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Thermodynamics of laminarinase partitioning in soya lecithin liposomes and their storage stability

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ABSTRACT

The goal of the present work is to define the partitioning behavior of laminarinase (EC 3.2.1.6) from *Trichoderma* spp. in soya lecithin liposomes using a thermodynamic approach based on the partitioning variation with the temperature. No information is available yet on use of laminarinase for microencapsulation in liposomes. An attempt has been made to define the stability of liposomes as well as free and immobilized enzyme during the storage under different conditions. The partition coefficients ($K_{o/w}$) were greater than 1, therefore the standard free energies of the enzyme transfer are negative, indicating an affinity of enzyme for microencapsulation in liposomes. The enthalpy calculation led to the conclusion that the process is endothermic. The transfer of laminarinase in liposomes is a entropy driven process attributable to positive value of entropy change. The presence of enzyme decreases the liposome storage stability from 70 days to an approximately 20 days at 25 °C and 40 days at 4 °C. Monitoring of the liposome's diameter demonstrates that their size and concentration decreases during storage. There was no evidence for liposome fusion process. The stability of immobilized enzyme in the buffer contained suspension did not increase in comparison with free laminarinase, however, stability increased in the soil and phytomass contained systems upon exposure to 254 nm UV-light for every 12 h. Thus the present study has theoretical input, as well as practical significance for enzyme application as biocontrol agent.

1. Introduction

Laminarinases (or β-(1 → 3)-glunacohydrolase, EC 3.2.1.6) are enzyme capable of hydrolyzing β-1,3- and β-1,4-glycosidic bonds [1,2], which displays its main hydrolytic activity on the β-1,3-glucose polymer laminarin. Fungi, such as *Trichoderma* spp., are known to possess laminarinases.

The importance of laminarinase (β-1,3-glucanase is a more general term for this enzyme) as well as chitinase, which are key enzymes responsible for the fungal cell and sclerotial wall lysis, has been reported [3,4]. Laminarinase has been produced by several fungi and bacteria during the biological control process of phytopathogens [5]. Constitutive elevation of extracellular lytic activity could improve the natural capability of *T. harzianum* to attack pathogens and its use as a biocontrol agent [1]. Mycoparasitism of plant pathogenic fungi by *Trichoderma* spp. has been well researched [6,7] and it is widely considered to be a major contributing factor to the biocontrol of plant diseases. Mycoparasitism may be defined as an antagonistic interaction between two fungi where the aggressor makes an intimate contact with its target before lytic enzymes are released to facilitate the degradation of the cell wall of the host organism. The use of antagonistic microbes seems to be one of the promising alternative approaches [8] to the chemical control which is based on use of fungicides. The accumulation of toxic compounds, potentially hazardous to humans and environment, and also resistance of the pathogens buildup are results of the chemical control approach [5]. Considering the problem of antagonistic microorganisms' adaptation to the environment, the application of mycolytic enzymes as antifungal treatment in the protection of some commercially important crops is promising.

Furthermore, the enzyme application *in situ* needs to increase its stability and protection against environmental factors [9]. Due to the prolonged lifetime of the catalyst the process costs is reduced; stability is an important parameter to be improved during the development of industrial enzymes. In some cases immobilization can be useful to achieve this goal [10]. The immobilization on solid supports can affect the enzyme mobility to fungi and increases system heterogeneity. Therefore, the immobilization in phospholipid vesicles (liposomes) can help to avoid the problems related to a

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union of enzyme on solid support. Liposomes are under investigation as models for biological membranes and as carriers for various bioactive agents such as drugs, diagnostic and genetic materials, and vaccines. The thermodynamics of enzyme molecules transfer may be studied by measuring the partition coefficient as a function of the temperature. This approach was widely applied in the case of various pharmacological preparations [11,12].

Enzyme encapsulation into liposomes is a promising technique to stabilize and prevent them from proteolysis and degradation [13]. The laminarinase encapsulation in liposome is not yet studied. To obtain liposomes the soya lecithin was applied in the present study. Soya lecithin is the commercial name for a naturally occurring mixture of phospholipids. Three main phospholipids in this mixture called "commercial soy lecithin". They are: the phosphatidylcholine at 33.0% (also called "pure" or "chemical" lecithin to distinguish it from the natural mixture), phosphatidylethanolamine at 14.1% (popularly called "cephalin"), and 16.8% of phosphatidylinositols (also called inositol phosphatides) as well as 0.4% of phosphatidylserine. Commercial soy lecithin also typically contains unrefined soy oil as well as additives insoluble in organic solvents [14].

