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ORIGINAL CONTRIBUTION

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Activity and stability studies of ultrafine nanoencapsulated catalase and penicillinase

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Abstract The enzymes catalase (bovine liver, EC 1.11.1.6) and α-penicillinase (bacillus cereus strain 569, type I, EC 3.5.2.6) were successfully encapsulated in the polyacrylamide matrix. The encapsulation was carried out in the water pool of water/aerosol OT/*n*-hexane reverse micelles. The polymeric particles of encapsulated enzymes were reasonably monodisperse and had diameters in the range of several tens of nanometers as measured from quasi-elastic laser light scattering. The activity– pH profile of the encapsulated enzymes in buffer followed the same pattern as that of free enzymes. However, the encapsulated enzymes were found to be less active than their free forms. The enzymes in the encapsulated form were more stable (both thermal stability and shelf-life) as compared to free enzymes. The activity of the encapsulated enzymes was found to be dependent on the degree of cross-linking of the polymer matrix. The greater the cross-linking in the matrix, the lesser were the activity of the encapsulated enzyme.

Key words Nanoencapsulation – reverse micelles – aerosol OT – catalase – penicillinase – enzyme kinetics

Introduction

Ever since their initial development, the possibility of the use of immobilized enzymes as therapeutic agents have been recognized and an enormous quantity of research has been carried out in this area [1–6]. The immobilized enzymes may be employed as therapeutic agents either to replace enzymes that are missing due to genetic or other malfunctions in the individual or as agents for the specific degradation of unwanted metabolites in the diseased individual. Such diseases can be controlled, at least in theory, by injecting soluble enzyme from an external source. But sometimes this approach is dangerous in the sense that a foreign enzyme protein will almost certainly cause an allergic response which may be fatal. In recent years active drugs have been linked as site substituent to a polymeric or oligomeric structure by means of cleavable bonds. Such derivatives of PEG have a host of uses, particularly in enzyme therapy because PEG-proteins are largely non-immunogenic and non-antigenic and have a greatly increased serum life-time [7, 8]. However efficacy of the modified enzyme as drug may be reduced and the complex may not serve the purpose of sustained and slow delivery of the enzyme as drug. Encapsulation of enzymes may overcome both these problems by preventing interactions between the enzyme and the body's immune response system and by stabilizing and protecting the enzyme. Incorporation of the enzymes in capsules generally increases the circulating life time of enzymes by protecting them from proteolytic activity in the serum. They may also enhance introduction of the enzymes into target cells, since water soluble enzymes can not penetrate through the plasma membrane.

Potentially the most useful form of encapsulation is entrapment of enzymes within a biodegradable, biocompatible non-antigenic polymer material. For therapeutic applications the particle size of the polymeric matrix must be accurately controlled to avoid blockage of capillaries of the body. Particles of diameter in the micron range are retained more or less indefinitely within the peritoneal cavity but certain substrates do not cross into the cavity and thus this route cannot be used in all cases. Submicron sized particles (usually known as nanoparticles) are always desirable for such studies. Numerous studies on nanoparticles have clearly established that they can be effective as drug carriers [9]. The preparative methods for the production of nanoparticles have been extensively reviewed in a number of books [10–12]. They are prepared from a variety of polymers: gelatin, dextran, etc. [13–15]. Some of the preparative procedures of nanoparticles are based on chemical polymerization or cross-linking reaction [16]. Other procedures involve solidification of the dispersed phase of fine emulsions without any chemical reactions [17]. Nanoparticles prepared by these methods are not monodisperse and have a mean particle diameter of 30–1000 nm [18] where an average size of an enzyme is 5–50 nm. Access of reactants to the encapsulated enzyme surface for chemical reaction may take place via two modes (i) either the enzyme comes out of the polymer matrix through biochemical degradation of the polymer or (ii) the diffusion of the reactant molecules through the pores of polymeric matrix. In both the cases the reaction is considerably and undesirably delayed if the volumetric ratio of the encapsulating particle to total enzyme encapsulated in the particle is large enough. To overcome such problems we have attempted to prepare ultrafine nanoparticles (usually of diameter < 100 nm) of entrapped enzymes using the aqueous core of reverse micelles as microreactor. Polymerization in such organized surfactant assemblies as reported by several authors [19, 20] can be an elegant system for delivering enzyme as drug and, at the same time, protecting the enzyme from direct interaction with the body's immune response system. The particles obtained by this method have been reported to have narrow size distribution as the reverse micellar droplets are highly monodisperse [18]. Khmelnitsky et al. [21] have entrapped α-chymotrypsin and laccase through reverse micelles of polymeric surfactants in nonpolar organic solvents and obtained highly monodisperse nanoparticle. α-Chymotrypsin derivatized with acryloyl chloride immobilized in nanogranules retained high catalytic activity and displayed enhanced thermal stability in both aqueous and organic solvents [22]. Several authors [23–25] have reported immobilization of enzymes in microemulsion based gels with retention of activity and stability. The size of these ultrafine nanoparticles can also be modulated with the change of water pool size of the reverse micellar droplets. This form of nanoencapsulated enzyme *in vivo* would give sustained release of enzyme [26], and also the rate of enzyme release can be regulated over a wide range by regulating the particle size. These nanoparticles may have potential use in immunological preparations to improve antigen-antibody response (adjuvant) [26].

