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Modelling the alcoholysis reaction of β-galactosidase with butanol in reverse micelles

A. Moreno-Beltrán [a], L. Salgado [b], R. Vazquez-Duhalt [b], A. Lopez-Munguia [b,*]

[a] *Facultad de Química, Cd. Universitaria, Universidad Nacional Autónoma de México, 04510 México D.F., Mexico*
[b] *Instituto de Biotecnología, Universidad Nacional Autónoma de México, Apdo. Postal 510-3, Cuernavaca, Mor. 62250, Mexico*

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Abstract

A theoretical thermodynamic model was applied to predict reverse micelle size in an octane–butanol/cetyl trimethylammonium bromide (CTAB)/water system where the alcoholysis of lactose and ONPG was carried out by β-galactosidase. Solvent concentration regions where the hydrolytic enzyme displayed only alcoholysis were found and assayed. The model allows the description of the experimental results, in terms of micellar concentration and size. It was also possible to define enzyme saturating conditions from the predictions of the empty and filled micelle volumes, which match the experimental results. Optimal conditions of the enzyme in a different system were also predicted introducing a parameter (ω_o'), which is a water/surfactant molar concentration ratio and independent of the surfactant used. Besides demonstrating the possibility of using β-galactosidase in reverse micelles for alcoholysis reactions with no hydrolysis even at high water content, it was also found that the enzyme is more stable in reverse micelles than in water saturated with butanol or water/butanol mixtures.

Keywords: β-Galactosidase; Reverse micelles; Alcoholysis; Modelling

1. Introduction

In recent decades, reverse micelles have been the focus of biotechnology research as vehicles to disperse enzymes in nonpolar media. This is due to their similarity with the microenvironment that enzymes find in the cell and the characteristic properties of this media (protection of proteins and genetic material from adverse factors, almost anhydrous conditions, etc.). As has been recently reviewed [1–3], there are many reports in the literature regarding the properties of proteins and their conformation related to reactivity and specificity of enzymatic reactions in various reverse micelle systems. It is now clear that in many cases the enzyme behavior differs substantially from that observed in aqueous media, the main differences arising in kinetic behavior. Several models have been proposed in an attempt to explain the diverse experimental kinetic results obtained, such as the phenomenon known as superactivity observed under certain reaction conditions [1,4–6]. In particular, the most influential factor affect-

* Corresponding author. Tel. +52-5-6227673; fax: +52-73-172388; e-mail: agustín@ibt.unam.mx.

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ing the activity of enzymes hosted in reverse micelles is micelle size, often expressed by ω_o (water/surfactant molar concentration ratio).

The models developed to explain the kinetic behavior of enzymes in reverse micelles can be classified as diffusional, nondiffusional or polydispersed, pseudophasic and intramicellar diffusion models [1]. Each of these models is based upon the control of one or more processes that occur as part of the global reaction mechanism and have been successfully applied to explain particular cases. Unfortunately these models are very specific to the cases observed, fitting only a few limited conditions. Nevertheless, it has been generally recognized that a critical aspect of the global description of enzymatic reactions inside reverse micelles is the determination of the micellar size and its effect on the observed reaction kinetics.

This problem has been approached both experimentally and theoretically. Several techniques have been developed to measure micellar size [7–10]. However, these techniques do not always provide a practical solution to the problem. In general, they are not simple and the results are sometimes misleading. For instance, by applying the ultracentrifugation technique the results suggest that the size of micelles do not change after the addition of the protein molecule [11], while other results indicate that the micellar structure changes dramatically after the protein addition [12].

Another alternative to explain experimental results is through models which would effectively predict the size of protein containing micelles. Some of these models are very efficient but their theoretical and mathematical complexity make them difficult to apply. This is the case in the model proposed by Rahaman and Hatton [13]. Other models are derived from a more adequate and simpler approach with approximations suitable for predicting the micellar size in enzymology. This is the case in the model proposed by Caselli et al. [14], which was derived to predict the size of protein-containing micelles, using the experimental results of Zampieri et al. [9]. This model is based on approximations derived from experimental observations, thermodynamic considerations, and geometric restrictions. The parameters needed to solve the model are relatively easy to obtain or estimate and the model may be solved by a simple numerical resolution. The objective of the work described in this paper was to apply a mathematical tool, based on the Caselli et al. model, to describe and explain the effect of the most influential parameters on the observed activity of enzymes in reverse micelles. In particular, the model was applied to alcoholysis reactions of β-galactosidase.

