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Journal of Molecular Catalysis B: Enzymatic 33 (2005) 15–21
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
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Stability and catalytic kinetics of microencapsulated β-galactosidase in liposomes prepared by the dehydration–rehydration method

José Manuel Rodriguez-Nogales *, Angélica Delgadillo

Chemistry Research Centre, Universidad Autónoma del Estado de Hidalgo, Carretera Pachuca-Tulancingo, Km 4.5, 42070 Pachuca, Mexico

Received 22 December 2004; received in revised form 21 January 2005; accepted 21 January 2005
Available online 11 February 2005

Abstract

Lipid vesicles prepared by the dehydration–rehydration method were used as carrier for the microencapsulation of β-galactosidase in order to permit the hydrolysis of the milk lactose following the lysis of liposomes in the presence of gastric fluid. Some characteristics of the liposomal and free β-galactosidase were compared. The kinetic behaviour of the enzyme was altered substantially in presence of lipid vesicles. The kinetic study indicated an decrease in both substrate affinity and maximum velocity when β-galactosidase was associated with phospholipid vesicles. Differences in the activity of free and liposomal β-galactosidase as a function of pH and temperature were found, although the optimum incubation temperature of free and entrapped enzymes remained similar. However, the optimum pH for liposomal enzyme was more acid than that for free enzyme. First-order kinetic analysis of thermal inactivation of enzymes showed that the activation energy with free enzyme was smaller than that with liposomal enzyme. In relation to the ΔH^* and ΔS^*, their values were greater than that those with free enzymes. These results confirm that β-galactosidase entrapped in liposomes showed superior thermal stability at all temperatures evaluated. Moreover, the proteolytic stability of the β-galactosidase was enhanced by encapsulation in liposomes.

Keywords: β-Galactosidase; Liposomes; Microencapsulation; Thermal and proteolytic stability

1. Introduction

β-Galactosidase (E.C. 3.2.1.23) is an enzyme widely distributed, mainly used to hydrolyze lactose into glucose and galactose, but it also utilized to catalyse the hydrolysis of terminal β-glycosidic bonds present in carbohydrate, glycolipids, glycoproteins and glycosamineglucans [1]. Problems with lactose fall within three main areas, heath, food technology and environment [2]. Concerning the health issue, more than 70% of the worlds population suffer from the inability to use lactose or lactose-containing products due to the lactose intolerance symptoms caused by the lack of β-galactosidase activity [3,4]. Because of these problems, the reduction of the lactose content in milk and dairy products is of prime importance and the enzyme β-galactosidase is commercially used for this purpose. An enzymatic hydrolysis of lactose is more adequate than a chemical hydrolysis because this process does not generate nasty flavours, odours and colours and the alimentary properties of dairy products are not modified [5].

Two alternatives exist for the enzymatic hydrolysis of milk lactose. It can be hydrolyzed by free or immobilized β-galactosidase to its constituent monosaccharides [6–11]. However, these methods face the problem that the hydrolyzed-lactose milk has a sweeter taste than whole milk [5,12] and some subjects dislike this taste. An approach to overcome this problem could be the microencapsulation of β-galactosidase in liposomes [13].

Interest in the use of microencapsulation technology in the food industry has been increasing during the last decade [14] and liposomes recently showed potential as support of enzymes in the food industry [15–17]. Liposomes are simply

* Tel.: +52 771 71 72000x6501; fax: +52 771 71 72000x6502.
E-mail addresses: rjosem@uaeh.reduaeh.mx, manolomexico@hotmail.com (J.M. Rodriguez-Nogales).

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doi:10.1016/j.molcatb.2005.01.003

vesicles in which an aqueous volume is entirely enclosed by a membrane composed of lipid molecules (usually phospholipids) and they can entrap and retain a wide range of active agents [18]. Lipid vesicles could be used as containers, which protect the enzymes from getting in immediate contact with the medium to which they are added [19]. For the hydrolysis of lactose milk, the enzyme-containing lipid vesicle is added to milk and is disrupted into the stomach by the presence of bile salts, allowing an in situ degradation of the lactose [20].