Keeping the above potential uses and advantages in mind, the enzymes such as catalase and α-penicillinase have been encapsulated in polyacrylamide matrix through reverse micellar solution and their activity and storage stability in buffer have been investigated.

Experimental

Materials

The surfactant, sodium bis(2-ethylhexyl) sulfosuccinate, i.e, aerosol OT (AOT) was of analytical grade and was a 99% pure product of Sigma (USA). *n*-Hexane procured from SRL (India) was of 99.5% purity and was used without further purification. *N,N'*-methylenebisacrylamide, acrylamide, ammonium persulfate and *N,N,N',N'*-tetramethylethylenediamine (TEMED) were products of Sigma, USA. Sodium monophosphate and sodium diphosphate were of AR quality from Glaxo, India.

Catalase (EC 1.11.1.6) was obtained from Centre for Biochemical Technology, New Delhi, and Penicillinase Type 1 (EC 3.5.2.6) from Bacillus cereus strain 569 was obtained from Sigma chemical company USA. Benzylpenicillin (Penicillin G) was obtained as a gift from Hindustan Antibiotics Ltd. and was used without further purification. Cholesterol, phenol red, and H_2O_2 were purchased from BDH (India).

Deionized double-distilled water was used to prepare buffer and reverse micellar solutions. The molar ratio of added water to AOT in reverse micelles was represented by W_0 ($= [H_2O]/[AOT]$) . The dialysis bag used was spectra/pore membrane with molecular weight cut-off 3000.

Methods

Figure 1 shows the flow sheet of the preparation of ultrafine nanoparticles of enzymes in polyacrylamide matrix in the aqueous pool of the reverse micellar droplet. An enzyme immobilization technique using acrylamide and *N,N'*-methylene bis(acrylamide) has been described by Hicks et al. [27]. In a typical experiment, in 20 ml of AOT/*n*-hexane solution 100 μl of a mixture of acrylamide and *N,N'*-methylene bis-acrylamide solution ([acrylamide] =

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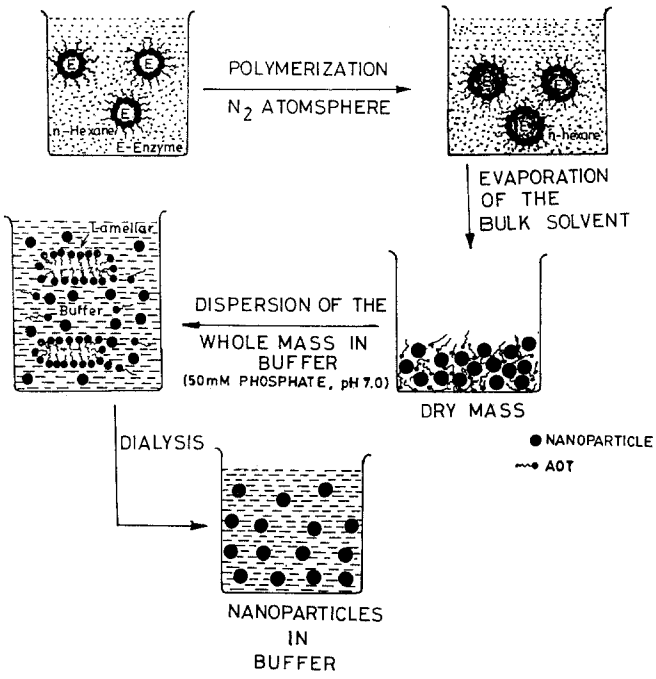