Recent research has been carried out on the synthesis of alkylglycosides using soluble and insoluble alcohols and a wide variety of carbohydrates. Shinoyama et al. [15] have compared the alcoholytic capacity of various enzymes such as α- and β-glucosidases in the presence of their corresponding sugar donors and alcohols, and particularly β-galactosidase from various sources [16]. Matsumara et al. [17] were able to synthesize η-alkyl-β-D-galactosides using ONPG or lactose as galactose donor and 1,8-octanediol, 1,10-decanediol, 1-octanol and 1-decanol as nucleophiles with excellent results. Stevenson et al. [18], also using β-galactosidase from *K. fragilis* and *K. lactis*, concluded that alcoholysis was more efficient with small alcohol acceptors than with high molecular weight linear alcohols, due to their low solubility. The use of cosolvents has been proposed to decrease water activity without increasing alcohol concentration. It should also be pointed out that in this particular case, one of the substrates is hydrophylic (sugar) while the other may be hydrophobic (long chain alcohols). Higher yields were obtained after optimizing reaction conditions and using cosolvents such as dioxane, acetonitrile, DMSO, DMF, or THF. However, some of these cosolvents may denature the enzyme. Beecher et al. [19] proposed a cosolvent reaction system based on DMSO to improve the carbohydrate solubility, and using trichloroethane for the hydrophobic alcohol. For this

purpose, the enzyme from *A. oryzae* was chemically modified with polyethylene glycol (PEG) for the synthesis of alkyl-galactosides using PEG of various molecular weights. The stability and solubility of the modified enzyme is a function of PEG molecular weight, and the derivative retained 50% of the original activity. It was concluded that the modified enzyme was very soluble, but a major disadvantage was its poor stability.

Although β-galactosidase has been studied in many biphasic systems, its behavior in reversed micelles has not been reported, particularly in the case of alcoholysis where two phases are present in the reaction system. Georgina et al. [20], studied the stability of β-galactosidase in an AOT/cyclohexane system. The enzyme had the same stability as in the aqueous phase when ω_o was high (21 or 37), but rapidly deactivated when ω_o felt to 9. In recent work, Shiomor et al. (21) reported the behavior of β-galactosidase from *E. coli* for ONPG hydrolysis in AOT/isooctane. Although both substrates and products are water soluble, the enzyme exhibited maximum activity values at $\omega_o = 10$ and at $\omega_o = 45$. The latter value corresponding to the size of the protein. The authors also found good stability at $\omega_o = 21$ and at $\omega_o = 37$, losing activity at the lower values.

Considering these data, the objective of this work was to study the behavior of β-galactosidase in reverse micelles for alcoholysis reactions. In the first approach, we used the cetyl trimethylammonium bromide (CTAB)/octane/water system with butanol as both cosolvent and substrate of the reaction. In this particular system one of the substrates, lactose or ONPG, is water soluble, while the butanol is not only hydrophobic, but part of the micellar system.

2. Theoretical model

The model used to predict the size of reverse micelles containing proteins is based on the transformation shown in Fig. 1, in which the initial state consists of 'empty' micelles of number N_o and radius r_o. By introducing N_p protein molecules, the system is rearranged in N_e 'empty' micelles of radius r_e and N_f 'filled' (protein containing) micelles of radius r_f.

This process involves an inherent change in Gibbs free energy ($\Delta G°$) which could be calculated through the appropriate considerations. Therefore, for any given system, the micelle size may be predicted from the values of N_f, N_e, r_f and r_e that minimize the function $\Delta G° = \Delta G°(N_f, N_e, r_f, r_e)$, that is, the conditions that give the system thermodynamical stability.

The model is based on the following approximations: (1) Monodispersion on 'empty' and 'filled' micelle size. (2) The micelle–protein ensemble can be modeled as a microcapacitor. The protein molecules are rigid dielectric spheres, with an average surface charge of σ_p. Protein and surfactant counterions are taken into account. (3) There is a one protein molecule capacity per micelle. (4) Intermicellar interactions are neglected. (5) For the purposes of this model, there is no distinction between water molecules near the micelle core, near the protein or bulk water. (6) The dielectric constant of the protein surface is a constant value ϵ_p which is independent of the protein.

