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Thermodynamics of Chitinase Partitioning in Soy Lecithin Liposomes and Their Storage Stability

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Abstract The goal of this study was to define the partitioning behavior of chitinase from *Trichoderma* spp. in soy lecithin liposomes, using a thermodynamic approach based on the partitioning variation with temperature. An effort has been made to define the liposomes, as well as free and immobilized enzyme stability during storage at 4 and 25 °C. The partition coefficients ($K_{o/w}$) were greater than 1; therefore, the standard free energies of the enzyme transfer were negative, indicating an affinity of the enzymes for encapsulation in liposomes. The enthalpy calculation led to the conclusion that the process is exothermic. The presence of enzyme decreased the liposome storage stability from 70 days to an approximately 20 days at 25 °C and 30 days at 4 °C. Monitoring of the liposome's diameter demonstrated that their size and concentration decreased during storage. The liposome's diameters ranged from 1.06 to 3.30 μm. The higher percentage of liposome corresponded to a diameter range from 1.06 to 1.34 μm. This percentage increased during storage. There were no evidences for liposome fusion process. The stability of immobilized enzyme was increased in comparison with free chitinase.

Keywords Chitinase · Microencapsulation · Soy lecithin liposomes · Storage stability · Immobilized enzyme

Introduction

Chitinases (β-*N*-acethyl-D-glucosaminidase, EC 3.2.1.14) are hydrolytic enzymes that break down glycosidic bonds in chitin [1]. Chitin composes the cell walls of fungi and exoskeleton elements of some animals (including worms and arthropods). Chitinases are generally found in organisms that either need to reshape their own chitin or dissolve and digest the chitin of fungi or animals. Chitin, like cellulose, has been thought of as abundant

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but difficult to digest. Fungi, such as *Trichoderma* spp., are known to possess chitinases. Previous studies revealed that chitinase has a variety of application potentials (e.g., preparation of fungi protoplast, cytochemistry location, antifungal agent, and transgenetic plant) [2]. The importance of chitinase and β-1,3-glucanase (laminarinase), as key enzymes responsible for the fungal cell and sclerotial wall lysis, has been reported [3, 4]. These enzymes are produced by several fungi and bacteria and could impact the biological control process [5]. Various modes of action have been associated with the ability of *Trichoderma* spp. to control plant pathogens [6] including antagonism to antibiotics [7] or cell wall-degrading enzymes [8], substrate competition, and the ability to colonize the ecological niche favored by the pathogen. The use of antagonistic microbes seems to be one of the promising approaches, an alternative to chemical control [9], which is based on intensified use of fungicides. Chemical control resulted in the accumulation of toxic compounds potentially hazardous to humans and the environment and also in the buildup of resistance of the pathogens [10]. Several fungicides based on *Trichoderma* spp. have been commercialized in the last few years. However, considering the slow adaptation of antagonistic microorganisms to the environment, the application of mycolytic enzymes as antifungal treatment in the protection of some commercially important crops may be promising.

Chitinase is one of the enzymes that are strong inhibitors of many important plant pathogens. Chitinases are able to lyse polysaccharides of mature hyphae, conidia, chlamydospores, and sclerotia. *Trichoderma* spp. enzymes are substantially more antifungal than other purified enzymes from any other source when assayed under the same conditions. They are more active than corresponding plant enzymes, effective on a much wider range of pathogens (e.g., *Fusarium oxysporum*), and are nontoxic to plants at high concentrations [11, 12]. Lytic extracellular enzymes markedly inhibited mycelial growth rather than spore germination and also caused lysis of pathogenic fungi mycelia and germ tubes [12, 13].

On the other hand, the enzyme application in situ needs to increase its stability and protection against environmental factors [14]. Stability is a beneficial parameter for biocatalysis due to the prolonged lifetime of the catalyst; as a result, it reduces the cost of the process. This makes stability an important parameter to be improved during the development of industrial enzymes. In some cases, immobilization can be useful to achieve this goal. The immobilization on solid supports can affect the enzyme mobility to fungi and increase system heterogeneity. Therefore, the immobilization in phospholipid vesicles (liposomes) can help to avoid the problems related to the union of enzyme on solid support.

Liposomes are under the investigation as models for biological membranes and as carriers for various bioactive agents such as drugs, diagnostic and genetic materials, and vaccines. In the food industry, liposomes have been used to deliver food flavors and nutrients and have been investigated for their ability to incorporate food antimicrobials that could aid in the protection of food products against growth of spoilage and pathogenic microorganisms [15].

Recently, we reported [13] the antifungal effect of chitinase and laminarinase encapsulated in soy lecithin liposomes studied in vitro testing by means of monitoring of radial growth of *F. oxysporum* on potato dextrose agar. The assay was carried out with and without free and immobilized enzymes, their mixtures as well as thiabendazole. Although the antifungal effect of the individual enzymes decreased after microencapsulation, the simultaneous application of both enzymes allowed a total inhibition of fungal radial growth. It was demonstrated that the use of free or microencapsulated laminarinase and chitinase led to a reduced level of the chemical fungicide from 2.5 to 1.25 mg/ml. Complete inhibition of

fungal growth was observed with 1.25 mg/ml of thiabendazole plus free or immobilized enzymes at 0.01 mg/ml. Microencapsulation in phospholipid vesicles did not modify system heterogeneity and allowed enzyme interaction with fungi unlike immobilization on solid supports [13].

Enzyme encapsulation into liposomes is a promising technique to stabilize and prevent them from denaturation and proteolysis. It was demonstrated for an acetylcholinesterase activity, which is the main target for pesticides [16]. The chitinase encapsulation in liposome was not yet studied. The thermodynamics of enzyme molecule transfer can be studied by measuring the partition coefficient as a function of temperature. Such data were used for the prediction of absorption, membrane permeability, and in vivo drug distribution in the case of various drugs [17, 18].

Different phospholipids may be applied to obtain liposomes. In the present study, the soy lecithin was used. Soy lecithin is the popular and commercial name for a naturally occurring mixture of phospholipids, which varies from light to dark in reddish-brown color. Soybeans are by far the most important source of commercial lecithin, and lecithin in its turn is the most important by-product of the soy oil processing industry due to its many applications in foods and industrial products.

