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DEVELOPMENT OF IMMOBILIZATION AND ENCAPSULATION TECHNIQUES FOR BIOACTIVE MOLECULES

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ABSTRACT

Bioactive molecules such as enzyme: aminoacylase I (EC 3.5.1.14), and natural colorants :anthocyanins extracts of red cabbage, red grape skin and curcuminoids extract of tumeric (Curcuma longa L.) were immobilized and encapsulated in polysaccharides. The enzyme aminoacylase was immobilized by (i) surface immobilization on chitosan beads and (ii) by complexation with chitosan for the production of L-phenylalanine from a racemic mixture of N-acetyl-DL-phenylalanine. The two immobilized enzyme systems were characterized in terms of thermal stability, effects of pH and temperature, kinetic constants and operational stability. The activity and optimal temperature of immobilized enzyme were higher than those of the free enzyme. The immobilized aminoacylase by complexation with chitosan was a better system. The activities were enhanced by about 400% at neutral and alkaline pH and at temperatures of 50 – 70 ° C. In addition, it afforded good operational stability when used as a plug-flow bioreactor and batch reactor. The natural colorants such as red anthocyanins extracts and curcuminoids extract were successfully encapsulated in calcium alginate beads and in chitosan beads respectively. The stabilities of anthocyanins and curcuminoids in the beads were comparable to those in solutions.

INTRODUCTION

The immobilization of enzyme to insoluble support has been a topic of active interests in enzyme technology. Techniques of enzyme immobilization have been developed and applied in commercial processes (Vorlop & Klein, 1983; Elcin & Elcin, 2000). The major objective of enzyme immobilization is to obtain an immobilized biocatalyst with high retention of activity, high operational stability and thus the process cost can be reduced.

Aminoacylase (E.C.3.5.1.14) catalyses the hydrolysis of *N*-acetyl-*L*-amino acids to produce *L*-amino acids. It is used for the optical resolution of racemic mixture of *DL*-amino acids. The amino acid that we are interested is *L*-Phenylalanine, which is one of the essential amino acids. It was used as an amino acid supplement for human and animal feed, for intravenous infusion for medical purposes and for the synthesis of low calorie dipeptide sweetener, aspartame (Lee et. al, 1993b).

We have successfully developed stabilized calcium alginate bead systems (Lee et .al. 1992 & 1993a) for the encapsulation of aminoacylase for the production of *L*-phenylalanine using chitosan as a surface coating memberane on alginate (Lee et. al 1992). Chitosan is the *N*-deacetylated (glucosamine) products of chitin which is a 1,4-linked *N*-acetyl- -*D*-glucosaminan.. They are natural amino polysaccharides, biodegradable and biocompatible. In this project, we extended the application of chitosan for immobilization of enzyme. The immobilized aminoacylase systems were prepared by (i) surface immobilization of aminoacylase onto chitosan beads and (ii) by forming an insoluble aminoacylase-chitosan complex. The immobilized systems were characterized in terms of thermal stability, effects of pH and temperature, kinetic constants and operational stability.

Other types of bioactive molecules of interest for encapsulation studies are natural colorants. Colour is one of the most important additives in food because of its intrinsic aesthetic value and as a basis for identification and quality judgement (Hendry & Houghton, 1996). The progressive reduction in the number of synthetic colours for use in food because of their toxicological effects has resulted in great interests in research into the availability and application of naturally occurring colorants in food. Natural colorants are usually unstable to light, pH and temperature (Hendry & Houghton, 1996). They are generally stabilized by the addition of various additives such as cellulose, polyethylene glycol, glycerine, collagen, gelatine and calcium alginates (Saito & Mori, 1995). In addition, colorants can be protected from photo-oxidation, air oxidation and other environmental factors by encapsulation processes and enhance the shelf life for these molecules (Saito & Mori, 1995). In this project, attempts were made to encapsulate the natural colorants namely anthocyanins and curcuminoids in beads prepared from alginate and chitosan. Anthocyanins were extracted from red cabbage and red grape skin and curcuminoids were isolated from tumeirc (*Curcuma Longa L.*).