These approximations are based on experimental observation and are reasonable for minimization of the Gibbs free energy of the micellar system. The $\Delta G°$ value is composed by four contributions involved in the process already

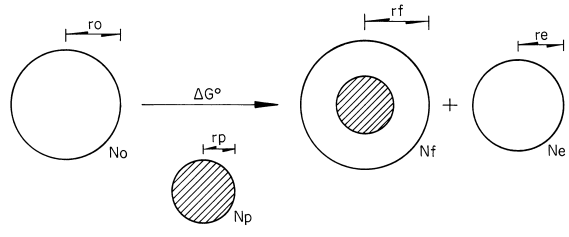

Fig. 1. Schematic representation of the intake of proteins in a reverse micelle system, generating two populations of micelles.

described: (a) An electrostatic term due to the interaction of the charged micelle wall formed by the ionized polar heads and the protein surface modeled as a microcapacitor. (b) The corresponding entropic contribution. (c) An entropic term due to the rearrangement of the insoluble ions inside the micelle. (d) A term related to the entropy of mixing of the micelles as macromolecular aggregates.

The four contributions are described by the equations reported by Caselli et al. [14]. At the same time, the system is subjected to restrictions to the total volume and total surface of micelles before protein intake:

$$N_o\left(4/3\pi r_o^3\right) = n_w v_w N V_s$$

$$N_o\left(4\pi r_o^2\right) = n_a S_a N V_s$$

and after the protein intake:

$$4/3\pi\left(N_e r_e^3 + N_f r_f^3\right) = N\left(n_w v_w + n_p v_p\right)$$

$$4\pi\left(N_e r_e^2 + N_f r_f^2\right) = n_a S_a N$$

Where n_w is the water concentration in the system and v_w is the molecular volume of water; n_a is the surfactant concentration in the system, while the surface of the surfactant polar head (S_a) was calculated from geometrical considerations: 24 Å^2 for CTAB and 55 Å^2 for AOT. All variables were transformed to a dimensionless form, using the protein radius (r_p) and the protein concentration (n_p):

$$y = r_e/r_p \qquad N_o' = \left(N_o/n_p\right)N$$

$$z = r_f/r_p \qquad N_e' = \left(N_e/n_p\right)N$$

$$g = r_o/r_p \qquad N_f' = \left(N_f/n_p\right)N$$

β-Galactosidase radius was 56 Å as calculated from the protein volume determined from its three dimensional structure, assuming a spherical geometry for the molecule. Other important parameters required are σ_p and ϵ_p, which are, respectively, the average surface charge of the protein, calculated from the number and charge of the surface amino acids of the protein divided by the area of the protein surface; and the dielectric constant of the protein surface, which is assumed to be a constant value of 30 for almost all proteins. The following auxiliary parameters are also defined:

$$b = n_a S_a/\left(n_p 4\pi r_p^2\right)$$

$$b_1 = \left(n_w v_w + n_p v_p\right)/\left(4/3\pi r_p^3 n_p\right)$$

The latter being a modification made to the Caselli et al. [14] model when consideration is taken that the total micellar volume is different before and after the protein intake. As will be shown later, this is an important consideration when analyzing the effect of enzyme concentration on reaction rate. It was therefore possible to write the function $\Delta G^\circ = \Delta G^\circ(N_f,\ N_e,\ r_f,\ r_e)$ as the function $\Delta G^\circ = \Delta G^\circ(N_f',\ N_e',\ r_f,\ r_e)$. Fur-