As a contribution to the generation of physicochemical and operational information about enzyme transfer properties, the objectives of this study were 1) to describe the partitioning behavior of chitinase in soy lecithin liposome system in the presence of different enzyme concentrations, by employing a thermodynamic approach based on the rational partitioning variation in respect to temperature; and 2) to define the stability of soy lecithin liposome systems as well as free and microencapsulated chitinase. The first objective of this study has theoretical input, while the second has practical significance for enzyme application as a biocontrol agent.

Materials and Methods

Soy lecithin was provided by PROQLIMS S.A. de C.V. (Mexico) as a commercially available product. In this study, the term “lecithin” is used in its broader sense, to refer to the natural complex. In the mixture called “commercial soy lecithin,” the main phospholipids were 33.0% of phosphatidylcholine (also called “pure” or “chemical” lecithin to distinguish it from the natural mixture), 14.1% of phosphatidylethanolamine (popularly called “cephalin”), 16.8% of phosphatidylinositols (also called inositol phosphatides), and 0.4% of phosphatidylserine. Commercial soy lecithin also typically contains unrefined soy oil as well as additives insoluble in organic solvents [19]. Lecithin was partially purified to separate additives insoluble in the mixture of $CHCl_3/CH_3OH$ 1:1 (*v*/*v*) before its use. All solvents were of analytical grade and provided by J. T. Baker (USA). All salts used in this study were of analytical grade and purchased from Jalmek (Mexico). Chitinase (Sigma Cat. no. C8241) from *Trichoderma* spp. and its substrate were purchased from Sigma-Aldrich Chemical Company (USA).

Liposome/Enzyme Solution Partitioning, Determination of Partition Coefficients, and Thermodynamic Functions of Transfer

Liposomes were prepared by a method similar to the Bangham method [17]. Thin film of soy lecithin was formed on the walls of 2-l round-bottomed flasks, followed by evaporation of 1 ml aliquot of methanol solution. Then, flasks were placed in the oven at 50 °C for 24 h.

The film was dispersed in 5 ml of enzyme solution (protein concentrations were 0.0041, 0.0074, 0.0126, or 0.0191 mg/ml) in 0.1 M sodium acetate buffer, at pH 5.4, or in the same buffer without enzyme at 4, 25, 27, 32, and 40 °C, and vortex-mixed until all film was removed from the walls of the flasks. The mixtures were incubated for 24 h at the same temperatures as indicated above. This resulted in the formation of multilamellar vesicles (MLVs), which was verified by microscopy, according to reported methods [18] described below.

The enzyme distribution was determined in 24-h temperature-equilibrated MLVs in 1.2 ml samples, followed by centrifugation (15,000×g for 60 min) at the specified temperature. Protein concentrations in the initial enzyme solution and supernatant, obtained after centrifugation and organic phase separation, were determined by means of spectrophotometric analysis described by Bradford [20]. The same treatments were carried out with enzyme solutions without lecithin to compare the activity and stability of free and immobilized enzymes.

The molal partition coefficients ($K_{o/w}$) were calculated by means of the reported method [21] using the equation: $K_{o/w} = W_{aq}(C_o - C_f)/\left(C_f\ W_{org}\right)$ where C_o and C_f are the initial and final concentrations of enzymes (milligrams per milliliter) in the aqueous buffer phase before and after the transfer of the protein from the aqueous phase to the organic medium, respectively [18], as well as W_{aq} is the weight (grams) of the aqueous phase, and W_{org} is the weight (grams) of lecithin phospholipids in the sample. The standard free energy of transfer ($\Delta G_{w\to o}$) from aqueous media to organic system was calculated using the equation [18]: $\Delta G_{w\to o} = -RT\ \ln K_{o/w}$ where $K_{o/w}$ is expressed in molality.

The temperature dependence of partitioning (van't Hoff method) was employed to obtain data on the enthalpy of transfer ($\Delta H_{w\to o}$), based on the next equation, assuming that $\Delta H_{w\to o}$ is independent of temperature over the range of interest. The values $\Delta H_{w\to o}$ were obtained from the slopes of $\ln K_{o/w}$ versus T^{-1} weighted curves, using linear regression with the least-squares method: $\Delta H_{w\to o} = R[(-\delta \ln K)/\delta(1/T)]_P$.

The entropy of transfer ($\Delta S_{w\to o}$) was obtained by means of equation $\Delta S_{w\to o} = (\Delta H_{w\to o} - \Delta G_{w\to o})/T$, assuming that this property does not change with the temperature in the studied range. The $\Delta H_{w\to o}$ and $\Delta S_{w\to o}$ are used to represent the change in enthalpy and entropy, respectively, when 1 mol of enzyme is transferred from the aqueous medium to the organic system at infinite dilution expressed in molality [21, 22].

Liposome Storage Stability Evaluation

The MLVs prepared at 4 and 25 °C were stored at the same temperature that was employed for their preparation. Optical light microscopy (×40) was performed according to reported methods [18]. Calculation of the liposome's number was performed immediately after their preparation and each tenth day during their storage at 4 and 25 °C. Moreover, during liposome storage, their linear size was estimated from measurements of vesicle diameters. The estimation was carried out by the use of photographs taken with Sony Cyber camera at 5.1 megapixel to a 3.0 zoom, from the image observed at the Neubauer chamber by the optical microscope Axiostar plus, Carl Zeiss (USA). To calculate liposome diameter, the real linear size of the smallest square side (0.005 cm) was taken into account. The calculations were realized by means of comparison of this value to the measurements obtained from the photography (rule of three). One hundred measurements were performed in each case. The assays were repeated three times. The obtained data were treated by Sturges' rule [23] in order to determine the desirable number of classes into which a distribution of observations

should be classified; the number of classes (k) was 8, as approximated from the equation: $1 + 3.3\log n$, where n equals 100 as the number of observations. The data range (r) was calculated from the smallest and highest diameter values, while the class width (w) was calculated as r/k.