Walde and Ichikawa have given an excellent review of methods that can be used for the preparation of enzyme-containing lipid vesicles (liposomes) and their applications [19]. Numerous methods of enzyme entrapment in liposomes have been published, however, the vesicles prepared by the dehydration–rehydration method (DRV) have the following advantages over other methods: the entrapping efficiency is high, it is simple and easy to deal with the product, and it can be used for large-scale production [20].

In this context, the present paper reports on several attempts to gain knowledge of the entrapment of β-galactosidase in liposomes prepared by the dehydration–rehydration method. The effects of microencapsulation on the catalytic efficiency of the enzyme, as well as its kinetic properties and stability, compared to those of its native counterpart were investigated.

2. Materials and methods

2.1. Reagents

Phosphatidylcholine (PC) type XII-E from egg yolk (60% of PC), cholesterol (Ch), β-galactosidase from *Escherichia coli* grade VII (specific activity 39 mg *o*-nitrophenol h^{-1} mg^{-1} protein at 37 °C), *o*-nitrophenol (*o*-NP) and *o*-nitrophenyl-β-D-galactosidase (*o*-NPG) were from Sigma Chemical Co (St. Louis, MO, USA). All other reagents were of analytical grade.

2.2. Liposomal encapsulation of β-galactosidase

Liposomes were prepared by the dehydratation–rehydratation vesicle (DRV) method described by Kirby and Gregoriadis [18]. Vesicles were prepared with cholesterol and phosphatidylcholine in a molar ratio of 0.53. The lipid mixture (100 mg) was dissolved in 5 mL of chloroform and dried by rotary evaporation under reduced pressure at 35 °C. The resulting film was rehydrated with 5 mL of 0.1 M phosphate buffer at pH 7.4. The formed multilamellar vesicles were disrupted with a Branson S250 sonicator (150 W) at 4 °C under N_2 atmosphere. The solutions were centrifuged at 7500 × *g* for 30 min at 4 °C to eliminate the larger lipid aggregates and titanium particles released from the sonicator probe. The resultant dispersion of small unilamellar vesicles was mixed with 5 mL of an enzyme solution in 0.1 M phosphate buffer at pH 7.4 to obtain an enzyme: lipid ratio of 13.7 (w/w). The mixture was freeze-dried and rehydrated with 5 mL of phosphate buffer. Prior to the enzyme assay, the liposomal β-galactosidase was separated from unencapsulated enzyme by ultracentrifugation (100,000 × *g* for 40 min at 10 °C) and washed four times with 0.1 M phosphate buffer at pH 7.4. The liposomal pellet was resuspended in the phosphate buffer at pH 7.4 to a total volume of 3 mL.

2.3. Enzyme assay

Enzymatic activity of free and liposomal enzyme were determined using *o*-nitrophenyl-β-D-galactoside (*o*-NPG) as substrate. An aliquot of 0.5 mL of free and liposomal enzyme was added to the mixture of 0.1 mL Triton X-100 (0.1 M) and 4.9 mL of phosphate buffer (0.1 M, pH 7.4). The liposomes were disrupted by the addition of Triton X-100 to allow β-galactosidase to be released to the dispersion medium. About 0.5 mL of the mixture containing β-galactosidase was reacted with 2.5 mL of 2.3 mM of *o*-NPG dissolved in phosphate buffer. After the incubation for 30 min at room temperature, the enzymatic reaction was stopped by adding 0.25 mL of 2 M Na_2CO_3 solution. The absorbance of the reaction mixture was then measured spectrophotometrically at 405 nm [20]. The standard curve was established using *o*-NP (0–20 mM).

2.4. Determination of kinetics parameters

The apparent kinetic constants of Michaelis for the free and liposomal β-galactosidase were determined by measuring the enzymatic reaction rates at different substrate concentration ranging from 0.5 to 5.5 mM and at pH 7.4. Michaelis constants were calculated by analysing the data according to the Hanes–Woolf equation [21].