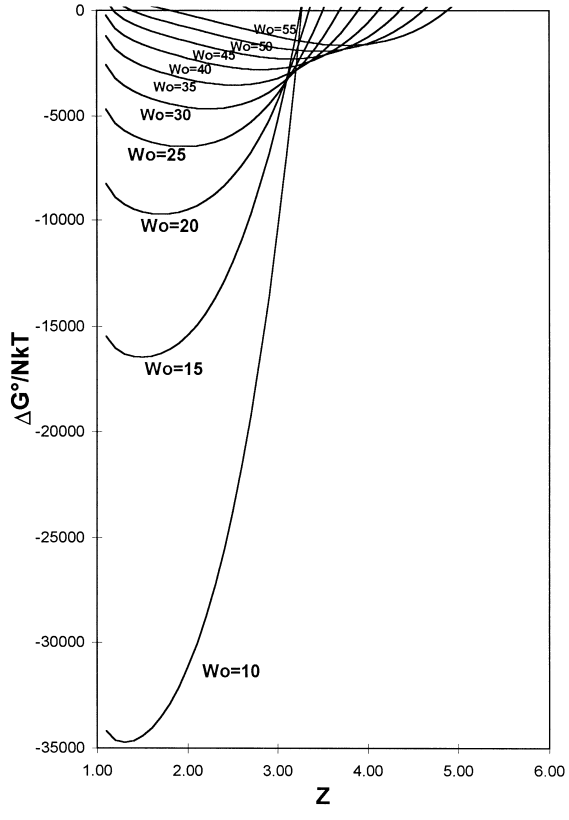

Fig. 2. Solution of the model by Caselli et al. [14], minimizing the ΔG° of protein intake by reverse micelles as a function of ω_o, the water/surfactant concentration ratio and z, the adimensional filled micelle radius.

thermore, based upon the assumption of a one protein molecule occupancy, $N_f' = 1$. It is also possible to demonstrate that:

$$y = \left(b_1 - z^3\right)/\left(b - z^2\right)$$

$$N_e' = \left(b - y^2\right)/z^2$$

Consequently, the function $\Delta G^\circ = \Delta G^\circ(N_f', N_e', r_f, r_e)$ can be written as $\Delta G^\circ = \Delta G^\circ(z)$, where z is the dimensionless filled micelles radius. The minimization of the Gibbs free energy is then carried out for a given ω_o value as a one variable problem as shown in Fig. 2. For each ω_o, the function was evaluated using the Newton's method with finite differences, for values of z given by $z^{\kappa+1} = z^k - \lambda \nabla * \Delta G^\circ(z)$ and the minimum was defined when $\nabla * \Delta G^\circ(z) = 0$, actually less than a given tolerance.

3. Materials and methods

β-Galactosidase from *E. coli* was purchased from Sigma and was studied both in water and reverse micelles using lactose and *o*-nitrophenyl galactoside as substrates, both in hydrolysis and alcoholysis reactions. When using lactose, in water or in reversed micelles reactions, the products were measured by HPLC equipped with a Waters 410 Refractive Index detector and a Novapack 4 mm (4.6 mm × 250 mm) column. When ONPG was the substrate, the activity was measured by spectrophotometry at 420 nm. Qualitative determinations of hydrolysis and alcoholysis were carried out by TLC on silica plates using acetonitrile–water (75:25) as solvent and developing with α-naphthol in sulfuric acid.

3.1. Reverse micelles

The reverse micelles system was formed by iso-butanol, iso-octane, and water containing the substrate in phosphate buffer 0.1 M pH 7.0. Micelle composition was the subject of an experimental design based on the Scheffe cubic model [21]. The data was analyzed with the statistical package NEMROD-Aix-Marseille-SURRPC-V8710. The micelles were prepared by the microinjection method: CTAB was added to the previously prepared organic phase (isobutanol and octane) and agitated to form an emulsion. Afterwards the substrate (either lactose or ONPG) was incorporated at the desired concentration in phosphate buffer 0.1 M pH 7.0, agitating for a few seconds until the micelles were formed. Finally, the reaction was started by addition of the enzyme solution. No differences were found when the addition of substrate was the last step in the procedure. The effect of micelle size and enzyme concentration on the initial alcoholysis rate was studied varying ω_o from 5 to 200 and enzyme concentration from 1 to 50 mg/l. The effect of both substrates on the initial alcoholysis rate was also studied in the range of 0–50 mM total ONPG or lactose concentration in micelles containing 5 mg/ml of enzyme.

3.2. Enzyme activity

β-Galactosidase activity was measured in water using either 25 mM lactose or 2.25 mM ONPG at 37°C including 1 mM of $MgCl_2$, and 0.11 M of mercaptoethanol, with a final enzyme concentration of 0.95 mg/ml, and following the release of products during the first 3 min. In the case of ONPG an extinction coefficient of 1.8469 $mM^{-1}\ cm^{-1}$ was calculated. In the case of reverse micelles, the final composition only for the activity essay was 55% butanol, 22% octane and 23% water (sodium phosphate buffer 0.1 M, pH 7.0), with a ω_o of 20 and 10 mM lactose or ONPG concentration. Substrate concentration is referred to the total micelle volume, in this case 1.76 ml. The reaction rate may be referred to the amount of butyl galactoside (BG) formed or to the substrate (lactose or ONPG) consumed.