Enzyme Activity and Storage Stability Measurements

Chitinase activity was quantitatively determined by colorimetric measurements assessing the nitrophenyl group of *p*-nitrophenyl-β-D-*N*-acetyl-glucosamide which served as the substrate [24]. A typical enzyme reaction mixture contained 0.45 ml of the same buffer, 0.1 ml of the enzyme-containing sample, and 0.1 ml of 0.5 mM of substrate in 0.1 M sodium acetate buffer, at pH 5.5. After incubation at 37 °C for 140 min, the reaction was stopped by adding 0.5 ml of 1 M Na_2CO_3. The reaction time was selected based on preliminary assay, which demonstrated the linear kinetic response for at least 150 min. The release of *p*-nitrophenol was monitored spectrophotometrically at 405 nm. One unit (IU) of enzyme activity was defined as the amount of enzyme that catalyzes the release of 1 μmol *p*-nitrophenol per min at 37 °C. The enzyme stability was evaluated by means of the above described technique and carried out each 7 days for 21 days.

To define the enzyme effect of encapsulation in liposome on the enzyme activity, the assay was carried out in the presence of nonionic surfactant Triton X-100, which was applied to free or immobilized enzyme preparation at 2% concentration before activity measurement for 30 min. By means of optical microscopy, it was demonstrated that liposomes were solubilized under this condition. The same water volume was applied to the control assay in order to calculate 100% of relative enzyme activity.

Enzyme activity was calculated by averaging activity the values obtained from at least two different repetitions in experiments performed three times. A variation of less than 10% was observed when replicated assays were compared.

Statistical Analysis

To evaluate the partition coefficients of chitinase, the factorial design was used considering enzyme concentration and the temperature as two different factors applied at the four and five levels, respectively. Also the operational characteristics of the system (activity and stability) were estimated under selected conditions, as described above. In this case, a completely randomized design was used. All assays were carried out three times with at least two different repetitions. The means and standard deviations of all obtained results were calculated. Data were analyzed by one-way analysis of variance. Significant differences ($p \leq 0.05$) between the means were determined by the DUNCAN multiple range tests [25].

Results and Discussion

Liposome/Enzyme Solution Partitioning, Determination of Partition Coefficients, and Thermodynamic Functions of Transfer

Table 1 summarizes the temperature dependence of the partition coefficients for chitinase in all studied systems. The word “partition” means that chitinase is distributed between two phases in a dynamic equilibrium. It is a heterogeneous equilibrium since the “solute” is

Table 1 Partition coefficients of chitinase in soy lecithin liposome system as a function of temperature (±0.1 °C), in molality (± standard deviation)

[Protein], μg/ml	$K_{o/w}$ at 4 °C	$K_{o/w}$ at 25 °C	$K_{o/w}$ at 27 °C	$K_{o/w}$ at 32 °C	$K_{o/w}$ at 40 °C
4.1 μg/ml	n.d.	82.2±6.2	66.6±5.6	29.9±2.5	7.0±0.5
7.4 μg/ml	69.5±3.4	28.9±2.1	20.57±1.9	15.9±1.1	9.3±0.7
12.6 μg/ml	20.9±1.8	14.0±1.1	12.5±1.2	11.3±0.9	9.2±0.7
19.1 μg/ml	8.6±0.7	6.9±0.7	7.1±0.6	6.8±0.6	6.6±0.6

n.d. no data

distributed between two distinct phases: water and liposome lipids. Data illustrating the decrease of the enzyme concentrations in the water containing phase after the liposome formation were the evidence that confirmed the existence of the distribution process between these two phases.

The partition coefficients of enzyme ($K_{o/w}$) were greater than 1 indicating an affinity of chitinase for microencapsulation in liposomes. The partition coefficients were decreased with the temperature increasing, as well as with the protein concentration escalation. The dependence of concentration led to the conclusion that $K_{o/w}$ was an apparent constant. The variability of $K_{o/w}$ with the concentration was reported in the case of encapsulation of some pharmacological substances [18, 26]. Data related to the lowest protein concentration (0.0041 mg/ml) microencapsulated at 4 °C were not taken into account because nearly all protein was immobilized (Tables 1 and 2). It was difficult to quantify exactly the non-

Table 2 Chitinase microencapsulation in soy lecithin liposomes (as 100% initial protein concentration was taken into account)

[Protein], μg/ml		Liposomes×10^{-7}/ml	fg of protein/liposome	% of immobilization
Initial	Immobilized			
At 4 °C				
4.1±0.3	≈4.1	463±83.2	0.885	≈100
7.4±0.2	6.3±0.1	288±53.4	2.18	85
12.6±0.4	9.5±0.7	264±54.0	3.60	75
19.1±0.4	12.2±0.1	266±71.9	4.59	64
At 25 °C				
4.1±0.3	3.6±0.1	0.683±0.188	527	88
7.4±0.2	5.4±0.3	0.300±0.090	1,800	73
12.6±0.4	7.8±0.3	0.403±0.201	1,930	62
19.1±0.4	8.4±0.3	0.317±0.088	2,670	44
At 40 °C				
4.1±0.3	1.6±0.2	n.d.	n.d.	39
7.4±0.2	3.5±1.2	n.d.	n.d.	47
12.6±0.4	3.6±0.9	n.d.	n.d.	29
19.1±0.4	5.6±1.7	n.d.	n.d.	29

± standard deviation

n.d. no data

encapsulated protein concentration due to its level, which was lower than the detection limit of the applied analytical technique [20].

Table 2 demonstrates the results obtained after liposome quantification by optical microscopy in the case of systems prepared at 4, 25, and 40 °C, as well as after the protein balance. Percent of immobilized protein was decreased with the increasing of the temperature and protein concentration. However, the liposome concentration decreased significantly with the rising temperature and only slightly with concentration increase, which led to the boost of protein concentration per liposome (Table 2).

Partition coefficients of chitinase in liposomes (Table 1) and the immobilization ratio (Table 2) were relatively lower at the higher protein concentration. The data analysis demonstrated that microencapsulation of chitinase followed a pseudo-saturation behavior (Fig. 1). Protein microencapsulated per liposome as a function of non-microencapsulated protein quantity was described by linear form of Langmuir-type isotherms (Fig. 1). Pseudo-Langmuir adsorption constants were estimated at 0.55 and 0.35 mg^{-1} while the maximum values of adsorbed protein were 5.74×10^{-12} and 3.62×10^{-9} mg/liposome for 4 and 25 °C, respectively. The Freundlich adsorption model did not describe the experimental data. Similar behavior was reported previously for drug containing liposomal systems [27].

