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Enhanced thermal and ultrasonic stability of a fungal protease encapsulated within biomimetically generated silicate nanospheres

Ashkan Madadlou [a,*], Daniela Iacopino [b], David Sheehan [c], Zahra Emam-Djomeh [a], Mohammad E. Mousavi [a]

[a] Department of Food Science & Engineering, Faculty of Technology, Campus of Agriculture & Natural Resources, University of Tehran, Karadj, Iran
[b] Nanotechnology Group, Tyndall National Institute, Lee Maltings, Cork, Ireland
[c] Laboratory of Protein & Proteomics, Department of Biochemistry, Faculty of Science, University College Cork, Lee Maltings, Cork, Ireland

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ABSTRACT

Background: Dendrimers are highly branched synthetic macromolecules with a globular shape. They have been successfully used for generation of nanospheres at mild conditions via biomimetic silicification. Encapsulation of enzyme molecules within these nanospheres during their synthesis is a promising method for rapid and efficient entrapment of several enzymes. However, encapsulation of proteolytic enzymes has been rarely done via biomimetic silicification. As well, the operational stability of encapsulated enzyme has not been systematically reported.
Methods: A proteolytic enzyme, either α-Chymotrypsin or a fungal protease from *Aspergilus Oryzea* was encapsulated along with iron oxide nanoparticles within particles yielded via biomimetic silicification of different generations of polyamidoamine (PAMAM) dendrimers. Stability of encapsulated enzyme was compared to that of free enzyme during storage at room temperature. As well, their thermal and ultrasonic stabilities were measured. Scanning electron microscopy, transmission electron microscopy and optical microscopy were used to investigate the morphology of nanospheres.
Results: Determination of encapsulation efficiency revealed that ~85% of fungal protease with concentration 1.4 mg mL^{-1} stock solution was immobilized within particles yielded by generation 0. Based on microscopic images the generated particles interconnected with each other and had spherical morphologies independent of generation. Kinetic analysis of encapsulated fungal protease demonstrated that Mechaelis-Menten constant (K_m) slightly increased.
Conclusion: PAMAM dendrimer generation 0 could be effectively used for rapid encapsulation of a fungal protease from *Aspegilus Oryzae.*
General significance: Encapsulation significantly enhances the thermal and ultrasonic stabilities of enzymes, suggesting a range of diverse applications for them.

1. Introduction

Amongst the several approaches to improve the operational stability of enzymes, enzyme immobilization can overcome some of major problems inherent with the use of free enzymes. It prevents from product contamination with enzyme molecules and simplifies their separation from the reaction mixture which also provides the possibility to reuse those [1]. The immobilized enzyme molecules do not contact with the external hydrophobic interfaces such as gas bubbles originated for example by harsh mixing, sonication or supplying the required gases. The hydrophobic interfaces otherwise will inactivate the soluble enzymes [2]. The immobilized enzymes however, commonly suffer from low specific activities compared to their free counterparts due to the mass transfer limitations for substrates toward their active sites [3]. Smaller porous particles owing to their shortened diffusion path of substrates can generally reduce the mass transfer resistance [4]. In theory, there is no attrition problem for a nanometric carrier. It is however almost impossible to separate them in a bioreactor by conventional methods like filtration [5]. Paramagnetic nanoparticles being an ideal alternative can be easily separated from the medium by using a magnetic field [4]. These particles retain no magnetism after removal of the magnetic field [6]. In general, enzymes are covalently attached on magnetic nanoparticles using several ligands [7]. It is worthy to consider that enzymes immobilized on nanoparticles are able to interact with the hydrophobic interfaces and even with enzyme molecules immobilized on other particles [2]. The enzyme molecules immobilized on magnetic nanoparticles have been therefore, coated with aldehyde dextran thus avoiding the enzyme inactivation by gas bubbles in a stirred system [1].

Encapsulation of enzyme molecules within nanospheres produced via bio-(mimetic) silicification is a promising method for rapid and

* Corresponding author. Tel.: +98 860380258, +98 9128614065.
E-mail address: Ashkan.madadlou@gmail.com (A. Madadlou).

