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ENHANCEMENT OF L-PHENYLALANINE PRODUCTION BY AMINOACYLASE-CHITOSAN COMPLEX

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ABSTRACT

Aminoacylase (E.C.3.5.1.14) catalyses the enantioselective hydrolysis of N-acetyl-L-amino acids. It is immobilized by forming an insoluble complex with chitosan to produce L-phenylalanine from a racemic mixture of N-acetyl-DL-phenylalanine. The aminoacylase-chitosan complex enhanced the yield of production of L-phenylalanine by about 400% at neutral pH, at 65 °C and at alkaline pH (7.5-8.5) at 37 °C.

Keywords: aminoacylase, chitosan, L-phenylalanine, enzyme, immobilization

1. INTRODUCTION

Enzymes have a wide variety of biotechnological, biomedical and pharmaceutical applications. They are used as catalysts for chemical or biochemical applications, and as biosensors, clinically as therapeutic agents, and as diagnostics devices, very often after being immobilized in variousmatrixes[1,2].Microencapsulation of enzymes has proved to be a useful immobilization technique[3-8]. Microcapsules containing enzymes can be easily prepared, separated from the reaction medium by filtration, and the immobilized enzyme systems can be used in several batch-wise reactions or continuously in a bioreactor[1,4].

The amino acid, L-phenylalanine, is one of the essential amino acids, frequently used as an amino acid supplement for

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human and animal feed, for intravenous infusion for medical purposes and for the synthesis of low calorie dipeptide sweetener, aspartame[9], which is about 180 times sweeter than sucrose.

Various methods of L-phenylalanine production have been reported. These include (i) direct fermentation from carbohydrates[10], (ii) microbial production of enzymes which converts a variety of chemical processes to phenylalanine[11], (iii) chemical synthesis, [12] and (iv) a combination of chemical and enzymatic methods[3,4,13]. The last method was used in this work. This method involves the optical resolution of a racemic mixture of chemically synthesized *N*-acetyl-DL-phenylalanine using the enzyme aminoacylase[3,4,13] (E.C.3.5.1.14) which catalyzes the enantioselective hydrolysis of *N*-acetyl-DL-phenylalanine producing L-phenylalanine. Previously, we have immobilized aminoacylase in stabilized calcium alginate beads[3,4] for the production of L-phenylalanine. Chitosan[14], a polycationic of polyglucosamine has been used for surface coating of polyanionic alginate beads.[3,5-8] for encapsulation of enzymes. In this work, chitosan was used to immobilize aminoacylase by forming an insoluble aminoacylase-chitosan complex for the production of L-phenylalanine. The thermal behaviour and the effect of pH on the enzymatic activity of the immobilized aminoacylase in chitosan complex were characterized and compared with the free aminoacylase enzyme.

2. MATERIALS AND METHODS

2.1 Enzyme and Chemical reagents

Acylase I from *Aspergillus* species was purchased from Sigma Chemical Co. It has a specific activity of 0.5 units/mg solid. One unit of enzyme activity is defined as the amount of enzyme that catalyses the hydrolysis of 1 µmol of *N*-acetyl-DL-phenylalaline-amino acids. *N*-acetyl-L-phenylalanine, L-phenylalanine, and *N*-hydroxyethylpiperazine-*N*-ethanesulphonic acid (HEPES) were purchased from Sigma. Ninhydrin, cobalt chloride, chitosan (low molecular weight), Tris hydroxymethylmethane, and stannous chloride were purchased from Fluka. Sodium alginate and calcium chloride were purchased from BDH. All chemicals were used without further purification.

2.2 Preparation of chitosan-aminoacylase complex

Forty ml of aminaoacylase solution (0.01 g/ml in distilled water) was added to 40 ml of 1.5 % chitosan solution (pH 5.6). The mixture was left at room temperature for 24 hours. The supernatant was removed by centrifugation and the precipitate was washed with distilled water, and was dried on a sintered glass funnel. The chitosan complex was kept at 4 °C until use.