Fig. 1 Schematic representation of the preparation of reverse micelles mediated nanoencapsulated enzyme

8 mg/ml; [*N*,*N*′-methylene bis-acrylamide] = 2 mg/ml) was added. 2 μl of ammonium persulphate (80 mg/ml) and 2 μl of *N*,*N*,*N*′,*N*′-tetramethylenediamine were added to it as initiator and activator respectively. 10 μl of aqueous stock solution of the enzyme penicillinase (230 μM) was added to the solution and $w_o = 14$ was maintained adding calculated amount of buffer. All the stock solutions were prepared in phosphate buffer (pH = 7.0, 50 mM). The above solution was taken in a round bottom flask and the contents were swirled vigorously in order to ensure the transparency of the solution. The reaction mixture was purged with N_2 gas and initiator was added last. The polymerization was done in nitrogen atmosphere by bubbling nitrogen gas in the reverse micellar solution for 8 h at 25 °C. The solvent *n*-hexane was, then, evaporated out using a rotary evaporator under low pressure when a transparent dry mass was obtained. This dry mass was dispersed in about 5 ml of buffer (pH = 7.0, 50 mM) and the dispersion was transferred into a dialysis bag. It was subjected to continuous dialysis in aqueous buffer for 2 days to separate the polymeric particles from the surfactant and other unreacted small molecules.

The dialyzed solution contained nanoparticles of encapsulated enzyme and was homogeneous and almost transparent. This was used as the stock solution for the kinetic studies.

Quasi elastic laser light scattering measurements

Dynamic (quasi-elastic) laser light scattering measurements for determining the size of the encapsulated enzymes dispersed in aqueous buffer were performed using Brookhaven 9000 USA Instrument with BI 200SM goniometer. Argon ion air cool laser was operated at 488 nm as a light source. The time dependence of the intensity autocorrelation function of the scattered intensity, $c(\tau) = \langle I(o)\ I(\tau)\rangle$ was derived by using 136 channel digital photon correlator. Intensity correlation data was processed by using the method of cumulants. The translational diffusion coefficient (σ_T) of the particle dispersed in aqueous buffer was obtained from a non-linear least square fit of the correlation curve using the decay equation.

$$c(\tau) = \langle I\rangle^2\ [1 + b\ \exp(-2\Gamma\tau)]$$

with $\Gamma = \sigma_T q^2$. Here b and q are known experimental constants and $\langle I\rangle^2$ is the $\tau \to \alpha$ value of the correlation function.

From the value of translational diffusion coefficient, the average hydrodynamic diameter, D_h of the scattering particles was calculated by Stokes–Einstein relationship:

$$D_h = kT/3\pi\eta\sigma_T\ ,$$

where k is Boltzmann's constant and is the viscosity of the solvent at an absolute temperature T. The diameter D_h represents equivalent spherical diameter of encapsulated enzyme polymer matrix.

Kinetic studies of free and immobilized enzymes

Catalase and penicillinase concentrations were determined spectrophotometrically respectively at 405 nm with $E_{405\ nm} = 3.25 \times 10^5\ M^{-1}\ cm^{-1}$ [28] and at 280 nm with $E_{280\ nm} = 2.6 \times 10^4\ M^{-1}cm^{-1}$ [29]. The kinetic experiments were performed on a DU-64 Beckmann Spectrophotometer under thermostatic control of $\pm$ 0.1 °C. Unless otherwise mentioned the temperature was fixed at 25 °C. In a typical experiment the kinetics of decomposition of H_2O_2 (25.6 mM in the reaction mixture) by catalase (60 nM in the reaction mixture) was followed by monitoring the decrease in absorbance at 240 nm due to depletion of H_2O_2 [30]. All catalase activity measurements were carried out at substrate concentration lower than the K_m of catalase [31], under conditions of first order kinetics. The kinetics of penicillinase catalyzed reaction was monitored by the change of color of dye put in the solution due to gradual increase of H^+ concentration produced from the enzyme catalyzed hydrolysis of

benzylpenicillin to penicilloic acid following the method suggested by Saz et al. [32]. In a typical kinetic experiment with nanoencapsulated enzyme penicillinase, to 25 ml of penicillin G (0.13 mM), 75 μl of 0.01% indicator solution was added. The pH of this solution was adjusted by adding very dilute H_2SO_4 or very dilute NaOH solution. 3 ml of this solution was taken each time in a cuvette in which 10 μl of the dispersion containing encapsulated enzyme was added. The calculated loading of the encapsulated enzyme in the cuvette was maintained at 1.56 nM, same as that maintained in the case of free enzyme. Addition of enzyme solution was taken as the zero time. The rate of the reaction was monitored for 120 s by the change of color of phenol red dye at 558 nM.