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efficient entrapment of several enzymes. A set of posttranslationally modified polycationic peptides called silaffins isolated from the walls of diatoms, single-cell algae; generate nanospheres within seconds when added to a silicic acid solution. There are seven highly homologous repeating units (R1 to R7) in the structure of silaffins [8]. In addition to biosilicification, the synthesized unmodified parent sequence of R5 unit (H_2N-SSKKSGSYSGSKGSKPRIL-CO_2H) is also capable to condense the silicic acid through self-assembling to larger structures [9]. This process, being appealing is called biomimetic silicification. Luckarift et al. [10] physically entrapped the 90% of butyrylcholinesterase within the nanospheres produced via biomimetic silicifiction of R5 peptide. The biomimetic silicification process is a one-pot procedure in which synthesis of silica and entrapment of enzyme molecules occur simultaneously at room temperature [11] and the peptides are captured within the precipitated nanospheres [12]. Other biological and biomimetic amine containing polymers have been also investigated to produce silica nanospheres. Dendrimers are highly branched synthetic macromolecules with a globular shape. They are structurally homogenous and biocompatible [13] polymers whose functionality can be tuned through the delicate choice of branching elements and terminal groups. Amine-terminated dendrimers, specifically polyamidoamine (PAMAM) and polypropylenimine (PPI) dendrimers have been successfully used for biomimetic silica synthesis. PPI dendrimers are the synthetic analogous to one of the major posttranslational modifications in the lysines of silaffins, while PAMAM dendrimers are reminders of the unmodified lysines within the R5 unit [12]. Based on the number of amino groups PAMAM and PPI dendrimers of different generations are possible, with each generation having double the number of groups as the former one [13].

The encapsulation of proteolytic enzymes has been rarely done via biomimetic silicification. As well, the operational stability of encapsulated enzyme has not been systematically reported. In the present communication, the encapsulation efficiency of two proteolytic enzymes within the nanospheres originated by three different generations (0, 2 and 4) of PAMAM dendrimers is compared. Then, the enzyme with the higher encapsulation efficiency is further studied for characteristics of nanospheres and its activity and stability is compared with those of free enzyme.

2. Materials and methods

2.1. Materials

PAMAM dendrimers with ethylendiamine cores of generations 0, 2 and 4 (20 wt% in methanol solution for generations 0 and 2 and 10 wt% for generation 4) were purchased from Aldrich and used without further purification. Fig. 1 presents the chemical structure of PAMAM dendrimer with ethylendiamine cores of generations 2. α-Chymotrypsin from bovine pancreas (a serine protease that hydrolyzes peptide bonds with aromatic or large hydrophobic side chains (Tyr, Trp, Phe, Met) on the carboxyl end of the bond) with molecular weight of 25 kDa and Flavourzyme (a protease from *Aspegilus Oryzae* that contains both endoprotease and exopeptidase activities) were purchased from Fluka and Sigma, respectively. N-Succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine *p*-nitroanilide and L-Leucine-*p*-nitroanilide were purchased from Sigma and Fluka, respectively. Iron oxide nanoparticles solution (5 mg mL^{-1} in toluene) with an average particle size of 5 nm was purchased from Aldich. It contained <0.1% oleic acid as stabilizing ligands.