As a contribution to the generation of physicochemical and operational information about laminarinase microencapsulation and properties, the objectives of this study are (1) to describe the partitioning behavior of laminarinase in soya 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 storage stability of soya lecithin liposome systems as well as free and microencapsulated laminarinase stability under different conditions. The first objective of this study has theoretical input, while a second has practical significance for enzyme application as biocontrol agent.

2. Experimental

Soya lecithin was provided by PROQLIMS S.A. de C.V. (Saltillo, Mexico) as a commercially available product. In this study term "lecithin" is used in its broader sense as a natural complex. Lecithin was partially purified to separate additives insoluble in the mixture of $CHCl_3$:CH_3OH 1:1 (v/v) before use. All solvents were analytical grades and provided by J.T. Baker (Phillipsburg, USA). All used salts also were analytical grades and purchased from Jalmek (Monterrey, Mexico). Laminarinase (Sigma Cat. No. C8241) from *Trichoderma* spp. and its substrate were purchased from Sigma–Aldrich Co. (Saint Louis, USA).

2.1. Liposome/enzyme solution partitioning, determination of partition coefficients and thermodynamic functions of transfer

Liposomes were prepared by a method similar to the Bangham method [11]. Thin films of soya lecithin were formed on the walls of 2-L round-bottomed flasks following evaporation of 1 mL aliquot of methanol solution. Then, the flasks were placed in an oven at 50 °C for 24 h. The films were dispersed in 5 mL of enzyme solution (protein concentrations were 0.0046, 0.007 or 0.012 mg/mL) in 0.1 M sodium acetate buffer, pH 5.4, or the same buffer without enzyme at 4°, 25°, 35° and 40 °C, and vortex-mixed until all the film was removed from the walls of the flasks [15]. The mixtures were incubated for 24 h at the same temperatures indicated above. It resulted in the formation of multilamellar vesicles (MLVs), which was verified by microscopy on Neubawer chamber, according to reported methods [12] described below.

The enzymes distribution was determined in 24 h temperature-equilibrated MLVs in 1.2 mL samples, followed by centrifugation ($15{,}000 \times g$ for 60 min) at the specified temperature. The protein concentrations in the initial enzyme solution and supernatant, obtained after centrifugation applied for organic phase separation, were determined by means of the spectrophotometric analysis described by Bradford [16]. This assay is based on a visible color change at 595 nm for an acidic solution of Coomassie Brilliant Blue G-250 when binding to protein occurs. The bovine serum albumin was applied to obtain the standard curve.

The same treatments were carried out with enzyme solutions without lecithin to compare activity and stability of free and immobilized enzymes.

The molal partition coefficients ($K_{o/w}$), were calculated by means of reported method [17] using equation: $K_{o/w} = W_{aq}(C_o - C_f)/(C_f W_{org})$ where C_o and C_f are the initial and final concentrations of enzymes (mg/mL) in the aqueous buffer phase before and after the transfer of the protein from the aqueous phase to the organic medium, respectively [18], W_{aq} is the weight (g) of the aqueous phase, and W_{org} is the weight (g) 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 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} curves, obtained 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 and expressed in molality [17,18].

2.2. 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 [12] for calculating the liposomes number immediately after their preparation and each tenth day during their storage at 4° and 25 °C. Moreover, during storage of liposomes their lineal size was estimated from measurements of vesicles' diameters that were carried out with photographs taken with camera Sony Cyber 5.1 megapixel to a 3.0 zoom from image observed on Neubawer chamber by optical microscopy. To calculate liposome diameter, the real lineal size of the smallest square side (0.005 cm) was taken into account. The calculations were performed by means of comparison of this value with the measurements obtained from photography.

The 100 measurements were performed in each case. The assays were repeated three times. The obtained data were treated by Sturges' rule [19] to determine the desirable number of classes; the number of classes (k) was 8, as approximated from the equation: $1 + 3.3 \log n$ where n equals 100 as numbers of observations. The data range (r) was calculated from smallest and highest diameter values, while the class width (w) was calculated as r/k.

2.3. Enzyme activity and storage stability measurements

The laminarinase (β-1,3-glucanase) activity was measured according to the method of Singh [3] with slight modifications, using 10 mg/mL laminarin from *Laminaria digitata* as substrate.