For the activity-pH profile studies four different types of indicator were used according to the pH of the solution and they are bromothymol blue (pH 6.0–6.5), bromocresol purple (pH 6.5–7.0), phenol red (pH 7.0–7.6), and phenolphthalein (pH 7.6–8.2). The velocity of the reaction was determined from the initial slope of the time-dependent absorbance change.

The activity of catalase was expressed in terms of turnover number ($v/[E]_t$) where v is the rate of reaction and $[E]_t$ is the total enzyme content in the solution. However, for penicillinase, the enzyme activity was expressed in terms of maximum turnover number, k_{cat}, calculated from the equation $k_{cat} = V_{max}/[E]_t$ where V_{max} is determined from Lineweaver–Burk plots.

Results and discussion

Size and dispersity of encapsulated enzyme particles

The dynamic laser light scattering data for the size of the encapsulated catalase and penicillinase enzyme containing particles and their size distributions in buffer (50 mM phosphate, pH = 7.0) are shown in Figs. 2 and 3. The mean diameters of the encapsulated particles have been found to be 126 nm and 36 nm for catalase and penicillinase respectively. It is known that only one enzyme molecule is solubilized into the aqueous core of the reverse micellar droplet [33]. The enzymes, catalase (diameter = 11 nm) and penicillinase (diameter = 6 nm) were encapsulated in the reverse micelle of water pool size of 12.4 nm ($w_o = 30$) and 8 nm ($w_o = 14$) diameter respectively [34] because at these w_o values of AOT reverse micellar solutions, the enzymes show maximum catalytic activity [35, 36] although Haber et al. [37] have found no 'bell-shaped curve' to describe the w_o of catalase activity in reverse micelles, with rate constant increasing monotonously up to $w_o = 50$. The sizes of the above nanoparticles formed in both the enzymes were found to be larger than those of the microreactors in which the polymeric reactions occurred. Clint et al. [38] had also observed that there is only a weak correlation between the particle size and droplet size in the preparation of 'Pt' nanoparticle *in situ* in water-in-oil microemulsion of H_2O/AOT/heptane system. They have reasoned that the effect of flocculating the microemulsion system (forming network) drastically perturb the size of the particle. Here also it is possible that the clustering of the droplets in the reverse micelle through interdroplet interaction is responsible for the larger growth of the particles during polymerization of acrylamide. In order to see the effect of interdroplet interaction and consequent droplet clustering on the particle size, enzyme hosted polymerization was done with reverse micellar systems containing various amount of cholesterol. The latter is known to reduce the interdroplet interaction and clustering by rigidifying the interfacial surfactant monolayer of the droplets [39]. Figure 4 shows the decrease of particle size with the increased concentration of cholesterol (defined by R_1 = [cholesterol]/[AOT]) in the reverse micelles. The size of the encapsulated catalase containing particle decreases from 126 to 45 nm with increase of cholesterol concentration from $R_1 = 0$ to $R_1 = 0.3$. Another important feature about the size of the particles is their narrow size distribution. Catalase-containing particles have a size

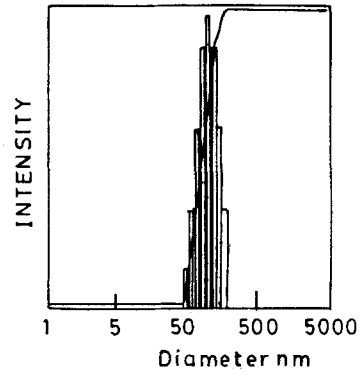


Fig. 2 Quasi elastic laser light scattering data of nanoencapsulated catalase

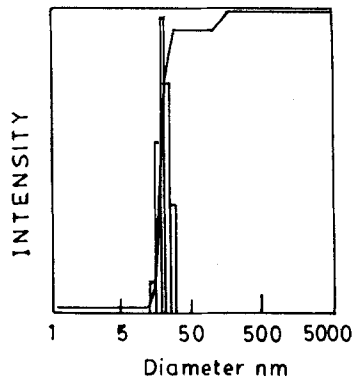


Fig. 3 Quasi elastic laser light scattering data of nanoencapsulated penicillinase