2.5. Determination of the pH–temperature/enzyme activity curves

The effect of pH and temperature of both free and microencapsulated β-galactosidase activity was studied using a response surface design [22]. A central composite design (2^2 factorial design) with 2 axial points was employed. The relative β-galactosidase activity (in %) was considered as dependent output variable. The factors were pH and temperature assayed at five levels (5.2, 5.6, 6.5, 7.4 and 7.8 for pH; 26, 30, 40, 50 and 54 °C for temperature). The design matrix of the central composite design chosen together with the results for the free and liposomal β-galactosidase are shown in Table 1. The design of the statistical experiments and the evaluation were performed using the computer program Statgraphics® Plus for Windows 4.0 (Statistical Graphics Corp., Rockville, MD, USA).

2.6. Enzyme stability towards thermal deactivation

Resistance of β-galactosidase to storage at elevated temperatures was examined by incubating samples of free and

Table 1
Design matrix and response of the central composite design for free and liposomal β-galactosidase

Run no.	Factors		β-Galactosidase activity (in %)	
	pH	T (°C)	Free	Liposome
1	5.6	30	55.05	100.00
2	7.4	30	100.00	47.30
3	5.6	50	1.04	26.37
4	7.4	50	1.95	31.20
5	5.2	40	32.69	63.17
6	7.8	40	94.59	33.65
7	6.5	26	49.23	84.33
8	6.5	54	0.30	27.67
9	6.5	40	15.61	59.33
10	6.5	40	15.52	59.34
11	6.5	40	15.61	59.29
12	6.5	40	15.60	58.96
13	6.5	40	15.58	58.72
14	6.5	40	15.60	59.36

liposomal enzyme at 30, 40, 50, 55, 60, 70 and 80 °C for 60 min and measuring the residual enzyme activity. Additionally, thermal stability of the free and encapsulated enzyme was also examined by incubating the enzymes at three temperatures (30, 35 and 40 °C) and withdrawing samples for assay at fixed intervals over an incubation period of 6 h. Thermal-decay constants (k_d) were determined from a linear regression analysis of the semi-logarithmic plot of percentage activity remaining versus time [23], calculated as:

$$\ln\left(\frac{A}{A_0}\right) = -k_d t + C_1 \quad (1)$$

where A is the enzyme activity at time t, A_0 the initial enzyme activity, t the treatment time, and k_d the thermal decay constant. The enzyme half-life was calculated from the values of thermal decay constants at different temperatures, following this equation [23]:

$$t_{1/2} = \frac{\ln(2)}{k_d} \quad (2)$$

The enzyme half-life represent the time required for enzyme to decline to 50% of its initial value of activity. To determine the activation energy (E_a) for thermal inactivation, an Arrehenius plot was constructed, and the line slope and intercept determined by linear regression analysis [23] of this equation:

$$\ln k_d = -\frac{E_a}{RT} \quad (3)$$

where R is the universal gas constant and T the absolute temperature. The thermodynamic data were calculated according to the Eyring absolute rate equation [24]:

$$\ln\left(\frac{k_d}{T}\right) = \ln\left(\frac{k_B}{h}\right) + \left(\frac{\Delta S^*}{R}\right) - \left(\frac{\Delta H^*}{R}\right)\left(\frac{1}{T}\right) \quad (4)$$

where k_B, h, ΔS^* and ΔH^* and R are Boltzmann constant, Planck's constant, entropy of activation and enthalpy of activation, respectively.

2.7. Enzyme stability towards proteolytic deactivation

The effect of protease from *Streptomyces griseus* (Pronase E, Sigma) on free and liposomal β-galactosidase was also studied. A reaction mixture containing 0.5 mL of native or liposomal enzyme with an activity of 10^{-3} mg *o*-NP ml^{-1} h^{-1} and 3.5 mL of protease (0.5 mg ml^{-1}) in 0.1 M phosphate buffer at pH 6 was incubated at 37 °C for 24 h. The reac-

