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Immobilization of cauliflower myrosinase on agar agar matrix and its application with various effectors

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Abstract: The purified and thermo stable myrosinase from cauliflower seedlings was immobilized by 3.5% agar agar matrix and immobilized myrosinase chips were stored in extraction buffer. After 48 hours 30% enzyme activity was exuded into extraction media from immobilized enzyme chips. Effect of some metal ions and organic solvents on the activity of immobilized cauliflower myrosinase was studied. Amongst selective metal ions (Sr^{2+}, Sn^{2+} and Ba^{2+}), Sr^{2+} at 4 mM concentration exhibited marked activating effect on the activity up to 3 fold. However, Sn^{2+} and Ba^{2+} increased the activity to a certain extent and then suppressed. Activation kinetics of myrosinase in presence of Sn^{2+} and Sr^{2+} were studied between 0-20min.The rate of reaction was almost constant till 15 min and then slight deactivation was recorded at various concentrations used. On the other hand, few heavy metal ions [Fe^{2+}, Fe^{3+}, Cu^{2+} and Zn^{2+}] strongly inhibited the activity even at lower concentrations. Several nonpolar organic solvents even at comparatively higher concentrations had detectable activation effects. Further, their activity was seen with respect totime (27 min). However, some protic polar organic solvents exhibited inhibitory effect with immobilized myrosinase except to butanol.

Keywords: Myrosinase, Cauliflower, Immobilization, Agar Agar, Metal Ions, Organic Solvent

1. Introduction

In spite of the broader use of enzymes, their applications are constrained by a number of practical problems. Besides the high cost of enzyme isolation and purification, the main problem is their fragility or sensitivity to harsh environmental conditions which results in restricted operational lifetime of enzymes and difficulty in their recovery in the active form after the process for reuse [1-4].

The most successful and the commonest way to overcome these limitations is the immobilization of enzymes [2, 4].The most important advantage of immobilization is that it leads to alteration of the original properties of enzymes which has remarkably opened the way for customizing enzyme properties for their specific applications.

Since myrosinases (thioglucoside glucohydrolase, EC 3,2,3,147)are of great significance in present day biotechnology with applications ranging from processed food, health products, baking, textile, paper industries, etc. hence several methods have been developed for the preparation of immobilized myrosinase, each having its own advantages and disadvantages specific to the method employed. Myrosinase was purified from *Sinapis alba* seeds and immobilized on different low cost solid matrices such as alumina, silica etc.[5].It was also immobilized on Nylon 6.6 and was used to build up a biosensor for total glucosinolate determination [6] and a bioreactor useful to produce a number of glucosinolate derivative products [7-9]. Myrosinase was also immobolized on egg shell membrane [10].

Immobilized enzymes, as compared to free or soluble enzymes, offer more advantages, e.g., enhanced stability against various denaturing conditions, higher catalytic activity, easier product and enzyme recovery, rapid termination of reaction, reusability and reduced susceptibility to microbial contamination [11-14].

The main limitations in application of immobilized enzymes include high cost and low yield [15, 16]. Thus, an efficient, easily available and inexpensive support like agar agar with good immobilization yields may be of more versatile applications.

Agar agar is a polysaccharide and is a mixture of two components: the linear polysaccharide agarose and a

heterogeneous mixture of smaller molecules called agaropectin. Its chemical structure gives agar agar the capacity to form very strong gels even at low concentrations. Not only being an excellent matrix, agar agar has a high porosity which leads to high capacity for protein entrapment. Additional advantages of using agar agar as a matrix are hydrophilic character, ease of derivatization, absence of charged groups which helps in preventing non-specific adsorption of substrate and products, and also commercial availability. In addition, the melting temperature of agar agar (85^{o}C) and solidifying temperature 32-40^{o}C is another significant factor which favours its applicability. This has a strong gelling ability. It is acid stable and shows no protein reactivity. Moreover, the cost of this material is also low as compared with other materials commonly used for immobilization.