The reaction mixture contained 1 mL of substrate solution in 0.1 M sodium acetate buffer, pH 5.5, and 1 mL of laminarinase. The reaction was carried out at 40 °C. It was observed at an independent assay that product concentration varied linearly with the time at least for 30 min. The reaction was stopped by boiling by means of tubes immersion into the water bath for 10 min. In each experiment replicate time zero measurements were compared with the replicate measurements of two other times, usually 0.2 and 0.4 h. A boiled enzyme control was run in parallel. No increase in reducing sugar was observed in the boiled enzyme control. Spectrophotometrically, the activity of laminarinase was determined by measuring the reducing sugar release, by the Somogyi–Nelson method [20]. Determination of reducing sugar by Somogyi–Nelson method is based on the absorbance at 500 nm of a colored complex formed between a copper-oxidized sugar and arseno-molybdate. The amount of carbohydrate is determined by comparison with a calibration plot obtained spectrophotometrically using dextrose. One unit (IU) of laminarinase activity was determined as 1 μmol of glucose per min at 40 °C.

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

The enzyme stability in buffer suspension was evaluated by means of technique described above and carried out on each seventh day for 21 days.

To evaluate the enzyme stability in the presence of soil and phytomass, 1 g of soil or cut glass biomass was placed in Petri dishes. Then, 1 mL of free or microencapsulated enzyme (at 0.012 mg/mL) was added. The Petri dishes were placed in the controlled area at the temperature of 25 °C and 40% humidity, and exposed to 254 nm UV light produced by short wave UV bench lamp. UV light was applied 12 h a day for 24 days, starting immediately after enzyme addition. Three dishes were dedicated to the measurements of the activity corresponded to each storage time, as well as to control without enzyme, by means of technique similar to described above. The reaction mixture contained 1 mL of substrate solution in 0.1 M sodium acetate buffer, pH 5.5, and 1 g of soil or phytomass with or without laminarinase. The reaction was carried out at 40 °C. In each experiment triplicate time zero measurements were compared with the triplicate measurements corresponded to 30 min of reaction time. The reaction was stopped by means of tubes immersion into water bath for 10 min. The mixture was centrifuged at 15,000 × *g* for 40 min at 4 °C. The supernatant was applied to reducing sugar determination. A control with soil or phytomass without enzyme was run in parallel. No increase in a reducing sugar was observed in control assays in comparison with zero time measurements. Measurements of the reducing sugar release in reaction catalyzed by laminarinase were performed spectrophotometrically at 500 nm by the Somogyi–Nelson method [20]. One unit (IU) of laminarinase activity was determined as described above.

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

2.4. Statistical analysis

The means and standard deviation of all obtained results were calculated. Data were analyzed by one-way analysis of variance (ANOVA). Significant differences ($p \leq 0.05$) between the means were determined by the DUNCAN multiple range tests [21].

Table 1
Partition coefficients of laminarinase in soya lecithin liposomes 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 35 °C	$K_{o/w}$ at 40 °C
4.6	5.5 ± 1.1	15.3 ± 1.5	30.4 ± 1.3	31.4 ± 2.2
7.0	15.4 ± 1.6	36.1 ± 1.4	74.3 ± 1.7	90.0 ± 2.0
12.0	19.1 ± 1.5	56.2 ± 1.9	74.8 ± 1.7	125.8 ± 1.8

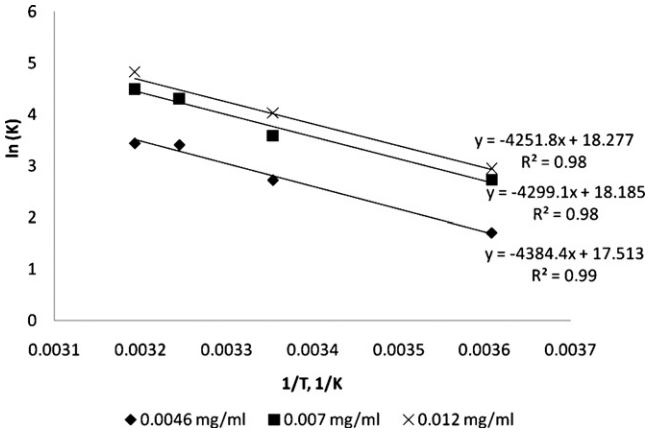

Fig. 1. Dependence partitioning on the temperature in van't Hoff coordinates for laminarinase in soya lecithin liposomes system.

