The effect of cross-linking time on immobilization was investigated. As shown in Figure 3, with the increase of cross-linking time, the relative activity of immobilized alliinase increased to a maximum value and then decreased. The optimum cross-linking time was 2 h. Longer immobilization process would cause denaturation of enzyme, so the immobilized enzyme activity fell after 2 h. Chen and Chang (1994) immobilized chitinase on a reversibly soluble-insoluble polymer and observed similar results regarding the effect of immobilization time.

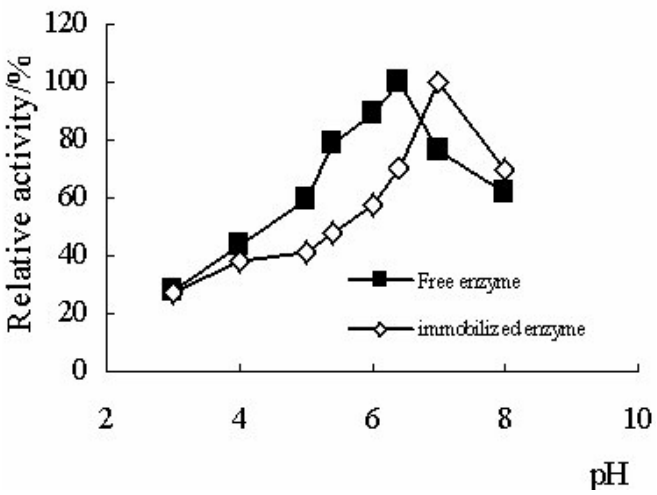

Figure 5. Effect of pH on activities of free and immobilized alliinase. Relative activities were calculated by using the highest activity of free and immobilized alliinase as 100%, respectively.

Effect of the amount of enzyme on immobilization

Figure 4 shows the effect of the amount of enzyme on immobilization. With the increase in the amount of alliinase, the relativity activity of the immobilized enzyme increased to a maximum value when the amount of alliinase was 20.2 u and then decreased slightly. Increasing the amount of enzyme in the immobilization medium resulted in a competitive binding of enzyme molecules through active chemical groups. It was assumed that the amount of enzyme bound into the carrier reached saturation at 20.2 u. The 20.2 u of alliinase was chosen for the preparation of immobilization. Teke and Baysal (2007) observed similar trend for the immobilization of urease into nylon-6-membrane. The immobilization efficiency peaked at an enzyme concentration of 10 mg/ml and then levelled of with the increase in urease concentration (from 10 to 20 mg/ml).

Properties of immobilized alliinase

Effect of pH on the activities of alliinase

The pH dependence of the activity of immobilized alliinase was studied, and the results were compared with those for free alliinase. Figure 5 shows the effect of pH on the activity of free and immobilized alliinase ranging between pH 3.0 and 8.0. The optimum pH was found to shift from 6.0 to 7.0 by the immobilization. Miron et al. (2006) reported similar changing in the optimum pH of alliinase after immobilization on beaded cellulose. In his work, the optimal pH for soluble alliinase activity was 6.5, whereas that of covalently immobilized alliinase ranged between pH 7.0 and 7.5.

Table 1. pH stabilities of free and immobilized alliinase. Both free and immobilized enzyme were incubated in pH 5.5, 6.0, 6.5 and 7.0 phosphate buffer for 3 h.

Alliinase	Residual activity*(%)			
	pH 5.5	pH 6	pH 6.5	pH 7.0
Free alliinase	59.8	78.7	97.6	89.9
Immobilized alliinase	96.0	102.9	100.0	107.0

*Residual activities were calculated by using the initial activity of immobilized alliinase as 100%.

Table 2. Thermostabilities of free and immobilized alliinase. Thermostabilities of free and immobilized enzyme were examined by incubation at 20°C, 25°C, 30°C, 35°C, 40°C and 45°C for 3h before the measurements.