3.3. Enzyme stability

β-Galactosidase stability was studied storing the soluble enzyme at 37°C and 25°C in various solutions: (a) buffer, (b) buffer saturated with butanol, (c) a mixture of 50% water and 50% butanol and d)reverse micelles. In the first three cases, samples were taken at different times and the residual activity essayed as already described for the aqueous soluble enzyme. In the case of reverse micelles, the sample was diluted in previously prepared empty micelles and the activity essayed by addition of the substrate.

4. Results and discussion

To define the composition of the butanol/octane/lactose in phosphate buffer 0.1 M pH 7.0 reverse micelles system, regions of the phase diagram containing different amounts of the three components were explored, keeping the lactose concentration (29 mM), and enzyme concentration (3×10^{-5} M) constant, and ω_o ($[H_20]/[\text{Surfactant}]$) = 20. Various regions were found where the enzyme displayed high alcoholysis rates without hydrolysis of the substrate. Using reverse micelles, it was possible to explore the limits of water concentrations at which the enzyme displays only alcoholysis activity. At a water content as high as 40% (w/w) in reverse micelles no hydrolysis products were observed. A high activity region was found between 14% < water < 32%, 14% < octane < 32% and 55% < butanol < 72% (w/w), as shown in Fig. 3. Using this region an optimal composition was found in terms of reaction rate and productivity, and described by the statistical model. This is a zone of a very high water content, but as it is shown also in Fig. 3, the water content may be considerably reduced without affecting the initial reaction rate.

The effect of ω_o ($[H_20]/[\text{Surfactant}]$) and enzyme concentration on the alcoholysis rate by

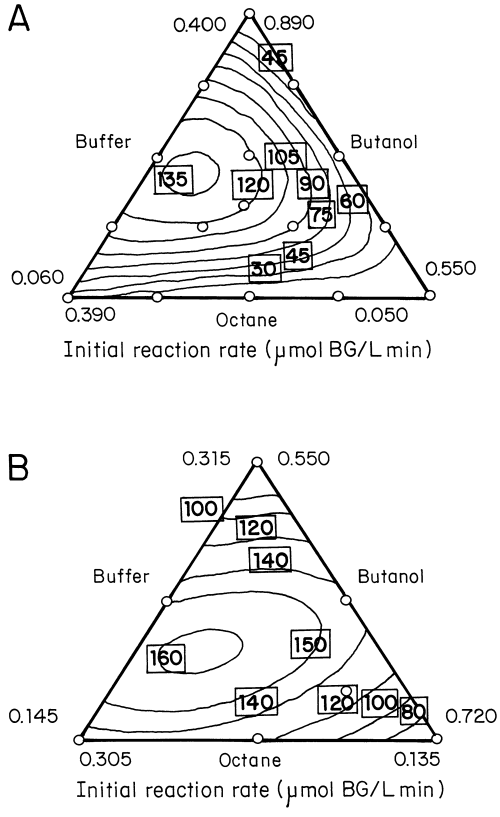

Fig. 3. Alcoholysis activity of β-galactosidase in reverse micelles in the system CTAB–butanol/octane/water (phosphate buffer 0.1 M). Reaction conditions: $\omega_o = 20$, $E = 3 \times 10^{-5}$ M, lactose 29 mM, pH = 7/37°C. Analysis in a wide concentration range. (A) Optimization in the highest activity region (B) $v_i = -6976X_1 - 1505X_2 - 928X_3 + 8659X_1X_2 + 13{,}169X_1X_3 + 4058X_2X_3$, where X_1 = buffer; X_2 = octane; X_3 = butanol (w/w). Selected composition: octane 22%, butanol 55% and water 23% (w/w).

β-galactosidase in the reverse micelle system was studied under reaction conditions where no hydrolysis took place, all galactose being transferred from lactose to butanol. BG was extracted in the continuous phase while lactose and glucose released remained in the aqueous phase, inside the micelle. The experimental results were compared with the theoretical predictions obtained with the Caselli et al. [19] model.

In Fig. 4, the initial alcoholysis rate is shown as a function of ω_o. As it may be observed, a maximum activity was obtained at $\omega_o = 20$.