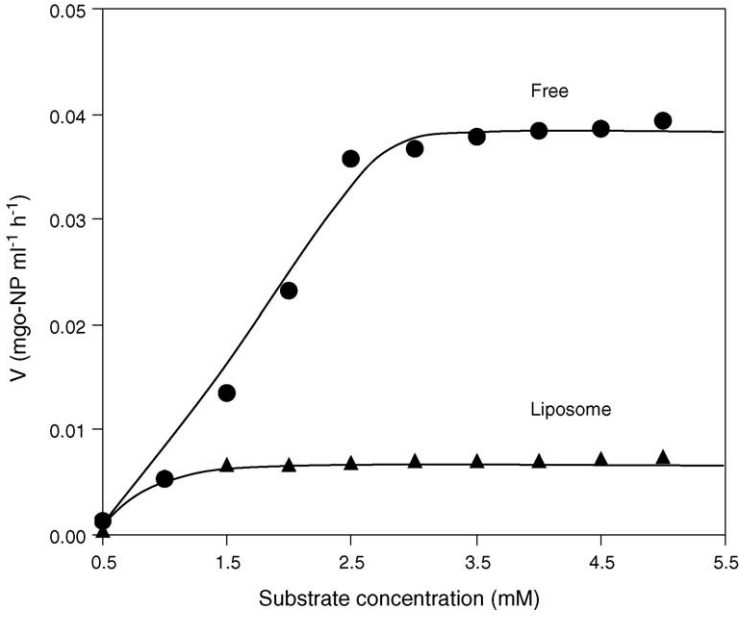

Fig. 1. Initial rate vs. substrate concentration plot for β-galactosidase free and entrapped in liposomes.

tion was stopped by reducing the temperature at 4 °C and the residual β-galactosidase activity was measured.

3. Results

β-Galactosidase was microencapsulated in liposomes with an entrapment efficiency of 28%. Entrapment efficiency was defined as the percentage amount of active enzyme entrapped in the vesicles in relation to the total amount of active enzyme present during the vesicle formation and entrapment procedure. The β-galactosidase activity corresponding to the amount of enzyme immobilized was 10^{-3} mg *o*-NP $ml^{-1}\,h^{-1}$.

3.1. Kinetic analysis

It has been assumed that the enzyme inside the vesicle follows Michaelis–Menten kinetics identical to the behaviour outside of the vesicle [25]. Free and liposomal β-galactosidase were assayed using different substrate concentration to determine the effect of the microencapsulation on the activity of β-galactosidase (Fig. 1). The Michaelis–Menten equation predicts a hyperbolic relationship between initial velocity and substrate concentration and the kinetic behaviour of free and liposomal enzyme was described by this relationship. The kinetic parameters of the free and liposomal β-galactosidase were calculated from Hanes–Woolf plot ($[S]/V$ versus $[S]$). In both cases the graphs were linear with a correlation coefficient (R^2) higher than 0.9892. Comparison of the kinetic parameters for a given free and immobilized enzyme provides information about interaction between enzyme and its support. In this sense, the K_m and V_{max} values were (i) 0.57 ± 0.01 mM and 26.1 ± 1.01 g *o*-NP $ml^{-1}\,h^{-1}$, respectively, using soluble enzyme and (ii) 3.38 ± 0.12 mM and 43.72 ± 2.20 g *o*-NP $ml^{-1}\,h^{-1}$, respectively, for liposomal enzyme.

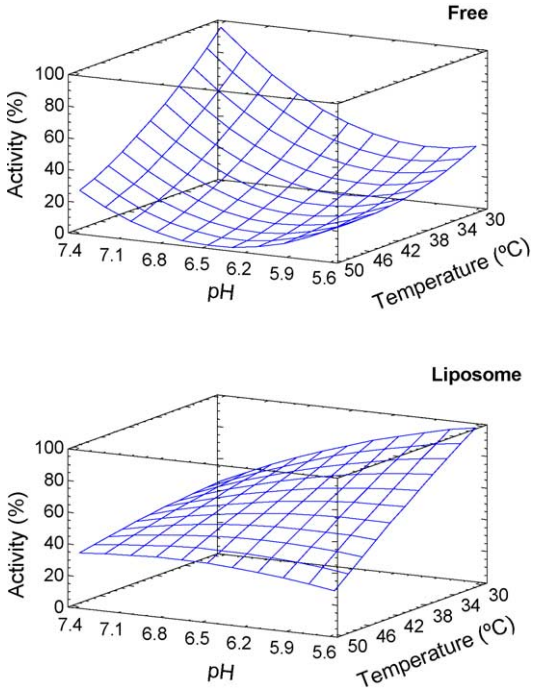

Fig. 2. Effect of pH and temperature on enzymatic activity for β-galactosidase free and entrapped in liposomes.