Data corresponding to liposome concentration at 40 °C are not presented due to agglomeration of liposomes. However, protein encapsulation in agglomerates was presented (Table 2).

Under this condition, the liposomes were not separated as single MLVs due to high membrane fluidity. For the reason that soy lecithin transition temperature is around 40 °C, liposomes are in a liquid crystal (fluid) state at 40 °C, whereas at 4 and 25 °C, liposomes are in a gel (rigid) state [22]. The transition of a membrane structure of tilted gel ($L_{\beta'}$) via ripple gel phase ($P_{\beta'}$) to a more fluid liquid crystalline phase (L_{α}) with temperature increase was reported by Sulkowski et al. [28].

The temperature dependence of partitioning for different chitinase concentrations in soy lecithin liposomes as ln$K$ versus $1/T$ (van't Hoff plots) is presented in Fig. 2. In all cases, straight lines with correlation coefficients (r) near 0.96 were obtained for evaluated

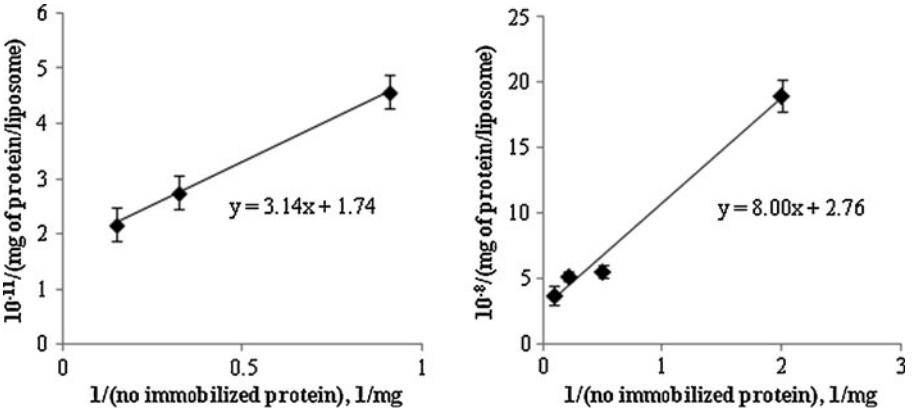

Fig. 1 A plot of linear Langmuir isotherm (reciprocal value of milligram of protein per liposome versus inverse protein quantity) obtained for chitinase microencapsulation in lecithin liposomes at 4 °C (*left*) and 25 °C (*right*). Correlation coefficient (R^2) was more than 0.98. Standard deviations of polynomial coefficients of linear functions were less than 10%

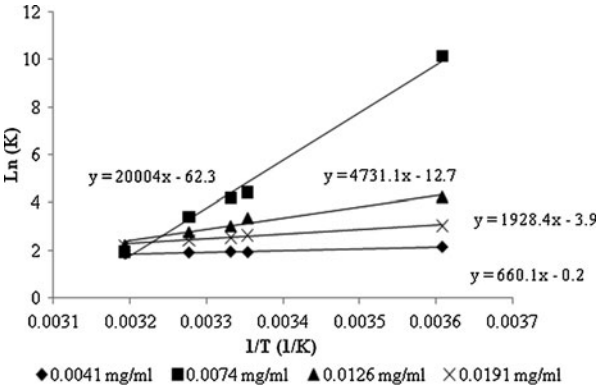

Fig. 2 Dependence of partitioning on the temperature in van't Hoff coordinates for chitinase in soy lecithin liposomes system. Correlation coefficients (R^2) were more than 0.96. Standard deviations of polynomial coefficients of linear functions were less than 10%

partitioning systems; therefore, the van't Hoff method was useful for the respective thermodynamic analyses [22].

Table 3 summarizes the thermodynamic functions related to the transfer of chitinase from aqueous media to soy lecithin liposomes. In all cases, values of $\Delta G_{w\to o}$ at 25 °C were negative, which indicated the preference of the enzyme for organic phase, confirming that chitinase transfer from aqueous media to organic system was spontaneous. However, the magnitudes of $\Delta G_{w\to o}$ decreased with concentration increase.

The parameters $\Delta H_{w\to o}$ and $\Delta S_{w\to o}$ (Table 3) were calculated from linear functions of Fig. 2 and $\Delta G_{w\to o}$ equation, using graphically estimated enthalpy value, as described above (Table 3). Magnitudes of both functions decreased with protein concentration increase. The plots describing the relation between thermodynamic functions $\Delta G_{w\to o}$, $\Delta H_{w\to o}$, and $\Delta S_{w\to o}$ versus protein concentration are presented in Figs. 3 and 4.

The tendency may be approximated with the hyperbolic functions $Y=a/(b+[\text{chitinase}])$, which give good visual correlation (Figs. 3 and 4) obtained using a and b parameters estimated from linearization of experimental data in inverse coordinates, as it is demonstrated in Fig. 3 (right). The estimated hyperbolic functions have only descriptive character and do not contribute to the mechanism definition.