Anthocyanindins are flavonoids or polyhydroxy and methoxy derivatives of a basic structure of the 2-phenyl-benzopyrylium or flavylium cation (Hendry & Houghton, 1996). Anthocyanins are glycosylated anthocyanidins. *Curcuma Longa (Domestica) L* belonging to the ginger family (Zingiberacease) are widely distributed in Malaysia.. Curcumin I bis (4-hydroxy-3-methoxyphenoxyl)-1,6-heptadiene-3,5-dione, is a natural yellow dye (Hendry & Houghton, 1996).

PROBLEM STATEMENT

1. Enzymatic processes are usually costly as enzymes are delicate and expensive.
2. Natural colorants are usually difficult to purify and they are usually not stable towards various environmental conditions.

OBJECTIVES

1. To develop economical enzyme systems for industrial processes.
2. To develop immobilized techniques for enzymes.
3. To develop encapsulation methods for natural colorant.

MATERIALS

Most of the chemicals were purchased from Sigma and they were used without purification. Red grapes and tumric were purchased from a wet market in Shah Alam. Red cabbage was purchased from a supermarket in Shah Alam, Selangor.

METHODOLOGY

Surface immobilization by physical adsorption of aminoacylase on chitosan beads

Chitosan solution was added dropwise into a gently stirred solution of tripolyphosphate. Beads formed instantaneously. The beads were then allowed to harden in the tripolyphosphate solution for 30 min, washed with distilled water and then suspended in 50 cm^3 of aminoacylase solution (0.01 g cm^{-3} distilled water) for 1 hour. The beads adsorbed with aminoacylase were then washed with distilled water a few times and they were kept at 4 °C in distilled water until use.

Preparation of chitosan-aminoacylase complex

Aminaoacylase solution (0.01 g cm^{-3} in distilled water) was added to chitosan solution. 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 sinted glass funnel. The chitosan complex was then kept at 4°C until use.

Method for standard activity assay of immobilized aminoacylase and native enzyme, methods of characterization of immobilized enzymes and native enzyme were carried out as reported by Lee at al., 1992.

Extraction and encapsulation of natural colorant

Anthocyanins were extracted from red cabbabge and red grape skin with solvent after homogenization. Curcuminoids were extracted from tumeric. TLC analysis were made on the extracts with appropriate eluent. Anthocyanins extracts were encapsulated in alginate beads according to the method of Lee et al (1993). Curcumin was encapsulated in chitosan beads by the method as described earlier for enzyme.

RESULTS AND DISCUSSION

Specific activity of aminaoacylase

The aminoacylase immobilized on chitosan beads and aminoacylase-chitosan comples were active with specific activity of 1.79 and 0.42 µmol/min/mg respectively, which were lower than that of the free enzymes (3.74 µmol/min/mg). It is possible that the strong electrostatic interactions of enzymes with chitosan have restricted the mobility of the enzyme molecules and may hinder the interaction with the substrates. Alternatively. the electrostatic interactions probably caused a detrimental effect on the enzyme as observed previously when polyethyleneimine or glutaraldehyde were used as a stabilizing agent for the calcium alginate beads (Lee et al, 1992).

Thermal stability

The effect of temperature on the stability of the native and the immobilized aminoacylase was investigated by incubating the samples at temperatures range of 25-80°C for 10 min. and the residual activities were then assayed a 37°C. The activity of the rate of hydrolysis at 37°C by each of the systems was taken as 100%. The thermal stability profiles are shown in Figure 1.

All the enzyme systems were stable at temperatures below 50°C. The two immobilized enzyme systems were more stable than the free enzyme at higher temperatures. At 62°C, the free enzyme lost 50% of its activity, but the immobilized enzyme however, still retained their full activities and lost about 50 % of its activity only at 70°C.