Alliinase	Residual activity* (%)					
	20℃	25℃	30℃	35℃	40℃	45℃
Free alliinase	80.9	68.9	31.3	22.5	4.0	5.8
Immobilized alliinase	87.0	75.0	48.8	36.7	20.0	9.8

*Residual activities were calculated by using the initial activity of immobilized alliinase as 100%.

pH stabilities of alliinase

For investigation of pH stabilities of the free and immobilized enzyme both of them were incubated in phosphate buffer (pH 5.5, 6.0, 6.5 and 7.0) for 3 h. As shown in Table 1, the immobilization of alliinase increased the pH stability of enzyme. The immobilized alliinase lost no activity after 3 h incubation in pH 6.0, 6.5 and 7.0 buffer. The activity maximum and the best pH stability of the immobilized alliinase were both at pH 7.0, so phosphate buffer pH 7.0 was selected as working pH of the immobilized alliinase.

Effect of temperature on the activities of alliinase

The effect of temperature on the activities of alliinase was also investigated ranged from 20 to 50℃. As depicted in Figure 6, the optimum temperatures for free alliinase and immobilized alliinase were 30 and 35℃, respectively. So, there was a shifting on the optimum temperature of the immobilized alliinase. Immobilization by means of glutaraldehyde cross-linking resulted in higher optimum temperature as glutaraldehyde reacts with -NH_2 groups of enzyme to form bonds akin to disulfide bridges (Sharma et al., 2001), leading to the increase of the rigidity of enzyme structure. Shift of optimum temperature to higher value after enzyme immobilization has been widely reported in other studies (Chen and Chang, 1994; Gabrovska et al., 2007; Glodek et al., 2002; Zhang et al., 2004).

Thermostabilities of free and immobilized enzyme

The thermostability is one of the most important properties of any immobilized enzyme for application. Thermostabilities of free and immobilized enzyme were also examined by incubation at 20, 25, 30, 35, 40 and 45℃ for 3 h before the measurements. These results are summarized in Table 2. After heat treatment, both free and immobilized alliinase lost activity, especially at high temperature. However, the thermostability of the immobilized enzyme was better than that of the free enzyme. The thermal stability of an enzyme was enhanced by immobilization, so the potential utilization of such enzymes would be extensive. According to the optimum temperature study, the best activity was monitored at 35℃ for the immobilized enzyme. However, following heat treatment at 35℃ for 3 h the immobilized

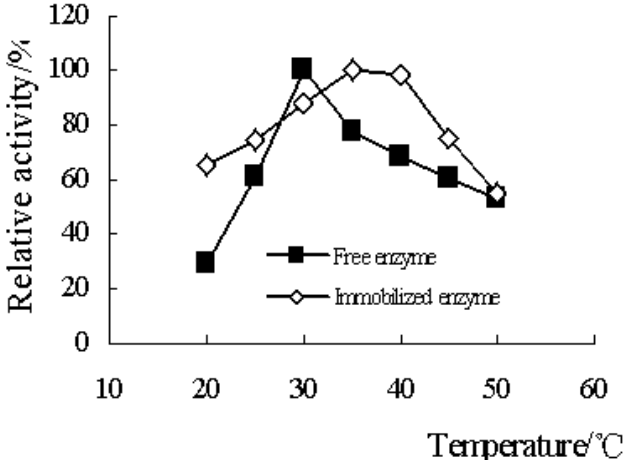

Figure 6. Effect of temperature on activities of free and immobilized alliinase. Relative activities were calculated by using the highest activity of free and immobilized alliinase as 100%, respectively.