3. Results and discussion

3.1. Liposome/enzyme solution partitioning, determination of partition coefficients and thermodynamic functions of transfer

Table 1 summarizes the temperature dependence of the partition coefficients for laminarinase in all studied systems. The partition coefficients of enzyme ($K_{o/w}$) are greater than 1 indicating an affinity of laminarinase for microencapsulation in liposomes. This parameter increases with the increase of the temperature as well as with protein concentration escalation. The dependence of concentration led to the conclusion that $K_{o/w}$ is apparent constant. The variability of $K_{o/w}$ with concentration was reported in the case of encapsulation of some pharmacological substances [12,22].

The temperature dependence of partitioning for different laminarinase concentrations in soya lecithin liposomes, as $\ln K$ versus $1/T$ (van't Hoff plots) is presented in Fig. 1. In all cases, straight lines with correlation coefficients (r) near 0.96 were obtained for evaluated partitioning systems; therefore the van't Hoff method is useful for the respective thermodynamic analysis [18].

Table 2 summarizes the thermodynamic functions related to the transfer of laminarinase from aqueous media to soya lecithin liposomes. In all cases, values of $\Delta G_{w\to o}$ at 25 °C are negative, which indicates the preference of the enzyme to the organic phase, confirming that laminarinase transfer from aqueous media to an organic system is spontaneous. Moreover, the magnitudes of $\Delta G_{w\to o}$ increases with the concentration increase.

Table 2
Free energy, enthalpy and entropy for the transfer of laminarinase from aqueous media to a 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))
4.6 ± 0.04	−6.7	36.4	144.9
7.0 ± 0.031	−8.8	35.7	149.7
12.0 ± 0.32	−9.9	35.3	152.1

The parameters $\Delta H_{w\to o}$ and $\Delta S_{w\to o}$ (Table 2) were calculated from lineal functions of Fig. 1 and $\Delta G_{w\to o}$, respectively, as described above. The enthalpy of laminarinase transfer ($\Delta H_{w\to o}$) is positive. Therefore, the process is endothermic. The entropies of transfer ($\Delta S_{w\to o}$) are positive.

Since initially the enzyme is present only in the water, it is necessary to create a cavity in the liposome organic phase in order to accommodate the solute after the transfer. This is an endothermic process, since an energy supply is necessary to separate the lipids molecules. When the enzyme molecules are accommodated in the organic phase, an amount of energy is released due to enzyme–phospholipids interactions, which is known as an exothermic process. On the other hand, after a certain number of enzyme molecules migrated from the aqueous to the liposome organic phase, the original cavities occupied by protein in the aqueous phase now are occupied by water molecules. This event is accompanied by the release of energy due to water–water interactions. However, it is also necessary to keep in mind that the water molecules can organize around the enzyme hydrophobic aminoacids (hydrophobic hydration). This event 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 linked among themselves by hydrogen bonding [23].

Attributable to the highly organized structure of phospholipidic layers, the energy requirement implies to a relatively high enthalpy of the transfer, in order to accommodate the enzyme molecules within organic phase. The same event can accompany by high increment in the system entropy due to disorder generated inside of the vesicle layers. Table 2 shows that the laminarinase transfer process from water to lecithin liposomes is endothermic, which is promoted with the increase of the temperature and implies high increments in the system net entropy. The increase in entropy of the laminarinase transfer to lecithin liposomes is possibly due to the disorder produced in the hydrophobic core of the lipid layers during separating the phospholipids hydrophobic tails to accommodate the protein molecules in liposomes. The obtained results indicate that the transfer of laminarinase is entropy driven due to positive value of entropy.

3.2. Liposome storage stability

Table 3 and Fig. 2 show the results of liposomes count after their preparation and during their storage at 4 and 25 °C as the temperatures employed for their formation.

At 40 °C the liposomes were not separated as single MLVs and agglomeration of liposomes were observed due to high membrane fluidity, however, agglomerates encapsulated added enzymes (Table 1). For the reason that soya 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 [18]. 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 [24].