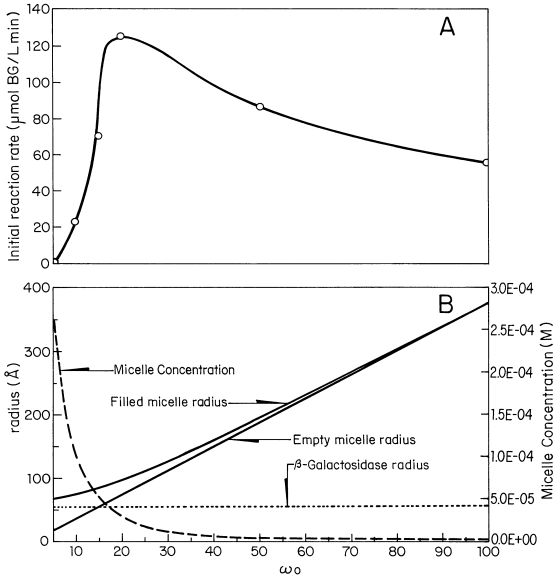

Fig. 4. Effect of ω_o on the initial alcoholysis rate in reverse micelles: 62% butanol, 26% octane and 12% water (phosphate buffer 0.1 M) with 3×10^{-5} M β-galactosidase and 7.3 mM lactose (pH = 7.0 and 35°). (A) Experimental results. (B) Theoretical analysis.

This behavior corresponds to the 'bell shaped' curve observed for other systems. The radius of the empty and filled micelles and the effective micelle concentrations were determined using the theoretical model under the same conditions as the experiments above. The results are shown in the same figure, where it should be noted that as ω_o increases there is a significant drop in the effective micellar concentration and micelles become large enough to host the protein molecule. This is due to the fact that the available surface decreases with ω_o leading to a rearrangement of the available material in bigger micelles.

On the other hand, as ω_o decreases, the space between the protein and micelle wall is reduced. This is also attributed to a rearrangement of material when the available surface is modified. When ω_o decreases, there is more surface available for a given micelle volume. This forces the system towards an arrangement of smaller and more abundant micelles. Both of these effects lead to a decrease in global activity: (i) if fewer effective micelles are available, less enzyme molecules will be active; and (ii) when the intramicellar space is reduced, there may be an steric hindrance of the enzymatic reaction.

Furthermore, alcohol diffusion from the micelle wall to the active site of the enzyme may be another determinant factor. Butanol is only slightly soluble in water, so the reaction may be controlled by the rate at which the system can supply the alcohol molecules to the active site. This means that the observed activity would increase when the distance required for butanol diffusion is decreased, i.e., the micelle radius is decreased.

Experimental results allow us to conclude that among the ω_o values explored, the highest alcoholysis rate was found at 20. This is very close to 17, the value at which the empty micelle radius is equal to the protein radius. Sev-

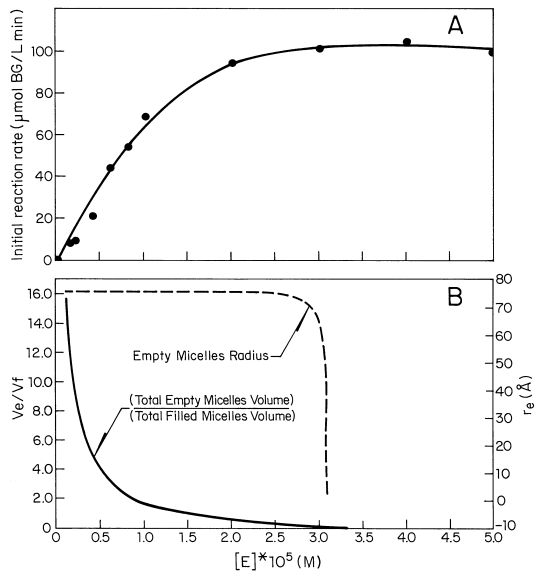

Fig. 5. Effect of enzyme concentration on the initial alcoholysis rate in reverse micelles: 62% butanol, 26% octane and 12% water (phosphate buffer 0.1 M) with 1.0 mM ONPG and $\omega_o = 20$ (pH = 7.0 and 35°C). (A) Experimental results. (B) Theoretical analysis.

eral authors have related activity in reverse micelles with the relation between protein and micelle size. In general, optimal values occur when they are similar [1]. This may also be the case for this system, although sufficient space for lactose and butanol diffusion is required for alcoholysis.