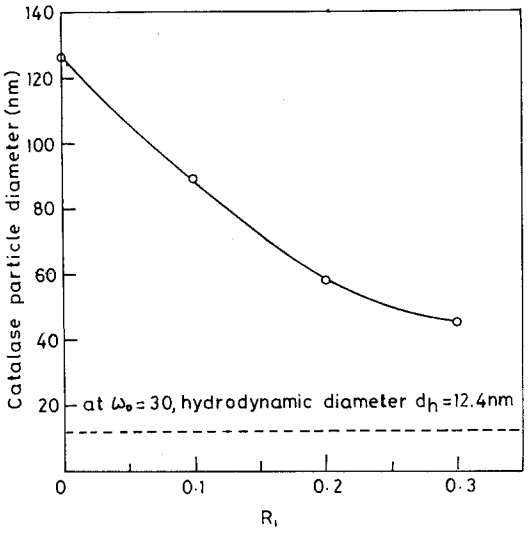


Fig. 4 Dependence of the diameter of nanoencapsulated catalase particle on the cholesterol content, R_1 = [cholestorol]/[AOT] reverse micelles, $W_o = 30$

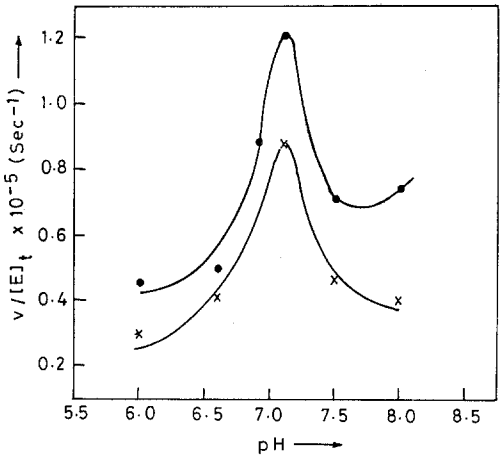


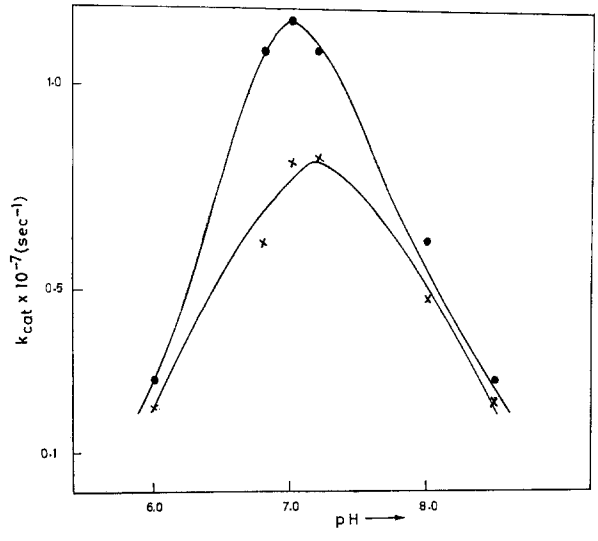
Fig. 6 Effect of pH on the activity of penicillinase free (–●–), encapsulated (–×–) [penicillinase] = 1.56 nM, [penicillin G] = 0.13 mM

distribution from 70 nm to 180 nm (Fig. 2) and penicillinase-containing particle size are in the range of 20–36 nm (Fig. 3).

Effect of pH on the activity of encapsulated enzyme

The plots of activity vs pH of the encapsulated as well as free form of enzymes (both catalase and α-penicillinase) are shown in Figs. 5 and 6 respectively. The results show that

Fig. 5 Effect of pH on the activity of catalase free (–●–), encapsulated (–×–) [catalase] = 60 nM, $[H_2O_2] = 25.6$ mM



the nature of pH profiles of free and encapsulated forms of catalase has been the same. Free and encapsulated penicillinase has also shown the same kind of activity-pH profile. The pH optimum for both forms of the enzymes also remained unaltered. The activity of the encapsulated enzymes was less compared to that of the free enzymes in the entire region of pH studied. Eryomin et al. [40] had also observed the reduction of activity when catalase was encapsulated in polyacrylamide matrix through AOT/toluene reverse micelles. Usually polyionic matrices will have the general effect of causing a partitioning of the protons between the bulk phase and the enzymes microenvironment [41]. Such partitioning of protons in encapsulated enzyme systems causes the shifting of pH optimum in encapsulated enzymatic reaction. In the present case a neutral polymer matrix has been used for encapsulation of enzymes and thus no proton partitioning was expected, hence no shift in pH optimum.