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2.3 Activity assay of free aminoacylase

To 0.2 ml (0.33 mg/ml) of enzyme dissolved in 0.1 M Tris buffer, pH 7.0, was added 0.2 ml 0.5 mM $CoCl_2$ solution. The mixture was brought to 37 °C and 0.4 ml 33.33 mM substrate, *N*-acetyl-DL-phenylalanine was added. The reaction was allowed to continue for 30 min and then stopped by placing the solution in a boiling water bath for an additional 30 min. The liberated L-phenylalanine was measured by the standard ninhydrin colorimetric method. The absorbance of the solution was measured at 570 nm with a Hitachi U-1100 UV-VIS spectrophotometer at room temperature. The amount of liberated L-phenylalanine was determined by preparing a standard curve with L-phenylalanine treated in the identical manner except that the enzyme and the substrate was replaced with Tris buffer. One unit of enzyme activity was defined as that amount required to liberate 1 μmole of L-phenylalnine per min at pH 7.0 and at 37 °C.

2.4 Activity assay of immobilized aminoacylase

A reaction mixture containing 3 mg of aminoacylase-chitosan complex, 0.6 ml of 0.5 mM $CoCl_2$, 2.4 ml of 25 mM *N*-acetyl-DL-phenylalanine in 0.1 M Tris buffer (pH 7.0) was incubated at 37 °C for 30 min. The reaction was then stopped by transferring the supernatant into another tube and boiled for 15 min. One ml of the solution was then used to determine the liberated L-phenylalanine by standard ninhydrin colorimetric method.

2.5 Determination of protein content in the complex

Aminoacylase–chitosan complex were suspended in 3.0 ml of 0.1 M acetate buffer (pH 5.0). The mixture was then centrifuged and the supernatant was used for protein determination by the Lowry method[15]. Chitosan beads that did not contain enzyme were used as a control. The enzyme in the aminoacylase-chitosan complex is estimated by taking the difference of the protein concentration before and after complexation.

2.6 Thermal stability study

Three mg of aminoacylase-chitosan complex were suspended in 0.6 ml of 0.5 mM $CoCl_2$ solution. The mixture was pre-incubated at various temperatures (25-80 °C) for 10 min, and cooled rapidly in an ice-bath for 1 min before the activity of the complex was assayed under standard condition.

2.7 Optimum temperature study

The aminoacylase–chitosan complex activity was determined under standard condition but the temperature of incubation was varied from 30 to 80 °C.

2.8 Effect of pH

The immobilized aminoacylase assay was done under standard condition except that the pH of substrate solution was varied. The *N*-acetyl-DL-phenylalanine was dissolved in 0.1 M Hepes buffer and the final pH was adjusted to 6.0 – 8.5.

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3. RESULTS AND DISCUSSION

3.1 Specific activity

Aminoacylase formed an insoluble complex with chitosan when it was allowed to mix freely with chitosan in solution. The complex formation may involve electrostatic interactions between aminoacylase and chitosan as well as non-ionic interactions of the enzyme with the carbohydrate backbone[16]. This resulted in an immobilization yield of about 6%. The immobilized aminocylase is active although its specific activity (0.42 _mol/min/mg) was lower than that of the free enzymes (3.74 _mol/min/mg).

3.2 Thermal behaviour

The thermal stability profiles and optimum temperature profiles of the free and immobilized aminoacylase are shown in Figures 1 and 2 respectively.

From Figure 1, it is observed that the aminoacylase enzyme in chitosan complex was apparently thermally more stable than the free enzyme. The immobilized enzyme showed higher activities at temperatures 50-65 °C and only lost about 50% of its activity at 70 °C. However, the free enzyme is almost completely deactivated at this temperature. It is noted from the optimum temperature profile (Figure 2) that the activities of the immobilized enzyme at 55 °C and 65 °C were enhanced by 350% and 400%, respectively as compared with the free enzyme.

The enhanced thermal stability and activity of immobilized aminoacylase in the chitosan complex at high temperatures (55-65 °C), may possibly be explained as follows. The non-ionic interaction with the carbohydrate backbone[16] and ionic interactions with chitosan may cause a change in conformation[17] that may stabilize the enzyme towards heating effect. With the greater progressive weakening of ionic interaction at higher temperatures, the increase in conformational flexibility may expose the microenvironment of the active site and thereby increases the accessibility of the substrate for the hydrolysis reaction. Concomitantly, ionic interactions of the substrate with chitosan may also be weakened at high temperatures. Therefore there is less diffusion restriction of the substrate towards the microenvironment of the active site of the enzyme.