Therefore, this work was devoted to the immobilization of cauliflower myrosinase on agar agar and to make a suitable study of different heavy metal ions and organic solvent on immobilized enzyme.

2. Materials and Methods

Cauliflower seed was arranged from Seed Technology section of Indian Institute of Vegetable Research (IIVR) Varanasi. BSA and sinigrin monohydrate were obtained from Sigma Chemical Co. USA. Potassium dihydrogen orthophosphate, Potassium hydroxide, Ascorbic acid, EDTA and Folin–Ciocalteu reagent were procured from Qualigens Fine Chemicals, Mumbai, India. Agar agar was obtained from Sigma Chemical Co., USA.

The percentage immobilization is calculated as the (total activity in immobilized gel/total activity of the soluble enzyme loaded) X 100.

2.1. Myrosinase

Enzyme was isolated from cauliflower (Brassica oleracea var. botrytis) seedlings and was purified to electrophoretic homogeneity by Native PAGE.

2.2. Immobilization of Myrosinase on Agar Agar

The purified myrosinase was immobilized in agar agar. The solutions having varying concentrations of agar agar were prepared in the extraction buffer (Potassium phosphate buffer, 20 mM, pH 7.4) by warming them at 50^{o}C. After cooling down to room temperature, 1.0 ml enzyme (containing 0.190mg protein/ml) was mixed with 9.0 ml of the matrix solution (the total volume of matrix and enzyme mixture being 10ml) and immediately casted on preassembled glass plates. After solidification at room temperature, agar agar gel was cut into small chips of 5 x 5 mm size and washed several times before use to remove any enzyme attached to the gel surface. These immobilized chips were stored in extraction buffer in refrigerator. The activity of these chips was assayed spectrophotometrically as described in the following section. The buffer, in which these chips were stored, was also periodically checked for the activity to assess the possible leaching of the enzyme.

2.3. Immobilized Myrosinase Assay

Myrosinase activity was determined by measuring the rate of decrease in absorbance at 227 nm resulting from the hydrolysis of sinigrin as per method [17]. Two immobilized agar agar chips (3.0-4.0 µg protein/chip) were used for enzyme assay instead of the soluble form of enzyme. Protein concentrations were determined with BSA as a standard [18]. Specific activities are expressed as units/mg of protein.

2.4. Optimization of Agar Agar Concentration for Immobilization of Myrosinase

In order to optimize entrapment, the concentration of agar agar was varied and the extent of immobilization was assessed.

2.5. Storage Stability of Immobilized Enzyme in Agar Agar

In order to explore storage stability and leaching of the enzyme from immobilized preparations, freshly prepared immobilized chips were stored in extraction buffer (20mM potassium phosphate buffer, pH 7.4) at 4^{o}C. The activities of the enzyme in chips and the storage media (for leaching) were assayed at different time intervals using standard assay protocol.

2.6. Effect of Metal ions on Immobilized Enzyme

It was of interest to explore the possible effects of metal ions on the immobilized myrosinase. To study this, a stock solution (50 mM) of the desired salt of metal ions (Fe^{3+}, Zn^{2+}, Cu^{2+} and Fe^{2+}) was made in potassium phosphate buffer (33mM, pH 6.0) and diluted with the same buffer as required.

2.7. Effect of Heavy Metal Ions on Immobilized Enzyme

Effect of Sn^{2+}, Sr^{2+} and Ba^{2+}ions was studied in the concentration range of 1.0-40 mM, added in the standard assay mixture and assayed the activity as described earlier.

2.8. Effect of Non Polar Organic Solvents on Immobilized Enzyme

It is interesting to know the behaviour of immobilized enzyme in the organic solvents. The effect of some organic solvents like toluene, benzene and chloroform on the immobilized system was studied in the concentration range of 10-50% (v/v) added in the standard assay system.

2.9. Effect of Protic Polar Organic Solvents on Immobilized Enzyme

In immobilized system, the effect of some protic polar organic solvents like methanol, ethanol, propanol and

butanol was explored in the concentration range of 10-50% (v/v) added in the standard assay system.