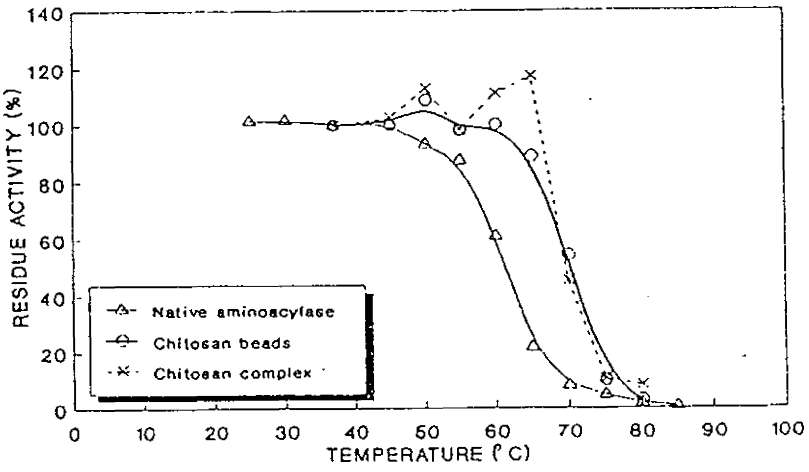


Figure 1. Thermal stability of native and immobilized aminoacylase

It is possible that the interaction of enzyme with chitosan leads to conformational changes where the enzyme active sites may be stabilized and protected and hence less susceptible to heating effect. Another possible consideration is that the electrostatic interactions is weaken at high temperatures and therefore exposing the active sites and increasing the accessibility to the substrates. The interactions of the substrates with chitosan are also weakened at high temperatures. Therefore there is less diffusional limitation of the substrates towards the active sites of the enzymes. The two peaks observed for the aminoacylase-chitosan complex seems to suggest the possibility of the existence of two active sites or two different conformations.

The immobilized aminoacylase remained active at high temperatures argues against that the low specific activity observed is a result of the detrimental effect of chitosan on the enzyme.

Optimum temperature study

The results of the effect of temperature on the activities of native and immobilized aminoacylase are shown in Figure 2. The profile of the temperature effect of the aminoacylase on chitosan beads was quite similar to that of the free enzyme. However, it is interesting to note that the aminoacylase-chitosan complex also showed two optima (55°C and 65°C) in this study and the activities were about three to four times higher as compare with the free enzyme and surface immobilized enzyme at temperatures in the range of 50-70°C.