The two peaks observed for the aminoacylase-chitosan complex in the temperature stability profile and optimum temperature profile seem to suggest the possibility that aminoacylase in the chitosan complex is heterogeneous with two different conformations.

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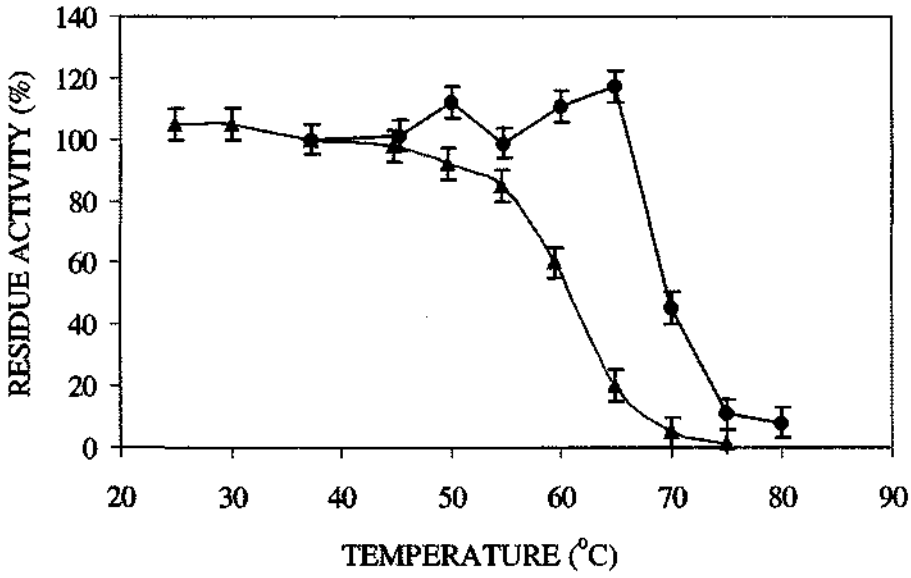

Figure 1: Thermal stability of free (-▲-) and immobilized aminoacylase (-●-). The enzymatic activity measured at 37 °C was taken as 100%. Data were mean of duplicate measurements.

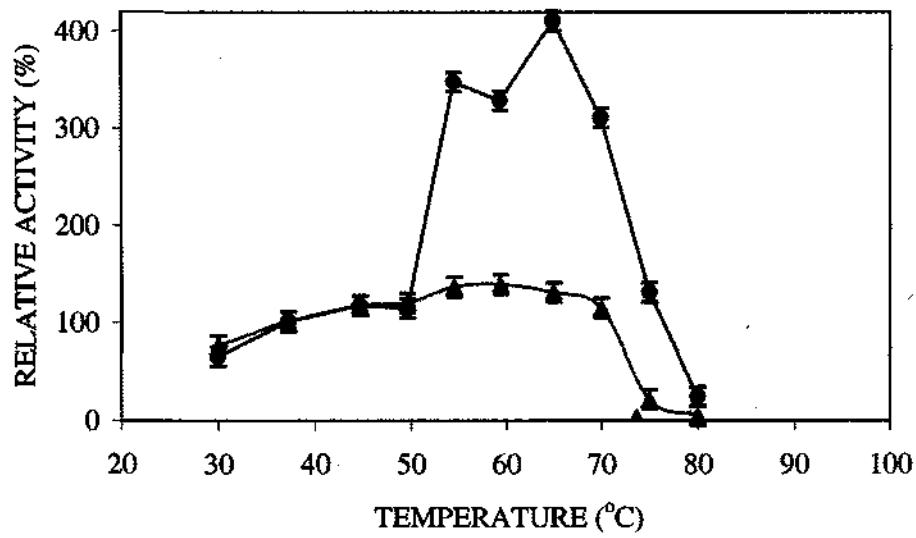

Figure 2: Optimum temperature study of free (-▲-) and immobilized aminoacylase (-●-). The enzymatic activity at 37 °C was taken as 100%. Data were mean of duplicate measurements.