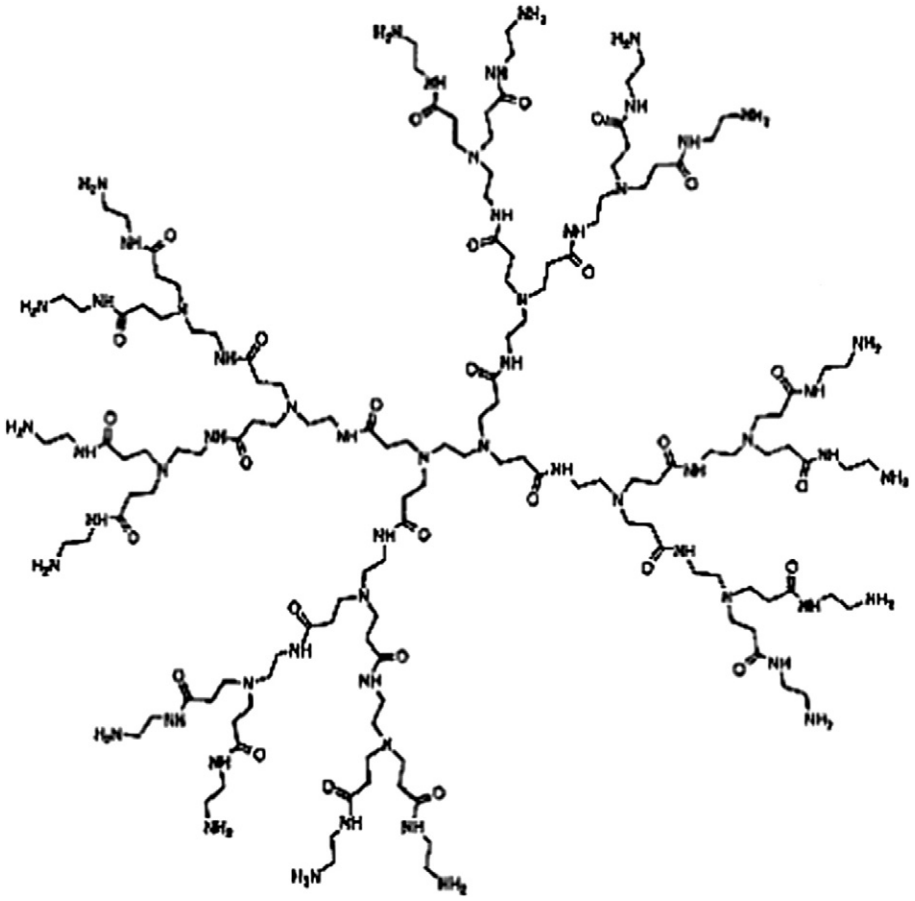

Fig. 1. Chemical structure of PAMAM dendrimer of generation 2 with ethylendiamine core.

2.2. Purification of iron oxide magnetic nanoparticles

1 mL of magnetic nanoparticles solution was washed three times with 20 mL of distilled water through shaking at 50 rpm for 30 min [14]. The particles were then accumulated with a magnet and suspended twice in absolute ethanol each of which for 15 min while shaking at 50 rpm to remove the stabilizing ligands [7]. The nanoparticles were subsequently resuspended in 3 mL deionized water and stored.

2.3. Enzyme encapsulation procedure

Dendrimer solutions of different generations were diluted with phosphate buffer 10 mM to a dendritic amine concentration of 80 mM. α-Chymotrypsin was dissolved in 10 mM phosphate buffer whilst, fungal protease being liquid was diluted with phosphate buffer 10 mM to destined protein concentrations. 265 μL enzyme aliquot was charged with 125 μL dendrimer and 60 μL iron oxide solutions. This was followed by adding 50 μL silicic acid resulting in rapid formation of silica precipitate. The generated silica particles were removed either by a magnet or through centrifugation (5 s at 20500 g), washed twice with deionized water and stored in phosphate buffer 10 mM. Silicic acid was obtained from a 60 min hydrolysis of 75 μL tetraethylorthosilicate (TEOS) in 425 μL 1 mM HCl [15]. For free enzyme treatments, TEOS was substituted with deionized water to prevent from the silicification of dendrimer templates. The encapsulation efficiency was determined by comparing the enzymatic activity of free enzyme and that of supernatant following the reaction [16]. For α-Chymotrypsin, N-Succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine *p*-nitroanilide 5 mM and for fungal protease L-Leucine-*p*-nitroanilide 40 mM were used as substrates. General protein content of enzyme solutions was quantified using the Bradford assay method.

2.4. Enzyme stability experiments

The residual activity of encapsulated and free fungal protease was measured during storage at room temperature for 10 days. For determination of thermal stability, free and encapsulated protease solutions were incubated at 30, 40, 50, 60 and 70 °C for 5 min and their residual activity was measured. For determination of ultrasonic stability, free and encapsulated protease solutions were sonicated at a frequency of 23 kHz using a MSE Soniprep 150 bench mounted ultrasonic disintegrator (Wolf Laboratories Ltd., York, England). The amplitude of titanium probe was adjusted either on ~4 or 22 μm for 15 min and 60 seconds, respectively. These resulted in actual power dissipated to solution of ~4.5 and 27 W, respectively. The power was measured as described previously [17]. Temperature of solutions was controlled over the sonoprocessing by an ice bath except for power measurements.