3. Results and Discussion

3.1. Optimization of Agar Agar Concentration for Immobilization of Myrosinase

In order to optimize entrapment, the concentration of agar agar was varied and assessed the extent of immobilization. As it is evident from table-1, there was only about 25% immobilization in 2.0 % (w/v) agar agar and the membrane also was very fragile and susceptible to damage during handling. At a concentration of 3%, more stable agar agar chips were obtained with immobilization of about 52%. Further, a concentration of 3.5% resulted in maximum immobilization (70%). Afterward, increase in agar agar concentration resulted in to poor immobilization and at 6%, the solidification of the gel occurred even before the addition of the enzyme. Therefore, 3.5% agar agar (w/v) was selected for further studies. In a recent study, a similar finding was reported in soybean α-amylase where 77% entrapment of enzyme obtained with 4% agar agar [19].

Table1. *Effect of varying concentration of agar agar on immobilization of myrosinase.*

Agar agar concentration (% w/v)	Immobilization %
2	25
3	52
3.5	70
4	65
5	34
6	Got solidified before the addition of enzyme
CD (5%)	27.85

CD= Critical Difference

3.2. Storage Stability of Immobilized Enzyme in Agar Agar

In order to explore storage stability and leaching of the enzyme from immobilized preparations. The activities of the enzyme in chips and the storage media (for leaching) were assayed at different time intervals using standard assay protocol. The result (Table-2) indicated that upon storage, the chips leached enzyme gradually in to the storage media. Thus on fourth day of storage, the chips retained about half of the initial activity and about half of the activity was exuded into the storage buffer.

Table2. *Relative activities of myrosinase in immobilized chips and in storage buffer.*

Days	% Relative activity in immobilized chips	% Relative activity in chip storage buffer
0	100	-
1	79	21
2	70	30
3	54	46
4	51	49
5	48	52
CD (5%)	21.13	21.13

The observations are suggestive of the fact that agar agar immobilized myrosinase can be conveniently used for 48 hrs and are not suitable of longer storage.

3.3. Effect of Metal ions on Immobilized Enzyme

The activity of the agar agar immobilized enzyme was determined in the presence of varying concentrations (1-10 mM) of Fe^{3+}, Zn^{2+},$Cu^{2+}$and Fe^{2+}added in the standard assay mixture. The results (Fig.1) revealed a concentration dependent inhibition of the activity by all the heavy metal ions studied. This observation was essentially identical to the one reported for the soluble enzyme [21].

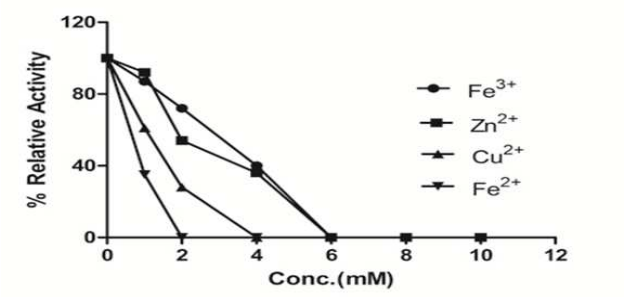

Figure 1. *Effect of heavy metal ions on agar agar immobilized cauliflower myrosinase activity.*

3.4. Effect of Heavy Metal ions on Immobilized Enzyme

These ions (Sn^{2+}, Sr^{2+} and Ba^{2+}) at lower concentration had strong activating effects on the immobilized enzyme (Fig.2). Amongst these three ions, Sr^{2+} had a strongest activation effect and activity continuously increased from 0-4 mM and maximum was recorded at 4mM (nearly 3 times of the control).After this activity decreased slowly and the enzyme became completely inactive at 40mM. However, Sr^{2+} ions were an inhibitor of bacterial myrosinase [20]. This difference in the behaviour might be due to the structural difference in the myrosinase. Plant myrosinase had a rather large molecular weight as

compared to the bacterial enzyme. Similarly, stannous ions had maximum activity (1.67 times of control) at 2mM concentration and beyond which there was a decrease in the activity, while no activity was recorded at 6mM onwards.