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3.3 Effect of pH

The effect of pH on the activity of optical resolution of the racemic mixture of *N*-acetyl DL-phenylalanine was also investigated. The buffer used in this study was 0.1 M Hepes buffer (pH 6.0–8.5). Figure 3 shows the pH profile of free aminoacylase and immobilized aminoacylase. The activities of the free aminoacylase were almost constant at pH 6-7 and reduced at higher pH. However, the aminoacylase in the chitosan complex showed approximately four fold increase in activity at pH 7.5-8.5.

At low pH, the basic amino acid residues in the microenvironment of the active site are electrostatically bonded to the positively charged amino groups of chitosan. As pH is raised above 7.0, the amino groups of chitosan become deprotonated and weaken the above interaction resulting in the exposure of the active site of the enzyme and therefore the enzymatic activity is enhanced. Concomitantly, the amino acid substrate is also free from binding to the chitosan, allowing it to diffuse more freely to the active site.

The wide optima pH range observed for the aminoacylase-chitosan complex suggests that more than one type of polar and ionizable amino acid residues may involve in the active site.

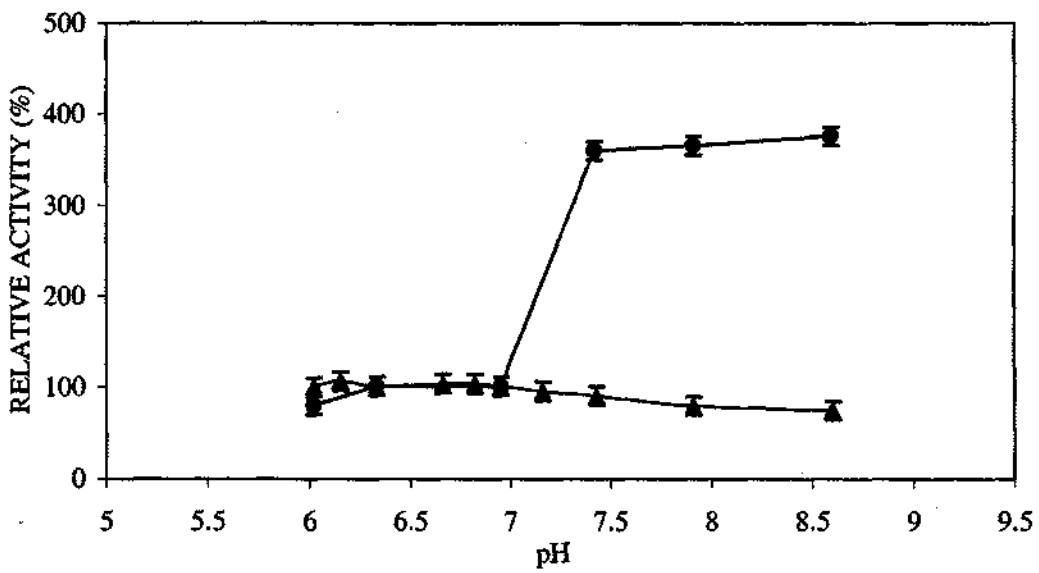

Figure 3: Effect of pH on the activities of free (-▲-) and immobilized aminoacylase (-●-). The enzymatic activity at pH 7.0 was taken as 100%. Data were the mean of duplicate measurements.

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The results of both thermal and pH studies seem to suggest that the weakening of ionic interactions of the enzyme and the substrate with chitosan lead to an enhancement of the immobilized enzymatic activity. The conformational change of the enzyme as a result of complexation with chitosan may also result in the increase in thermal stability and higher activities observed.

It is interesting to report that the immobilized system can be reused for at least ten cycles of reactions and also can be used continuously in a bioreactor for at least 21 days for the production of L-phenylalanine (data not shown).

4. CONCLUSION

Aminoacylase immobilized in the chitosan complex is more thermally stable and active. Production of L-phenylalanine is enhanced by about four fold by the aminoacylase-chitosan complex when operates at alkaline pH, at 37 °C as well as at temperatures of 55 °C and 65 °C.at neutral pH. These conditions are mild and readily accessible. Further work will be required to delineate the mechanism of the enhancement of activities observed.

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