2.5. Kinetic of fungal protease

The Mechaelis-Menten constant (K_m) of fungal protease was determined by the hydrolysis of L-Leucine-*p*-nitroanilide (0.1-100 μM) at room temperature. The absorbance increase at 410 nm due to release of *p*-nitroaniline was monitored using an infinite M200 microplate reader (Tecan Trading AG, Switzerland). For encapsulated enzyme, silica particles were removed by centrifugation before determination of absorbance [10]. No enzyme activity was observed at supernatant after removal of particles. Kinetic constant K_m was obtained through the non-linear regression analysis at 95% confidence level by using HYPER32.EXE software Version 1.0.0 (Liverpool University, Liverpool, UK).

2.6. Microscopic analyses

Scanning electron microscopy (SEM) images were acquired using a field emission scanning electron microscope (JSM-6700F, JEOL UK Ltd.) operating at beam voltage 7 kV. Solution of SiO_2/enzyme (diluted 1:20 with aqueous solution of sodium dodecyl sulfate (SDS)) was deposited on acid cleaned highly doped Si wafer. After solvent evaporation, a thin layer of gold was evaporated with a vacuum coating (Edwards RV3 scancoat 6, Edwards Laboratories, Milpitas, CA) in order to avoid sample charging during SEM imaging. For optical microscopy images, drop deposited from SDS aqueous suspension onto acid cleaned glass microscope slides, were acquired using a calibrated epi-fluorescence microscope (Zeiss Axioskop II Plus, Carl Zeiss U.K.) equipped with a 100 W halogen lamp and a CCD camera (Optronics DEI-750). Images were analysed using Image Pro Express software (Media Cybernetics Inc., USA). Dark-field images were acquired with the same microscope equipped with a dark-field condenser with numerical aperture (NA) 0.9 and working distance 1.5 – 2 mm. A Jeol 2100 Transmission Electron Microscope was used at an accelerating voltage of 200 kV to image the samples. A drop of dendrimer generation 0 sample diluted (1:20) with SDS was deposited for 5 min on a formvar/carbon coated copper grid (400 mesh). Excess solution was removed by wicking and the grid was dried in air.

3. Results and discussion

3.1. Encapsulation of enzymes

The encapsulation efficiency was compared for two types of proteases using different generations of PAMAM dendrimer. It is clear from Fig. 2 that α-Chymotrypsin had significantly lower encapsulation efficiency at any given generation of dendrimer. An increase in the concentration of -Chymotrypsin from 80 μg mL^{-1} to 4.5 mg mL^{-1} stock totally failed to encapsulate the enzyme. Both the PAMAM dendrimers and α-Chymotrypsin are positively charged at physiological pH as the dendrimers have a pK_a ~ 9.5 and α-Chymotrypsin has a *pI* ~ 9.1. The positively charged enzyme molecules repel the protonated surface of dendrimers, leading to minor electrostatic interactions between them. Under this condition, the enzyme is solely entrapped non-specifically in the aggregating silica matrix during precipitation of particles [16]. Of note, the fungal protease had an acidic *pI* and therefore, interacted extensively with the cationic surface of dendrimer causing a noticeable degree of encapsulation. A decrease in the concentration of fungal protease in stock from 6.9 to 1.4 mg mL^{-1} increased

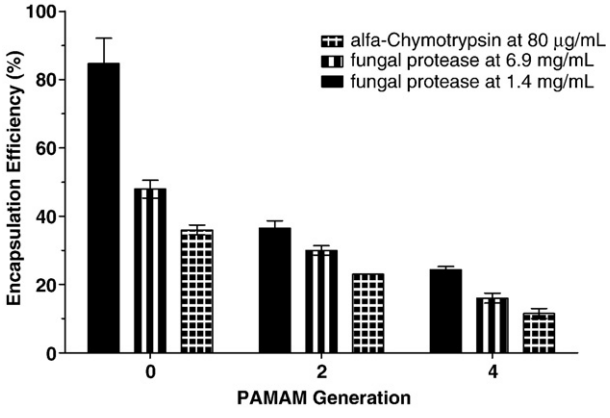

Fig. 2. Encapsulation efficiency of enzyme within silica spheres yielded via biomimetic silicification of different generations of PAMAM dendrimer.

the encapsulation efficiency suggesting a limited capability for silica particles to entrap the enzyme molecules within them. For both types of proteolytic enzymes, PAMAM template of generation 0 provided the highest encapsulation efficiency. An efficiency of ~85% was achieved for fungal protease with 1.4 mg mL^{-1} stock encapsulated via biomimetic silicification of PAMAM generation 0.