The enthalpy and entropic changes imply, respectively, to energetic requirements and the molecular randomness (increase or decrease in the molecular disorder), resulting in the net transfer of the enzyme from the water to the organic phase. In general terms, behavior presented in each phase should be considered independently before and after the partitioning process. The change in these parameters as a function of chitinase concentration reflects the consequence of modifications of the molecular interactions and the physical properties of liposomes during system saturation with the protein [22, 29–31]. Since initially the enzyme was present only in water, it was

Table 3 Free energy, enthalpy, and entropy for the transfer of chitinase from aqueous media to soya lecithin liposomes

Enzyme, mg/ml	$\Delta G_{w\to o}$, kJ/mol	$\Delta H_{w\to o}$, kJ/mol	$\Delta S_{w\to o}$, J/(mol×K)
4.1±0.3	−10.9±0.9	−166.3±9.9	−521.2±25.0
7.4±0.2	−8.3±0.5	−39.3±2.8	−103.9±9.1
12.6±0.4	−6.5±0.5	−16.0±1.3	−31.8±2.9
19.1±0.4	−4.8±0.3	−5.5±0.4	−2.3±0.2

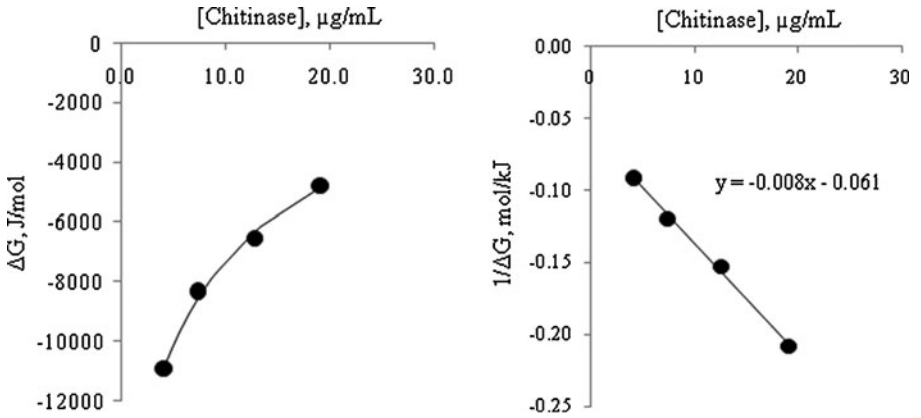

Fig. 3 Relation between $\Delta G_{w\to o}$ versus protein concentration applied during chitinase immobilization in soy lecithin liposomes: *left*, experimental data are presented as points, while theoretical line is obtained with the equation $\Delta G = -130,872.92/(7.93 + [\text{chitinase}])$; *right*, linearization of experimental data in inversed coordinates. Correlation coefficient (R^2) was more than 0.99. Standard deviations of polynomial coefficients of linear function were less than 10%

necessary to create a cavity in the liposome organic phase in order to accommodate the solute after transfer.

This is an endothermic process, since an energy supply is necessary to separate the lipid molecules. When the enzyme molecules are accommodated in the organic phase, an amount of energy is released due to enzyme–phospholipid interactions, which is known as an exothermic process.

The enthalpy of chitinase transfer ($\Delta H_{w\to o}$) was negative. Therefore, the process is exothermic. Negative enthalpy indicates the presence of a significant interaction between molecules of chitinase and soy lecithin phospholipids. Phospholipids can establish hydrogen bonding as a donor or acceptor of hydrogen [22]. On the other hand, after a certain number of enzyme molecules have migrated from the aqueous to the liposome organic phase, the original cavities occupied by the protein in the aqueous phase now are occupied by the water molecules. This event is accompanied by release of energy due to

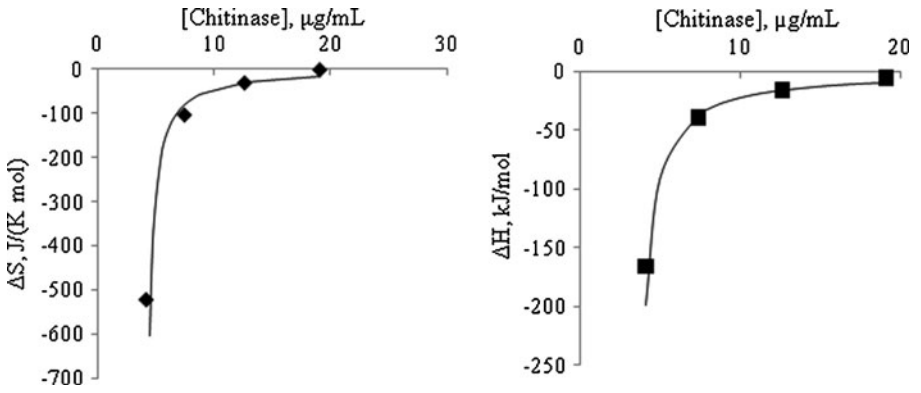

Fig. 4 Relation between $\Delta S_{w\to o}$ (*left*) and $\Delta H_{w\to o}$ (*right*) versus protein concentration quantified for chitinase immobilization in soy lecithin liposomes. Theoretical lines were obtained with the equations $\Delta S = -285.71/(-4.03 + [\text{chitinase}])$ and $\Delta H = -149,723.01/(-3.35 + [\text{chitinase}])$, respectively

water–water interactions. However, it is also necessary to keep in mind that the water molecules can organize around the enzyme hydrophobic amino acids (hydrophobic hydration). This process is accompanied by an intake of energy in addition to a local entropy increase related to separation of some water molecules which originally have been associated among them by hydrogen bonding [29]. The obtained results indicate that the transfer of chitinase is enthalpy-driven due to its negative value. The system entropy decrement can take a place due to ordination of enzyme and water environment. The changes of both thermodynamic parameters ($\Delta H_{w\to o}$ and $\Delta S_{w\to o}$) as protein concentration function described above led to a reciprocal change of $\Delta G_{w\to o}$, which was reflected in decreased partition coefficient as a function of the concentration of chitinase [30, 31].

The thermodynamics of chitinase transfer process was different to that detected in soy lecithin liposomes containing laminarinase [32]. In the case of laminarinase, the endothermic process took place and the transfer of laminarinase in liposomes was an entropy-driven process attributable to the positive value of entropy change. Such different behavior is related to the distinction in the primary structure of enzymes that leads to differences in the lipid–enzyme interactions [31, 32].

Liposome Storage Stability

Liposome storage stability was evaluated using optical light microscopy (×40) analyzing the image observed at the Neubauer chamber by the optical microscope Axiostar plus, Carl Zeiss (USA). Figure 5 demonstrates the example of the obtained photographs, which were analyzed as described above. Table 4 and Fig. 6 show the results of counting the liposomes after their preparation and during their storage at 4 and 25 °C as the temperatures employed for their formation. In Fig. 6, the standard deviation bars were higher in the first measurement, which was associated with the greater variability in the liposome concentration detected in this measurement made immediately after 24 h of incubation. It was possibly related to the presence of clusters of liposomes (Fig. 5, left), which were counted as a single liposome and later disappeared during storage.