Table 3 demonstrates that without enzyme liposomes concentrations were countable for 70 days at both temperatures, although at 25 °C their number was lower in comparison to the number at 4 °C (Table 3). Liposomes quantity decreased slowly at 4 °C, while at 25 °C the decrease is more considerable on 10th day of assay and

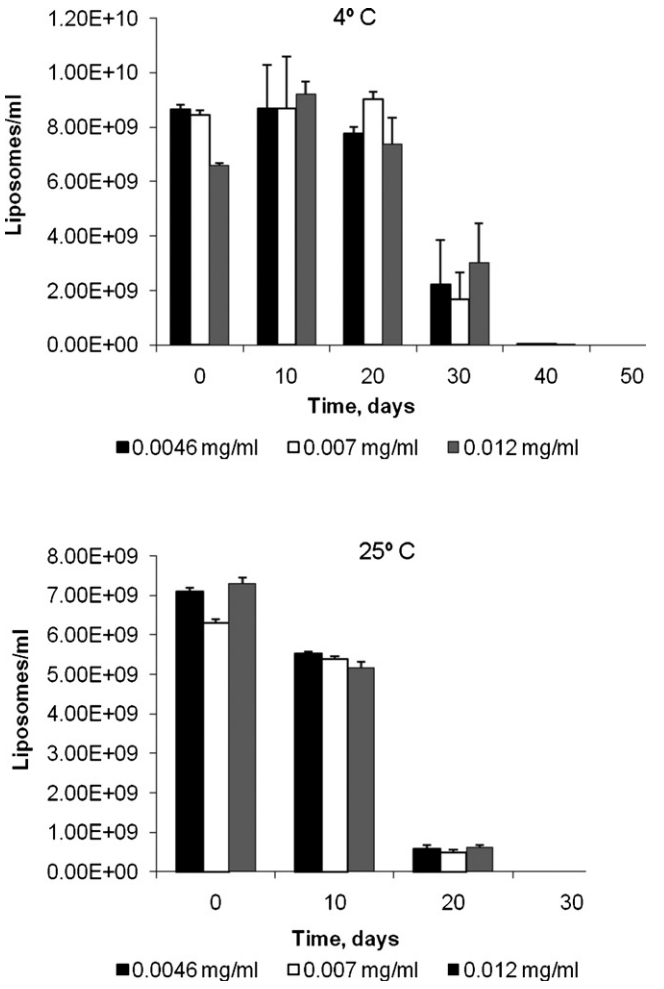

Fig. 2. Liposome storage stability at different temperatures: top, at 4 °C; bottom, at 25 °C in the presence of laminarinase at 0.0046 mg/mL (black bars), 0.007 mg/mL (white bars), and 0.012 mg/mL (grey bars).

followed by slower dropping in liposome concentration after 10 days of assay until 70th day.

The enzyme presence led to decrease in stability of liposomes detected at the prolong time of assay. Fig. 2 shows that in the presence of laminarinase the MLVs were undetectable on 50th day of their storage at 4 °C and on 30th day at 25 °C.

In the presence of laminarinase initial liposome concentrations (Fig. 2) were similar to ones quantified in the system without enzymes at 4 °C and 25 °C, respectively (Table 3). The liposomes concentration was drastically reduced at 4 °C on the 30th day (Fig. 2), while at 25 °C on the 20th day. The tendency of the system to keep the initial concentration at 4 °C for 10 days at the same level was observed with and without enzyme. However, at 25 °C the concentration decrease observed at the 10th day was lower than detected without enzyme on the same day of assay. It possibly signifies that at this temperature stabilization of liposome in the laminarinase presence happens only for first ten days of assay.

Table 3
Liposome storage stability at 4 °C and 25 °C without enzyme.

	Time (days)				
	0	10	30	50	70
Liposomes/mL (at 4 °C)	8.68 ± 0.16E+09	8.68 ± 0.26E+09	9.20 ± 0.42E+09	5.40 ± 0.13E+09	5.16 ± 0.22E+09
Liposomes/mL (at 25 °C)	6.48 ± 0.21E+09	8.38 ± 0.14E+08	9.20 ± 0.16E+07	5.29 ± 0.21E+06	4.28 ± 0.14E+06