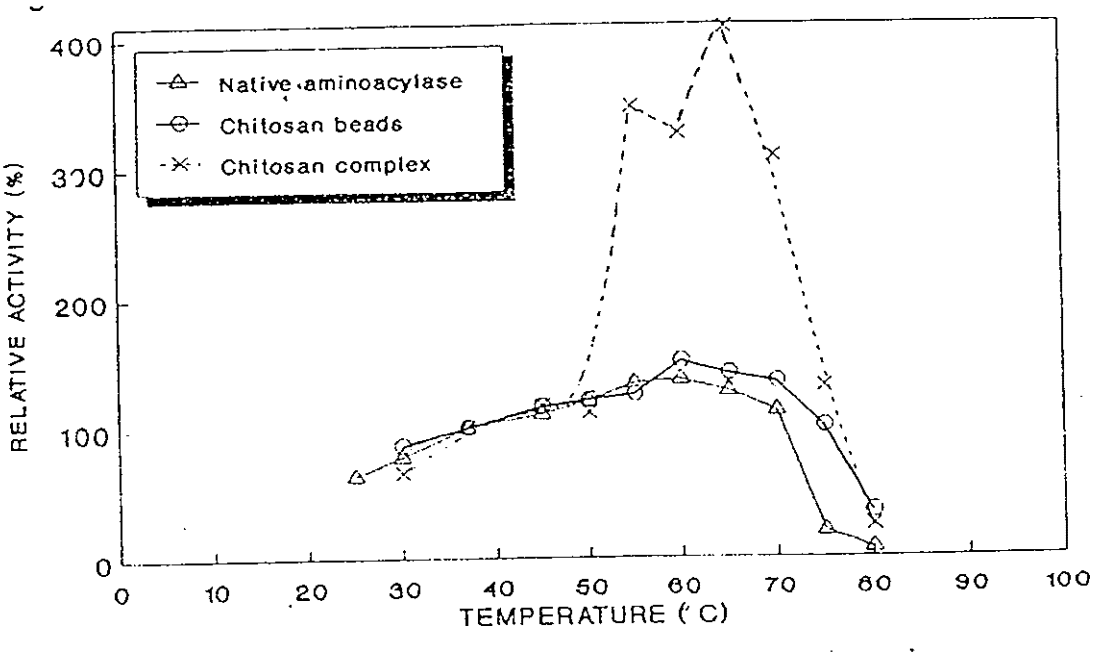


Figure 2. Optimum temperature study of native and immobilized aminoacylase

This result is consistent with that obtained for the thermal stability study which suggest the possible existence of two active sites on the enzyme when it is complexed with chitosan or that the aminoacylase in the chitosan complex is heterogeneous with two different conformations. Similar explanation as proposed for the dramatic increase in activity at high temperatures for the amnoacylase-chitosan complex observed in the thermal stability study may also be true in this study. The larger increase in the activity for the amnoacylase-chitosan complex as compare with the surface immobilized aminoacylase can possibly be explained by the local concentration of the substrates in the former, as there are more amino groups in the chitosan complex as a result of a larger surface area.

Effect of pH

The effect of pH on the optical resolution of the racemic mixture of *N*-acetylphenylalanine was also investigated. The buffer used in this study was 0.1 mol dm^{-3} Hepes buffer containing 10 mmol dm^{-3} (pH 6.0 – 8.5). Figure. 3 shows the pH profiles of native aminoacylase and immobilized aminoacylase. The activities of the native aminoacylase were not affected greatly by pH variation. However, at pH 7.0-8.5, the aminoacylase in the chitosan complex showed 400% higher in activity than the free enzyme and 200% higher in activity than the surface immobilized aminocaylase.

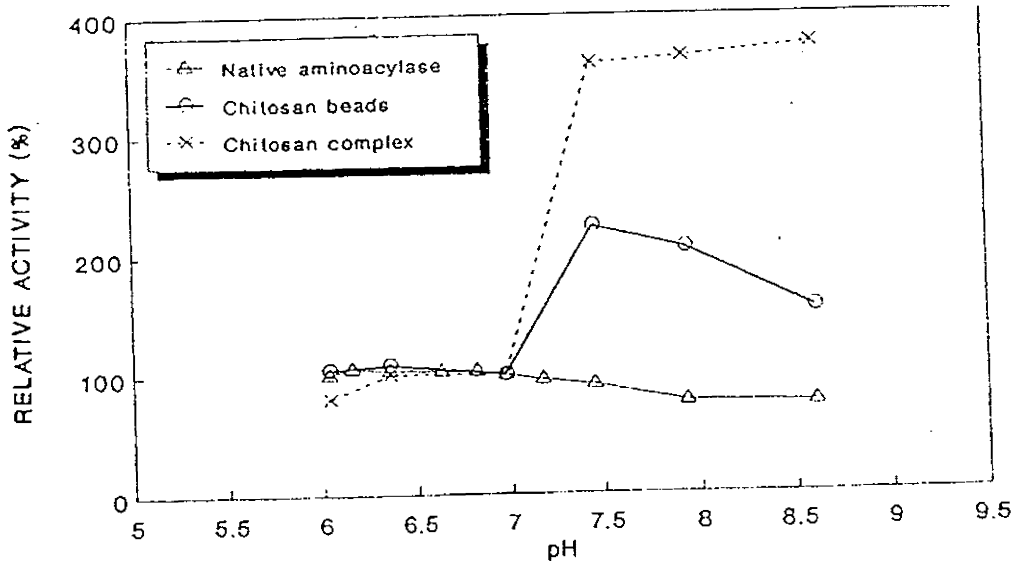


Figure 3. Effect of pH on the activities of native and immobilized aminoacylase

At low pH, the active sites are electrostatically bonded to the positively charged amino groups of chitosan. As pH was raised, the amino groups of the chitosan become deprotonated and probably liberated the active sites of the enzyme and therefore enhanced the enzyme activity. This was consistent with our observations for temperature studies for the aminoacylase-chitosan complex. The interaction of aminocylase with chitosan has resulted in conformational change and become more susceptible to heating effect and pH variation. These effects result in the exposure of active sites of the enzyme which leads to the observed higher activities. The wide optima pH range observed for the aminoacylase-chitosan complex also suggested the heterogeneity of amino acid residues involved in the active sites. However, anoptima at pH 7.4 was observed for surface immobilized aminoacylase.