It is found that the value of K_m with the immobilized enzyme was greater than that with free enzyme. Regarding the maximum rate of reaction, it was significantly low as compared to soluble enzyme. In this point, it is important to remark that the kinetic study was realized in the presence of 0.1 M Triton X-100 in order to release the enzymes from the interior of the liposomes. These modifications observed in the kinetic parameters once the enzyme has been microencapsulated, indicate that the liposomal enzyme has an apparent lower affinity for the substrate than that of free enzyme does, which may be caused by chemical and/or conformational changes in the enzyme structure provoked by an association of the β-galactosidase with the lipid vesicles. Sanchez and Perillo [26] related that two mechanisms may participate in the modulatory effect of phosphatidylcholine vesicles on the β-galactosidase activity. The first one would induce an inhibition through a sequestration of the enzyme–substrate complex by its penetration into the vesicular bilayer. The second mechanism might be a competition in the β-galactosidase-catalysed reaction exerted by the polar head group of the phospholipids.

On the other hand, a parallel kinetic study in the absence of Triton X-100 was realized. In this case, it was not possible to detect liposomal β-galactosidase activity at the different substrate concentrations (data not shown). This let us affirm that the hydrolysis of the substrate did not occur on the external surface of the vesicular bilayer and that the microencapsulation totally limited the accessibility of substrate molecules into the liposome where the enzymes were located.

3.2. Influence of the reaction pH and temperature on the enzyme activity

The pH and temperature influence the velocity of an enzyme-catalysed reaction. The active sites on enzymes are frequently composed of ionisable groups that must be in the proper ionic form in order to maintain the conformation of the active site. As the temperature increases, the expected increase in enzyme activity resulting from increased enzyme/substrate collisions is offset by the increasing rate of denaturation [22]. Results obtained on the effect of pH on enzyme entrapped are usually unpredictable: in some cases both the optimal pH and the pH/activity profile change upon immobilization, whereas in other cases they do not. Differences in the activity of free and liposomal β-galactosidase as a function of pH and temperature were investigated.

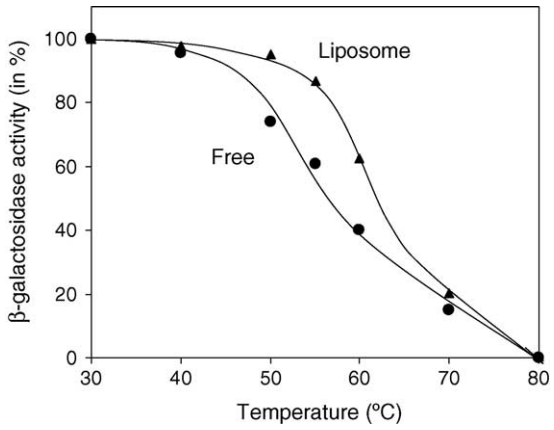

Fig. 3. Thermal stability of β-galactosidase free and entrapped in liposomes.

Fig. 2 indicates that pH and temperature promote different behaviour in the native enzyme in comparison with the entrapped one. Thus, native enzyme exhibits a maximum at pH 7.7 and 26 °C while liposomal enzyme shows a maximum at pH 5.2 and 26 °C. It was found, that the microencapsulation affects the optimum pH and that the pH activity profile of liposomal β-galactosidase was displaced to acid region with respect to that of free β-galactosidase. In the case of entrapped enzyme, the effect of the temperature is more important to acid values of pH. By contract, the influence of the temperature at basic pH on free enzyme seems to be higher than that on entrapped enzyme.

3.3. Thermal stability

Though immobilization does not necessarily lead to stabilization, there have been many reports on enzyme stabilization by immobilization [27–29]. Thermal stability of the liposomal enzyme was investigated by heating the samples for 1 h at seven different temperatures ranging from 30 to 90 °C, and the residual activity was measured. Microencapsulation of β-galactosidase in liposomes offers a noticeable increase in thermal protection. At 55 °C, liposomal enzyme retained 86% of its activity, however, only 65% of the activity of free β-galactosidase was observed at the same temperature (Fig. 3).