Table 4
Relative frequencies (±standard deviation) corresponding to diameters of 0.007 mg/mL laminarinase contained liposomes obtained and stored at 4 °C. (Classes were defined based on Sturges' rule applying $k = 8$, $r = 3.22E{-}3$ cm and $w = 4.24E{-}4$ 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
1.1	≤x<	5.4	79 ± 0.3	83 ± 0.3	94 ± 0.3	80 ± 0.5	97 ± 0.3
5.4	≤x<	9.6	19 ± 0.3	16 ± 0.2	6 ± 0.3	20 ± 0.5	3 ± 0.5
9.6	≤x<	13.8	0	0	0	0	0
13.8	≤x<	18.1	1 ± 0.3	0	0	0	0
18.1	≤x<	22.3	1 ± 0.2	1 ± 0.3	0	0	0
22.3	≤x<	26.5	0	0	0	0	0
26.5	≤x<	30.8	0	0	0	0	0
30.8	≤x<	35.0	0	0	0	0	0

Table 5
Relative frequencies (±standard deviation) corresponding to diameters of 0.007 mg/mL laminarinase contained liposomes obtained and stored at 25 °C. (Classes were defined based on Sturges' rule applying $k = 8$, $r = 2.56E{-}3$ cm and $w = 3.37E{-}4$ 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
2.22	≤x<	5.5	29 ± 0.4	44 ± 0.4	48 ± 0.3	0
5.59	≤x<	8.95	63 ± 0.3	48 ± 0.5	44 ± 0.4	0
8.95	≤x<	12.3	5 ± 0.4	5 ± 0.3	5 ± 0.4	0
12.3	≤x<	15.7	1 ± 0.4	1 ± 0.4	1 ± 0.3	0
15.7	≤x<	19.1	1 ± 0.5	1 ± 0.4	1 ± 0.3	0
19.1	≤x<	22.4	1 ± 0.4	1 ± 0.4	1 ± 0.4	0
22.4	≤x<	25.8	0	0	0	0
25.8	≤x<	29.1	0	0	0	0

Effect of enzyme on the stability of liposomes could be related to the interaction of proteins and lipids. It was characterized by the loss of stability at the prolong storage time at both temperatures. On the other hand, at 25 °C the stabilization took place for the first 10 days of interaction followed by rapid destabilization.

Thus, the soya lecithin liposomes are sensible to the enzyme presence and are better stored at the low temperature.

There are two aspects that have impacted 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 the 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, by aggregation or fusion. In the last case the size of liposomes should increase. The storage stability may be increased by the use of purified phospholipids [11,12]. In the present study commercially available soya lecithin containing various phospholipids was used.

The typical results describing relative frequencies (±standard deviation) corresponding to the diameters of laminarinase containing liposomes, which were obtained and stored at 4 and 25 °C are presented in Tables 4 and 5, respectively. The same results were obtained using different enzyme concentrations. Presented data demonstrates the general trend. Liposomes obtained at 25 °C are characterized by higher percent of vesicles with the greater diameter than those obtained at 4 °C. In both cases size of liposomes decreased during their storage. It was impossible to observe liposomes by optical microscopy on 30th and 50th day of the storage at 25 and 4 °C, possibly due to decrease of their diameters.

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

3.3. Enzyme activity and storage stability

Table 6 shows the laminarinase activity of free and encapsulated enzyme preparations quantified after immobilization. The lower activity of microencapsulated enzyme is related to partial enzyme encapsulation; therefore enzyme concentration is less in the microencapsulated form than is in free form. Moreover, the enzyme lost its activity during encapsulation at 25 and 40 °C that lead to lower activity values even for free enzyme. The reason for lower activity of encapsulated laminarinase may be related to entrapment of enzyme in the multilamellar vesicles. Chaize [13] reports that once encapsulated, the enzymes have encountered another problem: the permeability barrier of lipid membrane drastically diminishes the activity of the enzyme entrapped in the liposome by reducing the entrance rate of the substrate molecules and then reducing the substrate concentration inside the liposome. It could be the reason for decreasing of chitinase and laminarinase activity after their microencapsulation.

This hypothesis was tested in the assay performed in the presence of Triton X-100, which destroyed soya lecithin liposomes. Fig. 3 shows that free laminarinase lost about 80% of activity in the

Table 6
Laminarinase activity corresponding to the first measurement after immobilization.