Kinetic constants

The effect of substrate concentration on enzyme activity was studied to estimate the Michaelis constant (K_m) and maximum velocity (V_{max}). The kinetic constants for the native and immobilized systems determined from the Lineweaver-Burk plots were summarized in Table 1.

Table 1. Kinetic constants of native and immobilized aminoacylase.

Kinetic constants	Native aminoacylase	Aminoacylase-chitosan beads	Aminoacylase-chitosan complex
K_m (mM)	0.88	7.69	19.37
V_{max} (μmol/min)	0.021	0.045	0.124

The kinetic constants for the immobilized aminoacylase were higher that those of free enzyme. This is rather in contradictory to what one should anticipated as the carboxylic groups of the substrates is diffusionally limited by interacting strongly with the amino groups of chitosan. The likely explanation was that the complexation has resulted in conformational changes of the enzyme causing the enzyme active sites to be more accessible to substrates.

Batch operation stability study

To investigate the stability of the immobilized aminoacylase for extended operational condition, the immobilized aminoacylase was subjected to batch operational study. The immobilized enzyme activity was assayed after each cycle of reaction. The results are shown in Figure 4. It was obvious that the aminoacylase-chitosan complex was very active and stable throughout the ten cycles of operation while the aminoacylase-chitosan beads retained 60% activity after the 5th

cycles of operations. The loss of activity for the first few cycles of reaction was probably due to the desoprion of some weakly adsorbed enzyme from the beads surface. However, the aminoacylase-chitosan complex remained stable and active for at least 10 cycles of operation.

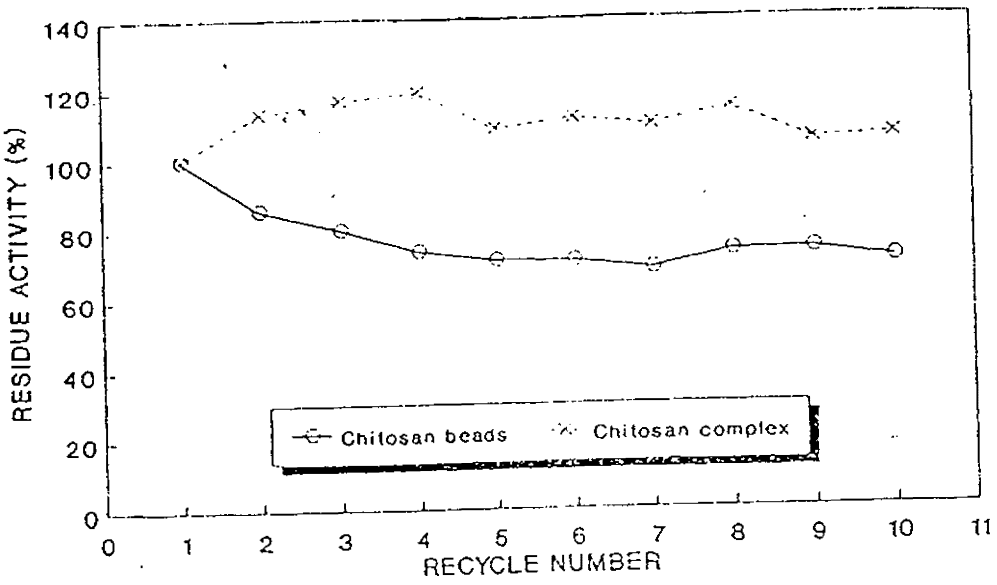


Figure 4. Batch operational stability of immobilized aminoacylase

Bioreactor-continuous operation

The two immobilized systems were packed into a bioreactor for continuous operation performance assay. The immobilized enzyme activity was assayed daily. It was observed that both immobilized systems were stable for continuous production of *L*-phenylalanine for at least 24 days.