The particles originated by different generations of PAMAM had similar microstructural features. Optical microscopy images of particles yielded by PAMAM template of generation 0 are demonstrated in Fig. 3a and b. It is observed that particles were extensively interconnected with each other forming a widespread network. Higher magnifications by scanning electron microscopy (Fig. 3c-e) revealed that particles had spherical morphology. It was also observed by SEM and TEM that a proportion of silica particles were partially coalesced whilst retaining their spherical shape (Fig. 3f and g). The average diameter of silica nanospheres yielded by different generations was measured by SEM and is reported in Table 1. Since the concentration of phosphate buffer has been recognized as a critical factor that influences the size of spheres [18], enzyme-free spheres were generated with the same concentration of phosphate buffer in final solution, i.e. deionized water was diluted with phosphate buffer 10 mM instead of liquid enzyme during sample preparation. The enzyme-free nanospheres yielded by generation 0 presented a bimodal distribution with smaller particle diameters averaging 198 nm and larger particle diameters averaging 405 nm. The former particles consisted ~25% in population while the latter ones did ~75%.

Table 1
Diameter of spheres yielded via silicification of different generations of PAMAM dendrimer.

Template generation	Molecular weight	Template diameter (Å)	Number of surface amino groups	Enzyme loaded	Nanosphere diameter ± SD[1] (nm)
0	517	15	4	No	198 ± 41(25%) 405 ± 54 (75%)
0	517	15	4	Yes	306 ± 79
2	3256	29	16	Yes	328 ± 83
4	14215	45	64	Yes	275 ± 69

1. Standard deviation.

The bimodal distribution of spheres yielded by PAMAM generations 0 and 1 has been previously reported and is not observed for higher generations [12]. This kind of distribution is probably due to the agglomeration of smaller particles leading to the rise of larger ones [16]. The enzyme containing spheres yielded by generation 0 presented a unimodal size distribution averaging 306 nm. This suggests that the presence of enzyme molecules within spheres prevented from their agglomeration. A number of uncharged surface amino groups of dendrimers [1], regarded as a generalized acid-base catalyst, accept a proton from monosilicic acid. The positively charged patches on the surface of dendrimers likely interact with negatively charged silica species, causing the condensation of these species on