The effect of β-galactosidase concentration on initial alcoholysis rate is shown in Fig. 5, where it may be observed that after the initial linear reaction rate a saturation behavior is exhibited. In the same figure it is shown by means of the theoretical model, that as the enzyme concentration increases, the ratio of the volume of empty micelles to filled micelles falls. This is an indirect measurement of the reverse micelles system capacity to produce filled micelles. Ishikawa et al. [22] reported that micelle size was not affected after the protein intake, this effect being attributable to the ratio of filled/empty micelles estimated to be about 0.01%, very far from a saturation state. This emphasizes the importance of the correction made to the original model of Caselli et al. [19] by an additional parameter b_1 when the protein intake has to be taken into account in the total micelle volume. It is also important to notice that saturation of the system (when the empty/filled volumetric ratio approaches zero), corresponds to the state at which the observed activity no longer increases with an increase in enzyme concentration. The saturation condition predicted by the model occurs when the enzyme concentration reaches $[E] = 3.15 \times 10^{-5}$ M, which is in good agreement with the experimental results.

We have also used this model to compare the results reported by Shiomori et al. [23] for the hydrolysis of lactose with β-galactosidase solubilized into AOT/isooctane reverse micelles. The authors obtained a local maximum in initial activity at $\omega_o = 43$. It is interesting to observe that when the appropriate geometrical corrections are made, it is possible to demonstrate that this maximum is equivalent to $\omega_o = 20$ for our system.

There is a linear relation between the micelle radius and the parameter ω_o which can be derived from the geometrical restrictions of the Caselli et al. [19] model. Given the total volume and surface relations already mentioned, we can state that: $r_o = (3n_w/S_a)\omega_o$; where n_w is the water concentration in the system. This can be written also as: $r_o = k\omega_o$, where k is a proportionality constant.

If we define a new variable as $\omega'_o = \omega_o/S_a$, the usual parameter becomes independent of the surfactant used. At the maximum activity, it was found that ω'_o for the AOT system was $\omega'_o = 45/55 = 0.82$, and for the CTAB system the same variable was $\omega'_o = 20/24 = 0.83$. This variable may be useful when comparing results obtained with different surfactants.

As far as other parameters in the model are concerned, a parameter sensibility analysis was carried out. It was found that all of the inherent system parameters (S_a, σ_p, ϵ_p, etc.) have little influence in the final results except for the surface of the surfactant polar head, which showed a strong effect on the quantitative final results.

Finally, from initial rate experiments the kinetic behavior and stability of the enzyme were studied. In a reverse micelles system containing 55% butanol, 22% octane and 23% water, with a $\omega_o = 20$ at 37°C, it was found that the data can be described by Michaelis–Menten kinetics with a K_m value for alcoholysis for lactose and ONPG of 3.8 and 2.4 mM, respectively. However these are only apparent values as the reaction involves two substrates, one of which (butanol), has to diffuse to the micelles and may become a limiting factor. When storing the enzyme at 37°C, it was also found that the half life of β-galactosidase decreases from 315 min in water, to only 22 or 16 min in water saturated with butanol and a 50% water/50% butanol mixture respectively. However, in reverse micelles, the stability is increased back up to 67 min. When the reaction temperature was reduced to 25°C, then the half-life of β-galactosidase in reverse micelles was also 315 min.

The protecting effect of the substrate is demonstrated by the fact that reactions in a system of the same composition and characteristics as mentioned above, reach 100% conversion of 25 mM lactose at 37°C in 4 h, resulting in 6 g/l of butyl-galactoside.

It may be concluded that the thermodynamic model described by Caselli et al. [14] may be a useful tool in predicting or describing the behavior of enzyme in reverse micelles, in particular the effect of enzyme concentration. This was here the case for β-galactosidase in octane–butanol/CTAB/water system, where the enzyme activity is restricted to alcoholysis, in this particular case, to the synthesis of butyl-galactoside. This reaction system is not appropriate for the alkyl-galactoside production due to the high water content, but at the same ω_o, if the water soluble substrate can be added continuously the system may become viable. Also, as a wide variety of alcohols may be used as cosolvents in this micellar system, the length of the alkylglycoside chain may be varied accordingly. It was also possible to compare the behavior of the enzyme in a different micellar system by the introduction of a parameter independent of the surfactant nature. This may be a valuable tool in the design and analysis of enzyme reactions in the common reverse micelle systems.

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