The enzyme presence and rising temperature for liposome formation led to a decrease in their number and stability. The liposomes without enzymes are more stable (Table 4) than in their presence (Fig. 6). Soy lecithin liposomes formed and stored at 4 °C were stable during more than 70 days (Table 4). At 25 °C, their number is drastically decreased in the absence as well as in the presence of enzymes (Table 4 and Fig. 6).

The number of chitinase containing liposomes obtained at 25 °C (Fig. 6) was significantly lower than the number of liposomes at the same temperature without

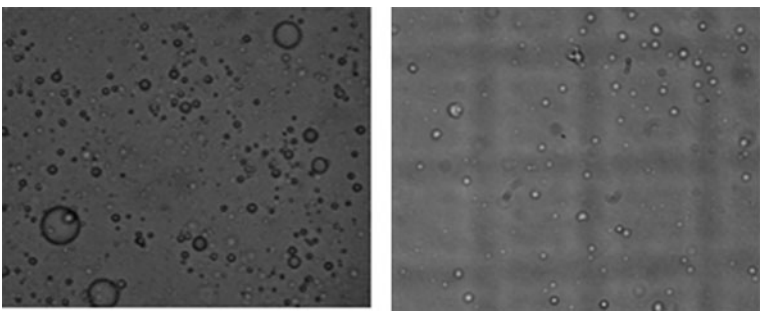

Fig. 5 Liposomes with chitinase at 0.0041 mg/ml obtained and stored for 0 days (*left*) and 30 days (*right*) at 4 °C

Table 4 Liposome storage stability at 4 and 25 °C without enzymes

Time, days	Liposomes×10^{-9}/ml (at 4 °C)	Liposomes×10^{-6}/ml (at 25 °C)
0	8.68±0.16	6,480±218
10	8.68±0.26	838±14.3
20	8.52±0.28	594±36.2
30	8.20±0.42	92.0±1.65
40	5.53±0.55	7.42±0.375
50	5.40±0.13	5.29±0.210
60	5.44±0.19	4.75±0.0632
70	5.16±0.22	4.28±0.144

enzyme (Table 4). In the presence of enzyme, the MLVs were countable for 30 days during their storage at 4 °C and for 20 days at 25 °C (Fig. 6). Thus, the soy lecithin liposomes are sensible to the enzyme presence and are better stored at low temperature.

There are two aspects that have affected stability of liposome systems: 1) the liposome component may degrade by hydrolysis and oxidation; chemical changes in the layer-forming molecules may affect physical stability; e.g., if phospholipids loss one of their acyl chains (turn into their lysoforms), the liposome structure is affected; and 2) the physical structure of the liposomes may be affected, by changes within the lipid layer, aggregation, or fusion. In the last case, the size of liposomes should be increased. The storage stability may be increased by the use of purified phospholipids [17, 18]. In the present study, the phospholipid mixture of commercially available soy lecithin was used.

The results describing relative frequencies (± standard deviation) corresponding to diameters of chitinase containing liposomes obtained and stored at 4 and 25 °C are presented in Tables 5 and 6, respectively. The same results were obtained using different enzyme concentrations. The presented data demonstrate the general trend. Liposomes obtained at 4 °C are characterized by a greater diameter than those obtained at 25 °C. The

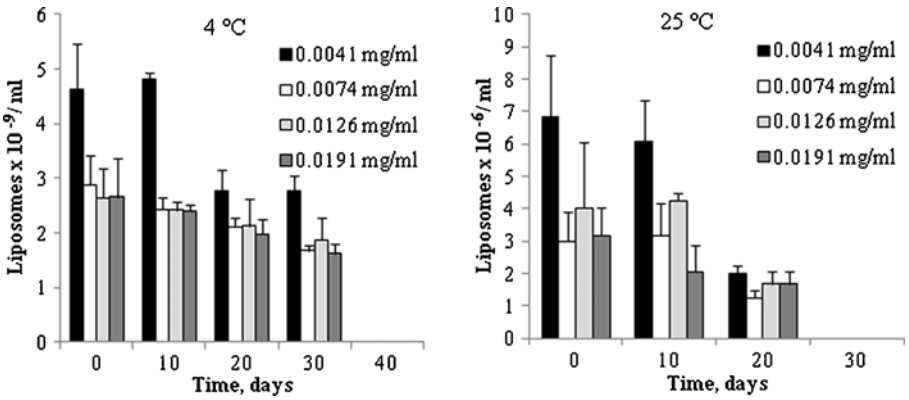

Fig. 6 Liposome storage stability at different temperatures: *left* at 4 °C; *right* at 25 °C in the presence of chitinase at 0.0041 mg/ml (*black bars*), 0.0074 mg/ml (*white bars*), 0.0126 mg/ml (*clear gray bars*), and 0.0191 mg/ml (*dark gray bars*)

Table 5 Relative frequencies (± standard deviation) corresponding to diameters of 0.0074 mg/ml chitinase containining liposomes obtained and stored at 4 °C (classes were defined based on Sturges' rule applying $k=8$, $r=2.13E{-}4$ cm and $w=2.80E{-}5$ cm)

Class of liposomes diameters, μm			Percent of liposomes with diameters corresponded to each class (± standard deviation)				
Low limit	Size	High limit	0 days	10 days	20 days	30 days	40 days
1.06	≤x<	1.34	56±2.3	59±1.5	72±1.4	94±1.2	0
1.34	≤x<	1.62	0	0	0	0	0
1.62	≤x<	1.90	0	0	0	0	0
1.90	≤x<	2.18	32±1.7	23±1.8	13±1.1	6±1.4	0
2.18	≤x<	2.46	11±1.2	12±1.4	11±0.9	0	0
2.46	≤x<	2.74	1±0.8	2±1.1	4±1.1%	0	0
2.74	≤x<	3.02	0	0	0	0	0
3.02	≤x<	3.30	0	4±1.0	0	0	0

size of a small portion of liposomes was increased after 10 days of storage at 4 °C. However, it decreased during the following several days of storage. Generally, the diameters of liposomes decreased during their prolonged storage that it was impossible to observe them by the optical microscope at the 30th and 40th day of storage at 25 and 4 °C, respectively.