Additionally, thermal stability kinetics of free and liposomal enzyme at 30, 35 and 40 °C were also studied for 6 h. The logarithm of the relative residual β-galactosidase activity, based on the initial enzyme activity, was plotted against the incubation time (Fig. 4). The deactivation step obeys an irreversible first-order kinetic (with correlation coefficient higher than 0.9969) and it suggests that a direct transition occurs from active enzyme to totally inactive protein. As can be observed in Table 2, the deactivation constants (k_d) of the free enzyme were higher than those of the entrapped β-galactosidase, noting that the thermal stability of the enzyme was improved by entrapping them in liposomes.

To understand the effect of the microencapsulation on the inactivation of β-galactosidase, the dependence of temperature on the rate of inactivation was observed. For most enzyme catalysed reactions, rate constants depend on changing temperature in accordance with the Arrhenius equation [30]. The relationship between the rate constant of reaction and the activation energy (E_a), can be given by this equation. The activation energies for free and liposomal β-galactosidase were determined in the temperature range of 30–40 °C. A lineal relationship between the k_d and the inverse of temperature ($1/T$) was found for free and liposomal enzyme. The coefficient of determination was high (>0.996) hence this linear model was appropriated for the inactivation data. The activation energies (E_a) for free and entrapped β-galactosidase were 100.6 ± 10.3 and $138.8 \pm 8.6\,\mathrm{kJ\,mol^{-1}}$, respectively. These values again suggest that the microencapsulation of β-galactosidase into lipid vesicles causes a significant increase in thermal stability.

In order to have more knowledge about the effect of the microencapsulation on the stability of β-galactosidase, the values of activation enthalpy (ΔH^*) and entropy (ΔS^*) were determined. The overall ΔH^* and ΔS^* values for soluble enzyme were $98.1 \pm 6.3\,\mathrm{kJ\,mol^{-1}}$ and $6.47 \pm 1.3\,\mathrm{J\,mol^{-1}\,K^{-1}}$, respectively. The ΔH^* and ΔS^* values obtained for free enzyme were noticeably lower than values of $134.3 \pm 6.4\,\mathrm{kJ\,mol^{-1}}$ and $20.0 \pm 3.7\,\mathrm{J\,mol^{-1}\,K^{-1}}$ estimated for liposomal enzyme. From here it can be seen that the enzymatic immobilization of β-galactosidase in liposomes declines the thermal inactivation. This behaviour could be attributed to the association of the enzyme with phospholipid vesicles, which provokes an increase of the thermal stabilization of the β-galactosidase. These positive ΔH^* and ΔS^* values obtained in this study are in general agreement with values expected for enzyme heat-inactivation [31] and provide a measure of the number of non-covalent bonds broken and the net enzyme/solvent disorder change associated with a thermal inactivation of enzymes [32].

From the point of view of applied enzymology, data of the half-life are more important than a detailed knowledge of the effect of temperature on catalysis [32]. The half-lives of the native and liposomal β-galactosidase are listed in Table 2. The encapsulation of enzyme in liposomes enhanced its stability with time at all temperatures. The relationship between the half-lives of the entrapped and free enzyme can be related as a stabilization factor. A value higher than one indicates a stabilizing action. These values were 2.1, 1.8 and 1.3 for β-galactosidase microencapsulated in liposomes at

Table 2
Thermal inactivation constants (k_d) and half-lives ($t_{1/2}$) for free and liposomal β-galactosidase

T (°C)	k_d (h^{-1})		$t_{1/2}$ (h)	
	Free	Liposome	Free	Liposome
30	0.07 ± 0.01	0.03 ± 0.01	4.67 ± 0.63	9.62 ± 0.73
35	0.13 ± 0.04	0.07 ± 0.01	2.34 ± 0.78	4.17 ± 0.13
40	0.23 ± 0.03	0.17 ± 0.02	1.33 ± 0.23	1.69 ± 0.28