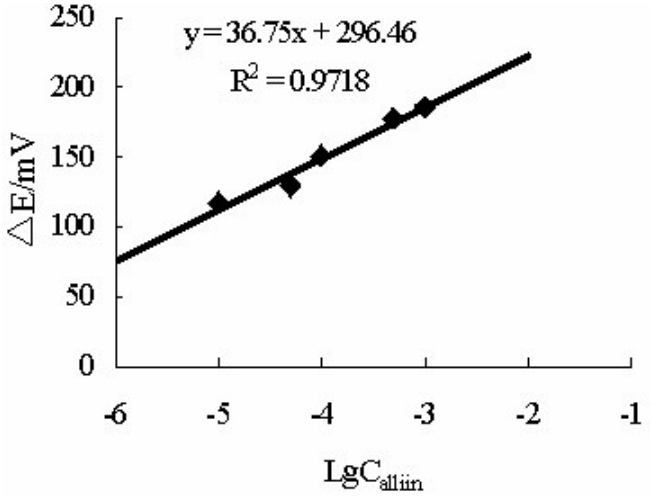

Figure 7. Calibration graph for the flow-injection enzymatic analytic-cal system. Working conditions: phosphate buffer pH 7.0, 10mM and 25℃.

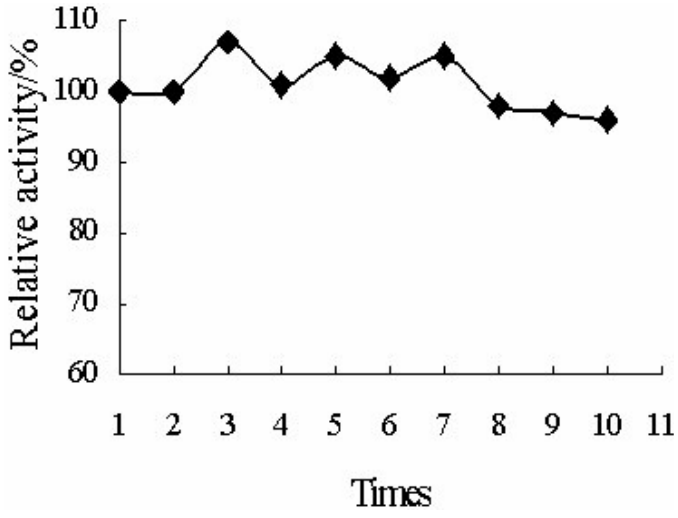

Figure 8. Operational stability of the system. Relative activities were calculated by using the initial activity of immobilized alliinase as 100%.

alliinase retained only 36.7% of its original activity. So, 25℃ was selected as the working temperature of the immobilized alliinase, for at the lower temperature the immobilized alliinase exhibited fairly well performance and maintained longer work time.

Application of immobilized alliinase

In order to develop a convenient and accurate method to measure the content of alliin, a flow-injection enzymatic analytical system was developed based on the immobilized alliinase and an ammonia gas electrode.

Calibration graph for the flow-injection enzymatic analytical system

In this study, the calibration graph of the flow-injection enzymatic analytical system was investigated. The system was operated at 25℃ using buffer solutions at pH 7.0 (10 mM). The concentrations of alliin were 0.01, 0.05, 0.1, 0.5 and 1 mM. The calibration graph for alliin standard is shown in Figure 7. Quantitative determination of alliin can be carried out in the linear range between 1×10^{-5} and 1×10^{-3} M.

Repeatbility

The repeatability of the flow-injection enzymatic analytical system was also studied by injecting 1 mM alliin (n = 7). The average value and the standard deviation (S.D.) were 1.1 ± 0.13 mM for the system, respectively, indicating that reproducible results can be obtained by this system.

Operational stability

Finally the operational stability of the system was investigated. In this study several measurements were carried out by flow-injection enzymatic analytical system. The result was shown in Figure 8. At the end of the 10th measurement, the immoblilized alliinase just lost 4% of its initial activity, indicating that the system exhibited good operational stability.

Conclusions

In this study, alliinase was immoblilized and its properties, such as temperature, pH dependence and stabilities, were investigated. For alliinase immobilized on chitosan microspheres, the optimum pH and temperature shifted to higher values, moreover, thermal and pH stabilities improved compared to those of free enzyme. Subsequently a flow-injection enzymatic analytical system was developed based on the immobilized alliinase and an ammonia-gas electrode. Under the optimal working conditions, the system exhibited excellent response performance to alliin and good repeatability. The flow-injection enzymatic analytical system is helpful to determine the content of alliin in plant material and can be applied for quality control of herbal remedies and screening of potential medicinal plants.

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