ENCAPSULATION OF NATURAL COLORANT

Red anthocyanins extract and blue anthocyanins extract from red cabbage were encapsulated in calcium alginate beads and curcumiinoids isolated from tumeric was encapsulated in chitosan beads.

Time course studies of the release of encapsulated red and blue anthocyanins from calcium alginate beads into buffer showed that the release of colorant was very rapid for the first 20- 30 minutes and reached a plateau in about 2 hours (Figures 5 & 6). Similar trend was also observed for the release of encapsulated curcuminoids from chitosan beads (data not shown). The beads were still remained coloured at the end of the studies. The rapid release of the colorant for the first 20-30 minutes probably is the result of desorption of molecules from the beads surface.

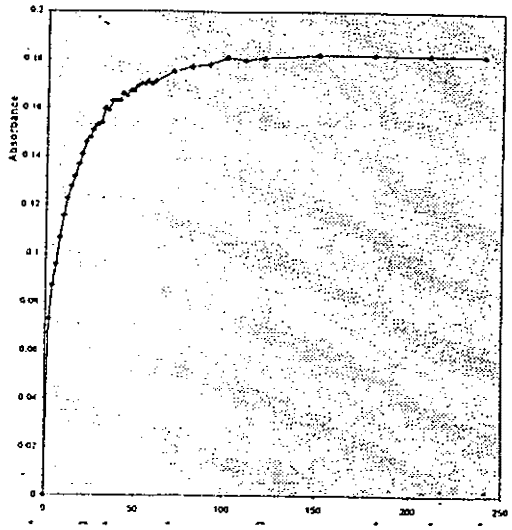


Figure 5. Time course study of the release of encapsulated red anthocyanins extract.

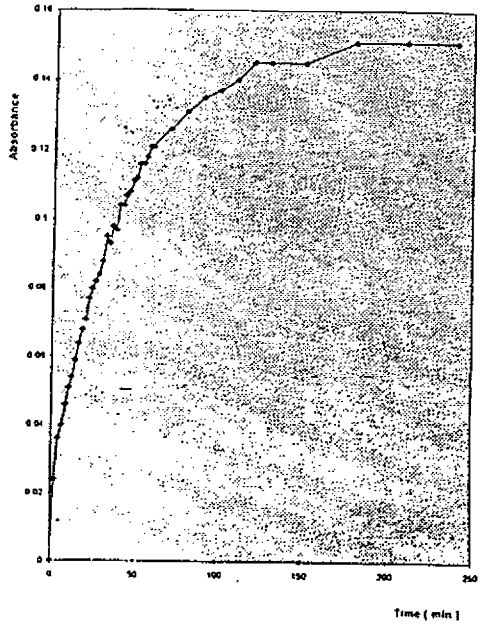


Figure 6. Time course study of the release of encapsulated blue anthocyanins extract

CONCLUSION

Bioactive molecules for instance enzymes, anthocyanins and curcuminoids extracts have been immobilized and encapsulated by polysaccharides.

The surface immobilization of aminoacylase on chitosan beads and aminoacylase-chitosan complex were active in producing *L*-phenylalanine from a racemic mixture of *N*-acretyl-*DL*-phenylalanine. The immobilization of aminoacylase by complexation with chitosan provided an efficient system for the production of *L*-phenylalanine. The aminoacylase-chitosan complex can operate at alkaline pH, and also at higher temperatures (about 70°C) which are not possible for the free enzyme system. Under these two conditions, the immobilized enzyme activities were increased by about 400% as compare with the free enzyme. The operational stabilities for this system were also excellent. No appreciable decrease in activity was detected for 10 cycles of reaction and the system can also operate continuously for at least 24 days when packed in a bioreactor. This system may be extended for immobilization of other enzymes and it may also have commercial potential.

Red and blue anthocyanins extracts have been successfully encapsulated in calcium alginate beads while curcumin were also successfully encapsulated in chitosan beads. However, methods for encapsulation to enhance the stability of encapsulated anthocyanins and curcuminoids colorants need further study.

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