Laminarinase (μg/mL)	4 °C		25 °C		40 °C	
	Free (IU/×10³ mL)	Encapsulated (IU/×10³ mL)	Free (IU/×10³ mL)	Encapsulated (IU/×10³ mL)	Free (IU/×10³ mL)	Encapsulated (IU/×10³ mL)
4.6	1.50 ± 0.01	0.63 ± 0.06	1.18 ± 0.39	0.49 ± 0.07	0.40 ± 0.04	0.09 ± 0.01
7.0	2.99 ± 0.1	1.98 ± 0.11	2.56 ± 0.86	1.44 ± 0.09	1.19 ± 0.08	0.54 ± 0.03
12.0	5.98 ± 0.35	2.71 ± 0.19	4.90 ± 0.23	3.23 ± 0.89	2.89 ± 0.51	1.38 ± 0.48

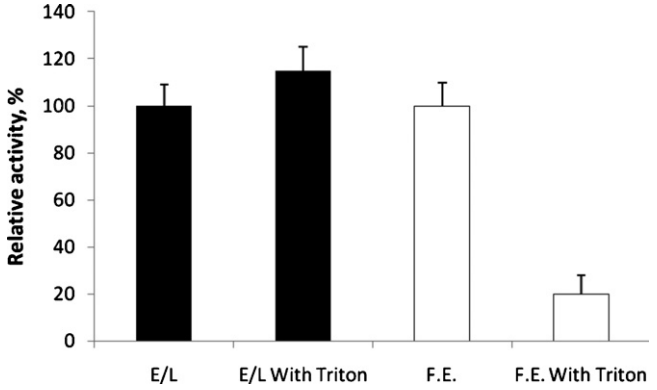

Fig. 3. Relative activity of free (F.E.) and encapsulated (E/L) laminarinase before (considering as 100%) and after 0.2% (w/v) Triton X-100 addition (preparations with 0.012 mg/mL of laminarinase were used).

presence of surfactant, while the activity in lecithin contained system was increased at 15% under the same conditions. The liposome solubilization with Triton X-100 caused the enzyme release that increased the detected enzyme activity. At the same time, the presence of solubilized lipids, which interacts with surfactant, protects the enzyme from inactivation due to Triton X-100 addition.

Moreover, the microencapsulation might lead to decrease in both substrate affinity and maximum velocity that was demonstrated in the case of β-galactosidase entrapped into phospholipid vesicles [15].

The results describing activity of free and encapsulated laminarinase during its storage in buffer contained system at 4 °C and 25 °C are shown in Fig. 4. The same observations were obtained using different enzyme concentrations. The presented data demonstrate the general trend. Laminarinase is a relatively stable enzyme: the enzyme from *Trichoderma reesei* retained 79% of its activity at −15 °C and 85% at 4 °C during the 10 days of storage [25,26].

Relative activities detected in the present study for the free and microencapsulated laminarinase were similar for 28 days of assay. The differences were not statistically significant. Descriptive statistics demonstrate that microencapsulated laminarinase activity is higher than free enzyme activity on the 14th day at 4 °C as well as at 25 °C (Fig. 4), while the behavior is reverse on 28th day. Thus,

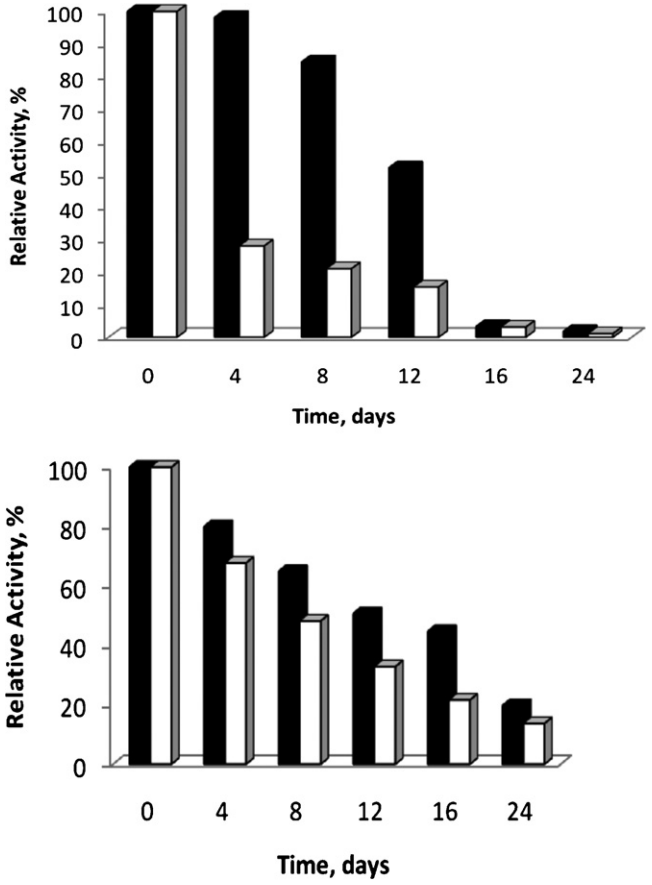

Fig. 5. Microencapsulated liposomes (black bars) and free (white bars) laminarinase (at 0.012 mg/mL) storage stability at 25 °C upon exposure to UV-light for every 12 h: the top, in the soil, and the bottom, in phytomass. (The first measured activity was used as 100%.)