The decrease in the activity of the encapsulated enzymes compared to that of free enzymes may be due to substrate diffusion constraints through the pores of the nanocapsule to the microenvironment of the enzyme. At the steady state of the reaction, the rate of internal substrate diffusion inside the polymer matrix at any point must equal its rate of removal by the enyzme. If the enzyme has a high intrinsic specific activity the substrate concentration gradient between bulk of the solution and inside the encapsulating particle will be steep. There will be a time when the enzyme may starve for the substrate and thus its activity will remain underutilized. If there exists such a substrate concentration gradient between the bulk and inside of the encapsulated enzyme, the enzymes effective activity will be formally reduced.

Effect of temperature on the activity of encapsulated enzymes

The dependence of turnover number on temperature for catalase catalyzed decomposition of hydrogen peroxide and penicillinase catalyzed hydrolysis of penicillin G to penicilloic acid are shown in Figs. 7 and 8 respectively. The activity-temperature curves of encapsulated catalase showed optimum temperature at 30 °C which is the same as that of free enzyme, whereas penicillinase showed an elevation of optimum temperature from 25° to 35 °C upon encapsulation. The figures show that the free forms of both the enzymes undergo a rapid decrease of activity after optimum temperature while the activity decay is considerably slower for both the encapsulated enzymes at post-optimum temperature. It showed that the enzymes entrapped in the polymeric matrix is more thermostable compared to the free enzyme. This is contrary to the observation made by Eryomin et al. [40]. It seems that the bulk medium in which reverse micelles solution is made to encapsulate the enzyme has a role to play towards the stability of the encapsulated enzyme. There is no general trend for the change of optimum temperature on encapsulation of the enzyme observed earlier also [42, 43].

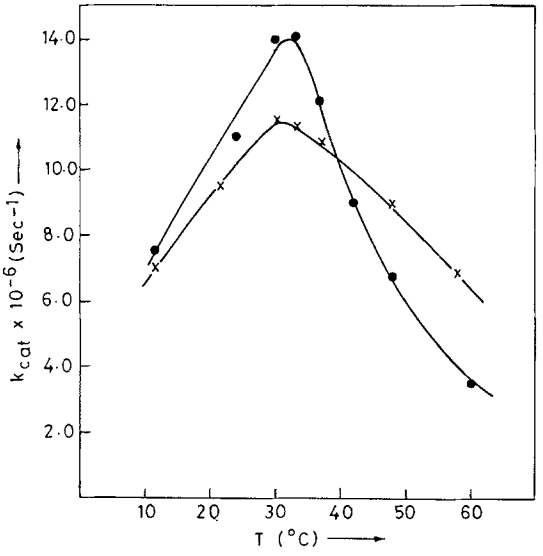
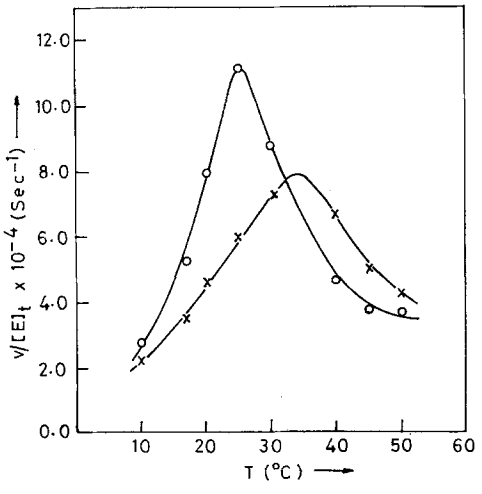


Fig. 7 Effect of temperature on the activity of catalase free (–●–), encapsulated (–×–) [catalase] = 57.7 nM, [H_2O_2] = 31.3 mM