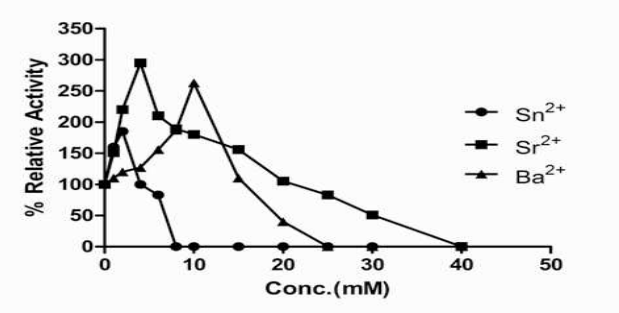

Figure 2. *Effect of heavy metal ions on immobilized cauliflower myrosinase activity.*

Similar observations of minor activation were also reported in bacterial myrosinase[20]. Ba^{2+}showed less activation amongst these metal ions and attained its maximumat 10mM.Above results suggest that heavy metal ions are excellent activators of the immobilized enzyme at lower concentrations, with soluble enzyme showing a similar trend with lower activation effect [21].

3.5. Time-Course of Effect of Sr^{2+} on the Activity of the Myrosinase

As described earlier (Fig.2), Sr^{2+} at 4.00 mM had maximum activating effect of immobilized myrosinase. In order to explore the rate of activation over time, three concentrations viz., 1, 2 and 4mM, were selected and effect of these were studied for 20 min. Fig.3 indicated that at 4mM concentration of Sr^{2+}, the rate of reaction was higher than its lower concentrations. Nearly till 15 min the trend of reaction was constant after which a little decrease in the activity was detected (remains 80% of maximum) till 20 min.

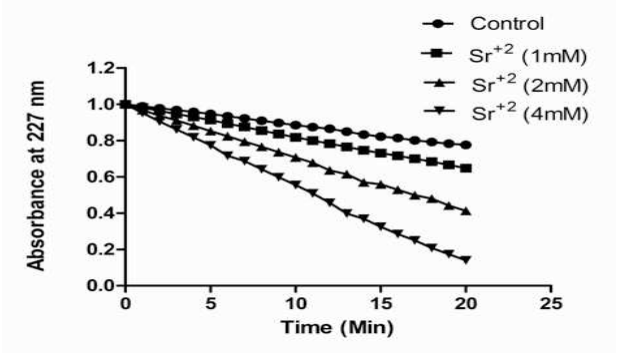

Figure 3. *Time-dependent hydrolysis of sinigrin by agar agar immobilized cauliflower myrosinase with specific concentration of Sr2+.*

3.6. Time-Course of Myrosinase Action in Presence of Sn^{2+}

As has been observed earlier in Fig.2, Sn^{2+}exhibited activation of myrosinase activity in the concentration range of 1-6 mM. Considering this, a time–course of effect of metal ion at the concentration of 1&2 mM was studied. The metal ion was added in the reaction mixture and the progress of reaction was studied for 20 min (Fig.4).The result revealed that rate of reaction was constant till 13 min and after which little decrease in the enzyme activity was seenupto 20 min in both the concentrations selected.

The above results suggested that heavy metal ions at suitable concentrations are probably stabilizing the enzyme such that the myrosinase continues to act on its substrate for a longer period of time, without significant decrease in the activity. In addition, a more significant increase in enzyme activity in case of agar agar immobilized chips than soluble myrosinase are indicative of greater stabilization of cauliflower myrosinase in immobilized system than the soluble one.