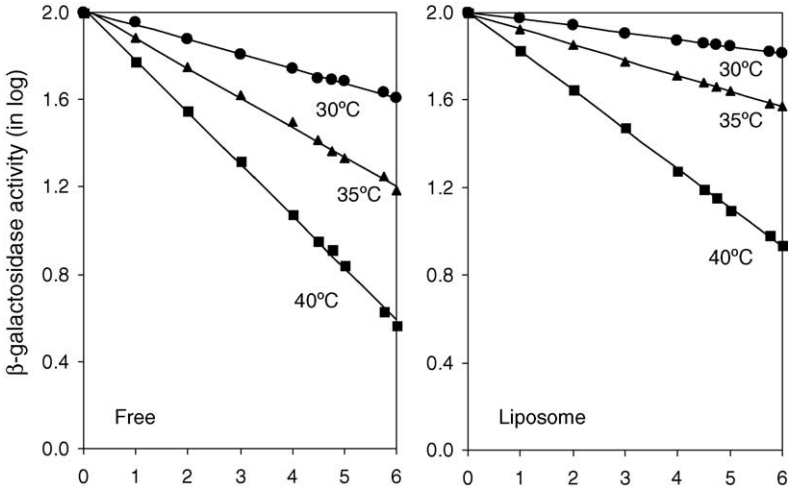

Fig. 4. Plots of the remaining activity of β-galactosidase free and entrapped in liposomes.

30, 35 and 40 °C. Similar results were found in a previous research with glucose oxidase immobilized in liposomes [34].

3.4. Stability towards proteolytic deactivation

In enzymatic reactions, inactivation of enzyme is often accelerated by contamination with proteolytic enzymes [33]. The encapsulation in liposome is increasingly recognised as a method of protecting biocatalysts from inactivation by proteolytic enzymes. Pronase from *S. griseus* was used for testing the resistance of the free and liposomal β-galactosidase to proteolysis [28]. About 70% of the native enzyme activity was lost after 4 h of exposure to protease and only 7% activity remained after 24 h (Fig. 5). The β-galactosidase entrapped in liposomes showed high resistance to proteolysis, retaining about 93 and 75% of its initial activity after 6 and 24 h, respectively. The immobilization in lipid membranes was very effective in protecting the enzyme from proteolytic deactivation. The loss of activity of liposomes after 24 h could be motivated by the fact that the liposomes were not stable enough at high incubation time or/and that there were inactive enzymes on the external surface of the liposomes vulnerable to the attack of the protease.

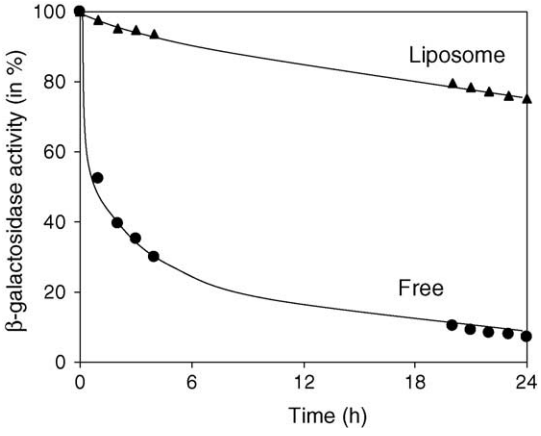

Fig. 5. Effect of pronase on the activity of β-galactosidase free and entrapped in liposomes.

4. Conclusion

Kinetic properties and thermal and proteolytic inactivation of β-galactosidase entrapped in liposomes were studied. Microencapsulation of the enzyme shifted pH optima from 7.7 to 5.5, however, the temperature optima was unaltered. Liposomal and free enzyme showed a typical Michaelis–Menten profile, but the entrapment altered the affinity of the enzyme to its substrate and the maximum rate of reaction. Half-life and energy of activation displayed by the liposomal enzyme indicated the thermostabilization of β-galactosidase by microencapsulation. Thermodynamic characterization led to conclude that encapsulation in liposomes improved both entropy and enthalpy of deactivation, thus, confirming the superiority of immobilized enzyme over the free one. Finally, proteolytic stability of liposomal β-galactosidase was demonstrated in the presence of a protease from *S. griseus*.

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