Fig. 8 Effect of temperature on the activity of penicillinase free (–○–), encapsulated (–×–) [penicillinase] = 2.12 nM, [penicillin G] = 0.142 mM



In the temperature range ~10–30 °C, plots show that with the increasing temperature the rate of increase of the encapsulated enzymic reaction is less compared to that of free enzyme. It is possible that the rate of the enzymic reaction is higher than the internal diffusion of the substrates to the surface of the enzyme. Therefore, the substrate concentration within the microenvironment of the enzyme should be lower than that in the bulk due to its depletion by the reaction. Effectively, this would reduce the standard free energy of the activation for the reaction relative to that catalyzed by the free enzyme in solution. This is exactly what we observed from Arrhenius plots. The activation energies determined from the straight line portion of the pre-optimum regions of Fig. 7 are 11.29 kJ/mol for free and 7.66 kJ/mol for the encapsulated forms of catalase. Similarly, the activation energies calculated for free and encapsulated penicillinase are 28.47 kJ/mol and 17.91 kJ/mol respectively. The lower activation energies for both the encapsulated enzymes than their corresponding free forms supports the proposition that the rate is limited by diffusion constraints within the polymer matrix in the above temperature range.

Effect of degree of cross-linking of the polymer matrix on the reaction rate of encapsulated enzyme

In our experiments, the entrapment of enzymes within the pores of polymeric matrix has been made. The substrate molecules reach the surface of the particle from the bulk through external diffusion. But in order for all the enzymes to be utilized, substrate must also diffuse within the pores of the particle in order to reach the enzyme surface. The pores within the particles exert the internal diffusional resistance to the substrate which approaches the enzyme surface from the particle surface. In the present experiment, internal diffusional constraint has been provided by increasing the degree of crosslinking of the polymeric matrix [44].

Let us assume that (i) the entraped enzyme is located centrally within the nanoparticle, (ii) the Michaelis–

Menten model describes the kinetics, (iii) the system is operating under the steady-state condition and is isothermal, (iv) the diffusion of the substrate and the product obeys Fick's law and the effective diffusivity is constant throughout the particle, (v) there is no external diffusional resistance, and (vi) neither partition nor inhibition occurs.

The differential equation describing the steady-state diffusion will be [45, 46]:

$$D_s\left[\frac{d^2[S_r]}{dr^2}+\frac{2}{r}\frac{d[S_r]}{dr}\right]=\frac{V_{max}[S_r]}{K_m+[S_r]}, \quad (1)$$

where $[S_r]$ is the substrate concentration at a radial distance r inside the particle and D_s is the diffusion coefficient inside the particle.

The net reaction rate inside the particle in the steady-state can be obtained by taking the integral of the flux of materials into the particle over the external surface area $4\pi R^2$ where R is the radius of the particle. In the steady-state of the reaction the enzyme is consuming substrate as fast as it enters the sphere. Thus the actual reaction rate (v) per unit area (in moles/sec. cm^2) will be

$$v = D_s \quad d[S_r]/dr|_{r=R} \quad (2)$$

Unfortunately, a general solution of Eq. (2) has not been found. Analytical solutions may be explored for the extreme cases in which $[S_r] \gg K_m$ or $[S_r] \ll K_m$. In the latter case ($[S_r] \ll K_m$)

$$v=\frac{D_s[S]_R}{R}[\sqrt{\alpha R^2}\,\mathrm{Coth}(\sqrt{\alpha R^2})-1], \quad (3)$$

where $\alpha = V_{max}/D_s \cdot K_m$ and is referred to as enzyme loading factor. Thus, reaction velocity is related to the substrate diffusion in a very complicated way. With the increasing degree of cross-linking in the polymeric matrix, the substrate would experience more diffusional constraint to reach the enzyme surface as the porosity would decrease.

In the limit of a very small particle ($R \rightarrow 0$), there will be no diffusional resistance to mass transfer and the above equation shows the reaction rate to be very high and approaching that in aqueous solution.

Since α is directly related to porosity of the polymeric matrix, a plot of turnover number against the amount of cross-linking agent added to the system shows (Fig. 9) that the higher the degree of cross-linking, the lower the enzymatic activity irrespective of the bulk substrate concentration.