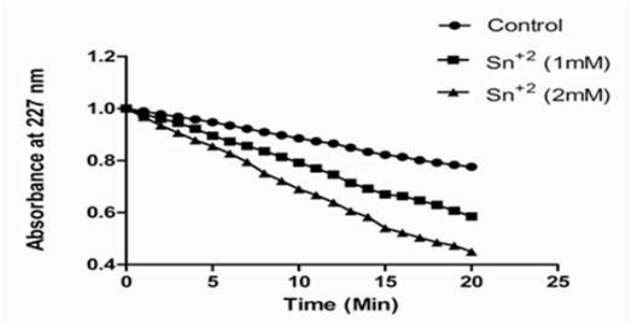

Figure 4. *Time-dependent hydrolysis of sinigrin by agar agar immobilized cauliflower myrosinase with selective concentration of Sn2+.*

3.7. Effect of Non Polar Organic Solvents on Immobilized Enzyme

It is interesting to know the behavior of immobilized enzyme in the organic solvents. The effect of some organic solvents like toluene, benzene and chloroform (which indicated an activation in the activity of soluble enzyme) on the immobilized system was studied in the concentration range of 10-50% (v/v) added in the standard assay system. Fig.5 indicated activation effect in immobilized chips over all selected concentrations. Activation pattern was toluene>benzene>chloroform at 50% (v/v) concentration. The enhancement was higher in immobilized myrosinase than the soluble enzyme.

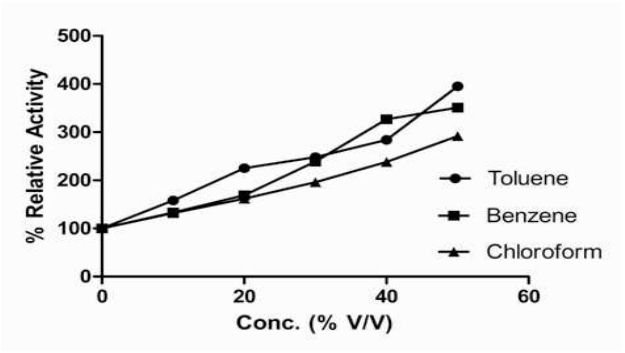

Figure 5. *Effect of non polar organic solvent on agar agar immobilized cauliflower myrosinase activity.*

3.8. Time-Course of Effect of Toluene on the Activity of the Immobilized Myrosinase

As evident earlier (Fig.5), toluene in the concentration range of 10-50 % (v/v) had activation effect on immobilized myrosinase. In order to explore the time-course of activation, two concentrations viz., 10 and 20% (v/v), were selected and studied for 27 min. Fig.6 indicated that at 10% concentration of toluene, the rate of reaction was lower than 20%. Time-dependent rate of reaction was recorded at these concentrations. Nearly till 19 min the rate of reaction was constant after which a small decrease in activity was detected up to 27 min.

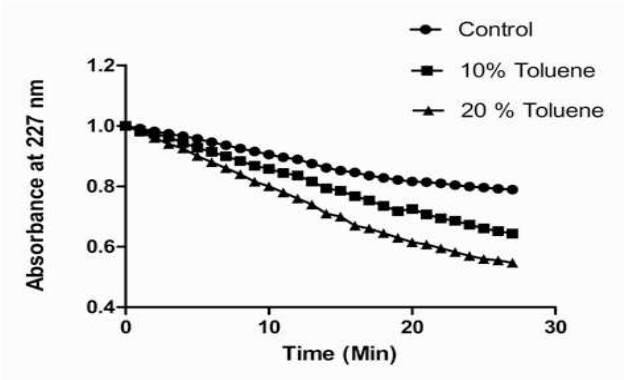

Figure 6. *Time-dependent hydrolysis of sinigrin by agar agar immobilized cauliflower myrosinase.*

3.9. Time-Course of Immobilized Myrosinase Action in Presence of Chloroform

As has been described earlier in Fig.5, chloroform also exhibited activation pattern in immobilized myrosinase. Considering this, a time–course of effect of chloroformat the concentration of 10 & 20% (v/v) was studied. The selected concentrations of chloroform were added in the reaction mixture and the progress of reaction was monitored up to 27 min (Fig.7). The result revealed that rate of reaction was constant till 16 min and after which little reduction in enzyme activity was noted till 27 min in both the concentrations selected.