Thus, monitoring of liposome diameter demonstrated that their size was not increased while their concentration decreased during storage. There was no evidence for liposome fusion process.

Enzyme Activity and Storage Stability

Table 7 shows the chitinase activity of free and encapsulated enzyme preparations quantified after immobilization. The microencapsulated enzyme was less active than the

Table 6 Relative frequencies (± standard deviation) corresponding to diameters of 0.0074 mg/ml chitinase containing liposomes obtained and stored at 25 °C (classes were defined based on Sturges' rule applying $k=8$, $r=2.13E{-}4$ cm and $w=2.80E{-}5$ cm)

Class of liposomes diameters, μm			Percent of liposomes with diameters corresponded to each class (± standard deviation)			
Low limit	Size	High limit	0 days	10 days	20 days	30 days
1.06	≤x<	1.34	84±1.20	89±1.00	100±1.40	0
1.34	≤x<	1.62	0	0	0	0
1.62	≤x<	1.90	0	0	0	0
1.90	≤x<	2.18	10±1.05	11±1.30	0	0
2.18	≤x<	2.46	6±1.30	0	0	0
2.46	≤x<	2.74	0	0	0	0
2.74	≤x<	3.02	0	0	0	0
3.02	≤x<	3.30	0	0	0	0

Table 7 Chitinase activity corresponding to the first measurement after immobilization, using 100% in storage stability assay calculations

Chitinase μg/ml	IU/ml×10^3 at 4 °C		IU/ml×10^3 at 25 °C		IU/ml×10^3 at 40 °C	
	Free	Encapsulated	Free	Encapsulated	Free	Encapsulated
4.1	5.9±0.06	3.9±0.3	1.9±0.1	1.1±0.1	1.1±0.1	0.6±0.05
7.4	14.2±0.1	7.9±0.6	3.13±0.1	1.40±0.05	1.8±0.1	1.6±0.1
12.6	24.1±1.1	11.8±1.4	5.3±0.2	2.3±0.3	3.2±0.1	2.0±0.1
19.1	36.4±1.1	15.3±1.1	8.0±0.4	2.3±0.3	4.9±0.3	3.3±0.2

free enzyme due to the encapsulation effect. In the presence of liposomes, the chitinase activity was approximately 50% less than the free enzymatic activity. Moreover, the enzyme lost its activity during encapsulation at 25 or 40 °C that led to the lower activity values even for the free enzyme, which was submitted under the same procedures applied during the microencapsulation. The reason for the lower activity of encapsulated chitinase may be related to entrapment of enzyme on the multilamellar vesicles. This hypothesis was tested in the assay performed in the presence of Triton X-100, which destroyed soy lecithin liposomes.

Figure 7 shows that free chitinase lost about 15% of activity in the presence of surfactant, while the activity in the lecithin-contained system increased at 50% under the same conditions. Triton X-100 is a nonionic surfactant, which has a hydrophilic polyethylene oxide group and a hydrocarbon lipophilic or hydrophobic group. In general, this detergent is routinely used in protein extraction and purification, as well as electrophoretic analyses of proteins [33]. Its effect on the enzymatic activity depends on the type of enzyme and concentrations used [34, 35]. In this study, solubilization of liposomes was observed in the presence of 2% Triton X-100. It is a relatively high concentration, which may be the cause of the partial loss of free enzyme activity. At the same time, the liposome solubilization with Triton caused the release of encapsulated enzyme increasing enzymatic activity.

The results describing the activity of the free and encapsulated chitinase during its storage at 4 and 25 °C are shown in Figs. 7 and 8, respectively. Due to the chitinase microencapsulation, relative activity of immobilized enzyme was higher in comparison to a

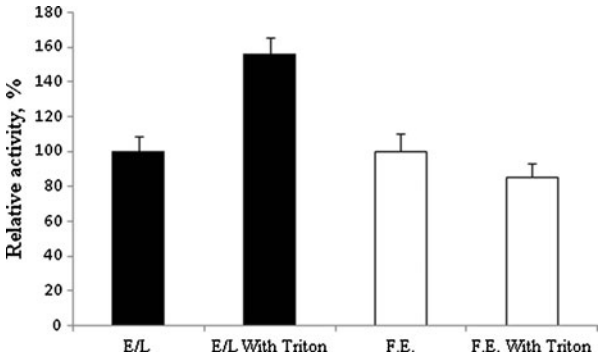

Fig. 7 Relative activity of free (*F.E.*) and encapsulated (*E/L*) chitinase before (considering as 100%, see Table 7) and after 2% (*w/v*) Triton X-100 addition (preparations obtained at 4 °C with 0.0191 mg/ml of chitinase were used)