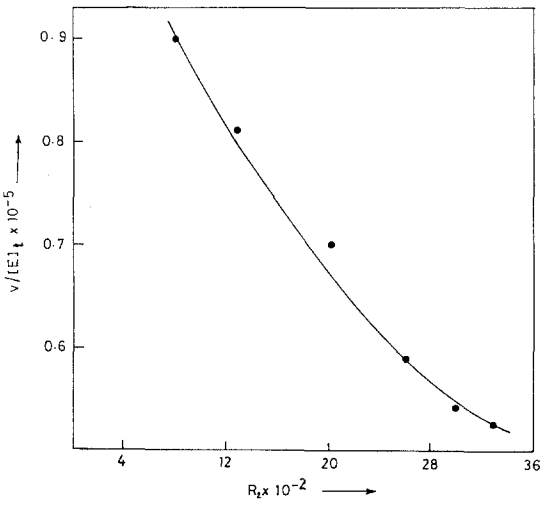


Fig. 9 Dependence of activity of penicillinase on the degree of entrapment (R_2). R_2 = [*N*,*N*′-methylene-bis-acrylamide] /[acrylamide] [penicillinase] = 1.56 nM, [penicillin G] = 0.13 mM

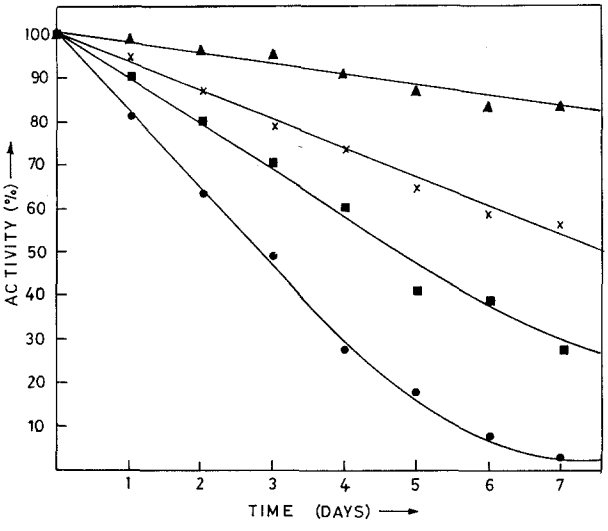


Fig. 10 Time dependence stability of catalase and penicillinase, Activity was expressed relative to initial activity (100%). Catalase: free (–●–), encapsulated (–×–) Penicillinase: free (–■–), encapsulated (–▲–). [catalase] = 60 nM, [H_2O_2] = 25.6 nM. [penicillinase] = 1.56 nM, [penicillin G] = 0.13 mM

Storage stability of encapsulated enzyme

Figure 10 shows the time-dependent stability of both catalase and penicillinase in their free as well as entrapped forms in phosphate buffer (pH 7.0, 50 mM). The enzymes were stored in a stoppered glass container in an incubator at 25 °C. The storage stability of the enzymes was determined by monitoring the relative activity (with respect to original activity) of the enzymes at different times. Comparison of the time-dependent storage stability of the enzymes, free and encapsulated, showed that the encapsulated enzymes are more stable than the free

enzymes. It was observed that the free catalase is completely inactivated at 25 °C in a period of 7 days but the entrapped form retained about 60% of its initial activity even after 7 days time. Similarly, the free form of penicillinase showed 80% loss of its activity under the same conditions while the encapsulated form maintained practically 100% of its activity during the same period of time. The enzyme catalase in free form was more susceptible to its external environment compared to the enzyme penicillinase in free form. Many explanations have been put forward for better storage stability of the enzyme in encapsulated form [41]. It had been argued that fixing an enzyme to a polymer matrix which was less affected by heat or pH changes provides the enzyme with a protective shell, preventing it from altering its conformation in response to such changes. It had also been thought that stability of an enzyme depended upon the enzyme or even total protein concentration. The free enzyme is usually in dilute solution while the local concentration of the encapsulated enzyme may be relatively high. This may be another reason for better storage stability of enzyme in the nanoencapsulated form.

Conclusions

Our results showed that the aqueous core of the reverse micellar droplet could be effectively used as a nanoreactor for solubilizing and encapsulating the enzymes like catalase (bovine liver) and α-penicillinase in polyacrylamide matrix. The entrapped polymeric particles are reasonably monodisperse and had submicron size. They formed stable dispersed systems in the buffer. Both the enzymes on encapsulation show enhanced thermal stability and shelf-life.

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