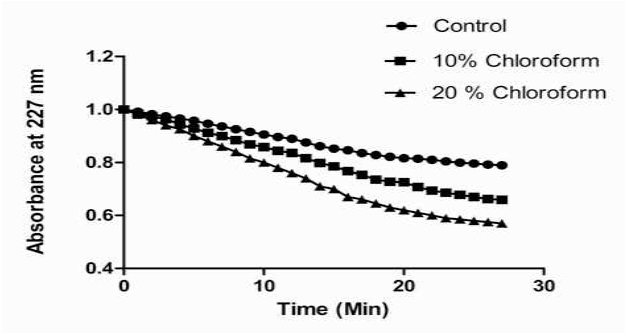

Figure 7. *Time-dependent hydrolysis of sinigrin by agar agar immobilized cauliflower myrosinase.*

However, the fact that immobilized myrosinase exhibited significant stability in some organic solvent is suggestive for its industrial application viz., health and processed food industry. Similar to our finding, a remarkable stability of α-amylase (isolated from Nsterenkonia sp.) was found towards organic solvents like cyclohexane, benzene, toluene and chloroform [22]. The significant activity of soybean α-amylase was reported in organic solventsviz., toluene, benzene and chloroform [23].

3.10. Effect of Protic Polar Organic Solvents on Immobilized Enzyme

In immobilized system, the effect of some protic polar organic solvents like methanol, ethanol, propanol and butanol was explored in the concentration range of 10-50% (v/v) added in the standard assay system. With the exception of butanol, these solvents have already shown inhibition effect in soluble enzyme. In this study, results (Fig.8) also indicated a similar decreasing effect in activity of immobilized chips except in the presence of butanol. Methanol, ethanol and propanol were exhibiting ≤ 50% activity at 30 % (v/v) concentrations and complete loss inactivity were seen at 50% (v/v). The activity of myrosinase was deceased in presence of several organic solvents, like simple alcohols, but these solvents are not able to prevent reaction taking place in case of the soluble enzyme [24].

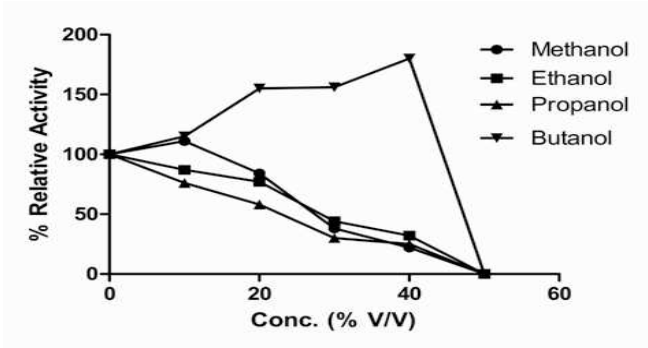

Figure 8*. Effect of protic polar organic solvent on agar agar immobilized cauliflower myrosinase activity.*

4. Conclusion

This preliminary work demonstrates that myrosinase, which is contained insignificant amounts in cauliflower seedlings and easily binds to an inexpensive solid support such as agar agar with interesting absorption and immobilization yields, with good chemical characteristics. The stability of this enzyme was better at low concentration of metal ions increasing its possibilities for application. From the technological point of view, immobilized enzymes can easily be separated from their action liquid and make laborious separation steps unnecessary. Additional benefits arise from stabilization against harsh reaction conditions which are deleterious to soluble enzyme preparations. Due to the wide variation in the properties of

the individual enzyme species and due to the varying requirements of reaction technology for the target compounds it is advisable to exploit fully the wealth of methods and techniques of immobilization. So agar agar immobilized myrosinase is suitable for both the technical requirements and the overall business framework.

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