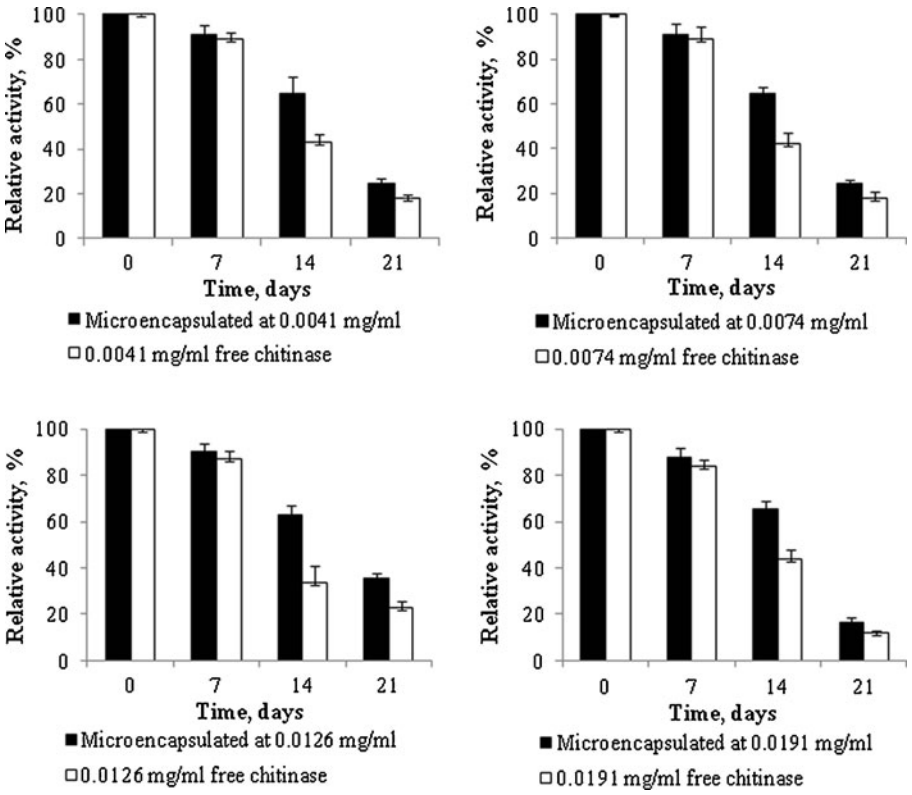

Fig. 8 Chitinase storage stability at 4 °C for microencapsulated in liposomes (*black bars*) and free (*white bars*) enzyme applied at different concentrations (as 100% the first measured activity was used)

free enzyme during their storage at different temperatures (4 and 25 °C) for 21 days, which demonstrated an increase of chitinase stability (Figs. 8 and 9).

These results differ from the reports for the laminarinase microencapsulated on the soy lecithin liposome system [32]. In the case of laminarinase stored in buffer containing suspension, the stability of immobilized enzyme did not increase in comparison with free laminarinase [32].

Chitinase is known as an unstable enzyme, which normally must be stored at temperatures lower than −15 °C. For example, the half-life at 4 °C was 9 days for the free enzyme produced by *Pseudomonas aeruginosa* [14, 36]. The lyophilized chitinase of *Trichoderma* is stable at 4 °C for 1 month [1].

Although the data of the present study were obtained in in vitro assays under controlled conditions, our other results of soil testing and in assay with tomato plant growth in the presence of fungus [37] showed that the enzyme microencapsulation protected protein structure against the environmental factors of soil such as different pHs and ion presence. The stability of the microencapsulated enzyme in soil was increased even in the presence of the chemical fungicide thiabendazole [37]. Moreover, the simultaneous application of encapsulated enzyme mixture (chitinase and laminarinase) with thiabendazole also allowed at least the double decrease of chemical fungicide concentration (to 0.006 mg/g of soil) to obtain inhibition of *F. oxysporum* growth in soil

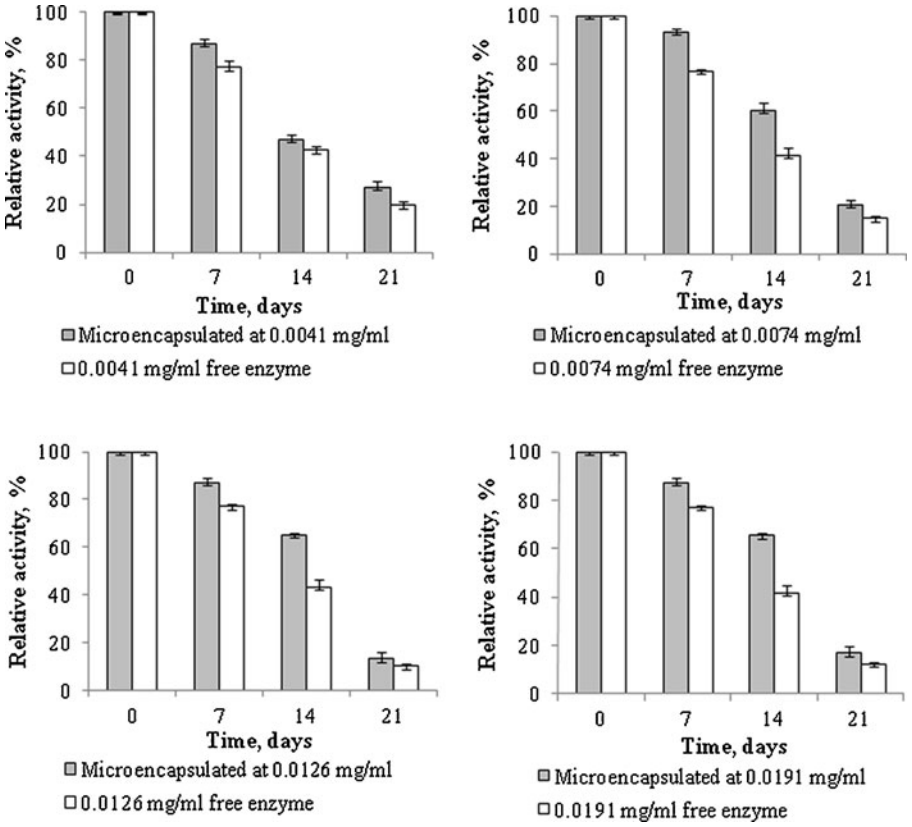

Fig. 9 Chitinase storage stability at 25 °C for microencapsulated in liposomes (*gray bars*) and free (*white bars*) enzyme applied at different concentrations (as 100% the first measured activity was used)

[37]. These results as well as the present study demonstrate the advantage of enzyme microencapsulation on enzyme stability.

Conclusions

The important contributions of the present study are the findings on the microencapsulation of enzymes in liposomes that can be considered for process optimization in future studies and applications. From the previously presented analysis, it could be concluded that chitinase has an affinity to soy lecithin liposomes. Chitinase transfer is enthalpy-driven due to its negative value. The greater percentage of immobilized chitinase was obtained at lower enzyme concentration and temperature. Moreover, the stability of microencapsulated enzyme preparations was increased, while the enzyme presence decreased the stability of soy lecithin liposomes. In summary, liposomes appear to be suitable carriers for chitinase. Future investigation will focus on the effects of chitinase microencapsulation and on the kinetic parameters of catalytic process as well as on the study of the synergy between different microencapsulated enzymes in order to contribute into the improvement of the biocontrol process.

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