stability of immobilized enzyme did not significantly increase in comparison with free laminarinase in buffer contained suspension. However, it was greater in soil and phytomass contained systems upon exposure to 254 nm UV-light (Fig. 5).

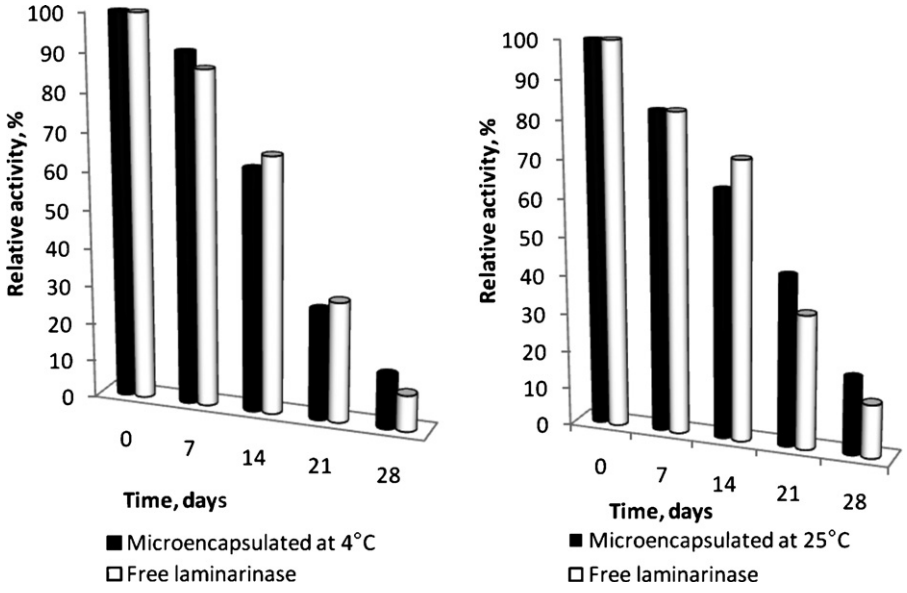

Fig. 4. Laminarinase storage stability at 4 °C (left) and 25 °C (right) for microencapsulated liposomes (black bars) and free (white bars) enzyme using 0.012 mg/mL. (The first measured activity was used as 100%.)

Fig. 5 shows the laminarinase activity of free and encapsulated enzyme preparations added to the soil and phytomass and stored in presence of UV-light. In both cases the microencapsulated enzyme had a higher residual activity than free enzyme, which was inactivated more drastically in soil than in the presence of phytomass. Thus, the encapsulation of laminarinase in liposomes protects the enzyme against environmental factors, such as UV-radiation and possibly against the presence of proteases, salts, etc.

The effect of microencapsulation to increase the enzyme stability at different temperatures as well as the stability against proteolytic activity, was reported previously in the case of β-galactosidase entrapped in liposomes [15].

4. Conclusion

From the presented analysis it could be concluded that laminarinase has an affinity to soya lecithin liposomes. Laminarinase transfer is endothermic and entropy driven process. The enzyme microencapsulation is stimulated with temperature and protein concentration increase. The protein presence decreases the soya lecithin liposomes stability during their prolonged storage. Although the encapsulation did not increase enzyme stability in the buffer contained suspension, liposomes are able to protect the enzyme against environmental factors, such as UV-radiation and others, in soil and phytomass contained systems. The important contribution of the present study of laminarinase microencapsulation in liposomes is findings in physicochemical parameters of process and operational properties of microencapsulated laminarinase. Obtained data, described for the first time in this paper, can be considered for this process optimization in future studies and applications. The future investigations will be focusing on the effects of the microencapsulated preparations on the phytopathogenic fungi's growth in order to contribute into the improvement of the biocontrol process.

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