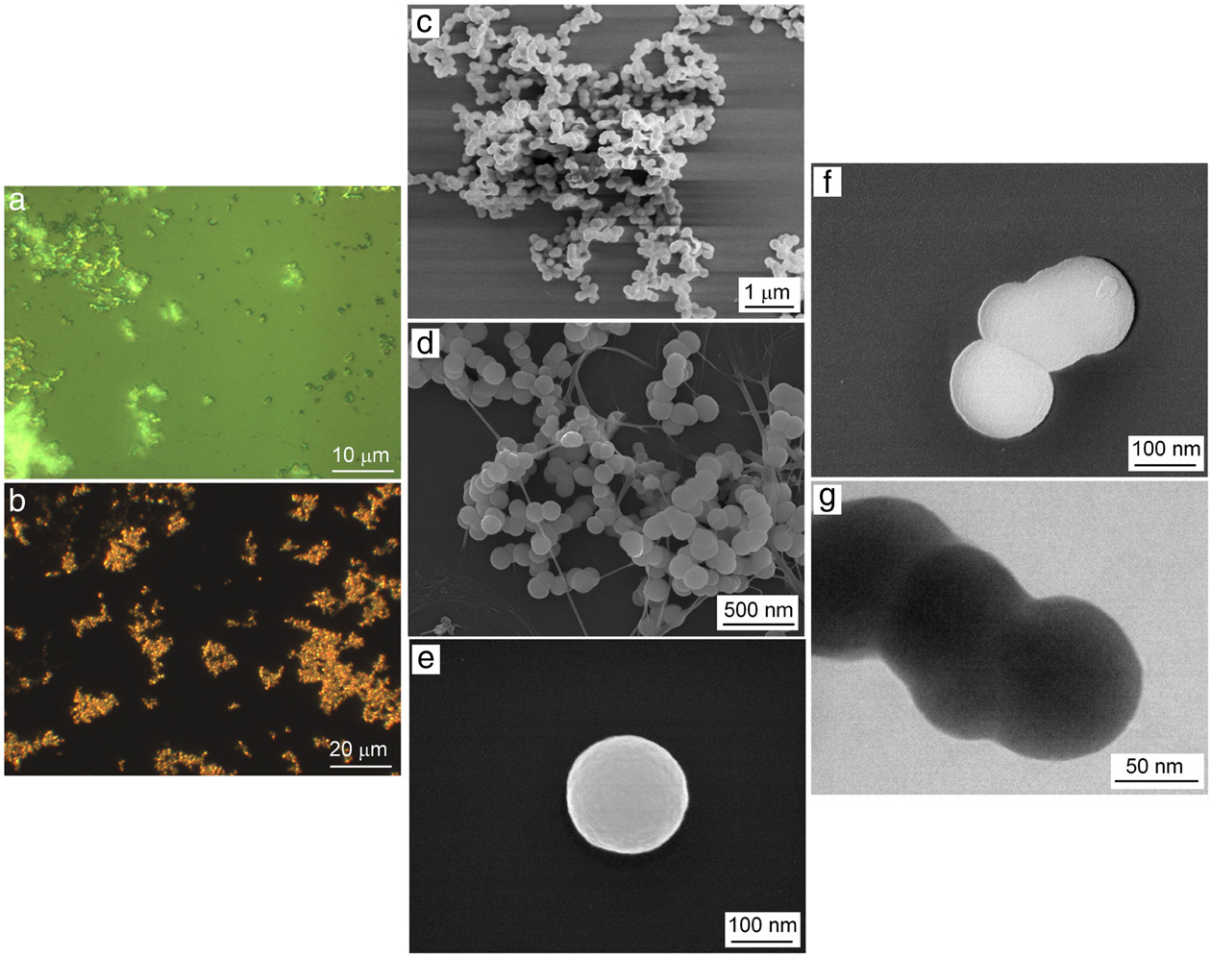

Fig. 3. Bright field (a) and dark field (b) optical microscopy images of spheres containing protease from *A. Oryzae* with 1.4 mg mL^{-1} stock yielded via biomimetic silisification of PAMAM dendrimer of generation 0; c-f) SEM images of spheres; g) TEM image of spheres.

the surface of dendrimers [12]. Neutralization of growing negatively charged silicate surface is required for further growth and agglomeration of particles. This neutralized surface is formed through interaction of cations with silicate surface which decreases the electrostatic repulsions thus permitting the particles to grow and agglomerate to larger sizes [18]. The negatively charged molecules of fungal protease likely interacted with cationic surface of dendrimer templates, followed by their physical entrapment in the growing silica spheres. The enzyme molecules not only made the silica matrix discontinuous but also repelled the silica species and prevented from the extensive agglomeration of silica particles.

3.2. Kinetics and stability of encapsulated protease

Fungal protease with concentration of 1.4 mg mL^{-1} stock solution encapsulated via silicification of PAMAM generation 0 was further studied for enzymatic function. Fig. 4 shows the representative hyperbola obtained for free and encapsulated fungal proteases. The entrapment of protease within nanospheres increased the K_m value from 6.515 ± 1.257 to 9.606 ± 2.341 mM, indicating a decreased affinity of enzyme for its substrate [19] because of an increase in mass transfer limitation for substrate through silica matrix [4]. A minor change in the structure of protease due to encapsulation within spheres could also play a role in the increased K_m. The increase in K_m was however small whilst, that of sol-gel entrapped enzymes undergo a significant increase from 250% [20,21] up to 600% [16]. This suggests that silica matrix did not significantly hinder the access of substrate to active site of enzyme or release of product by creating a diffusion barrier in the system [16].

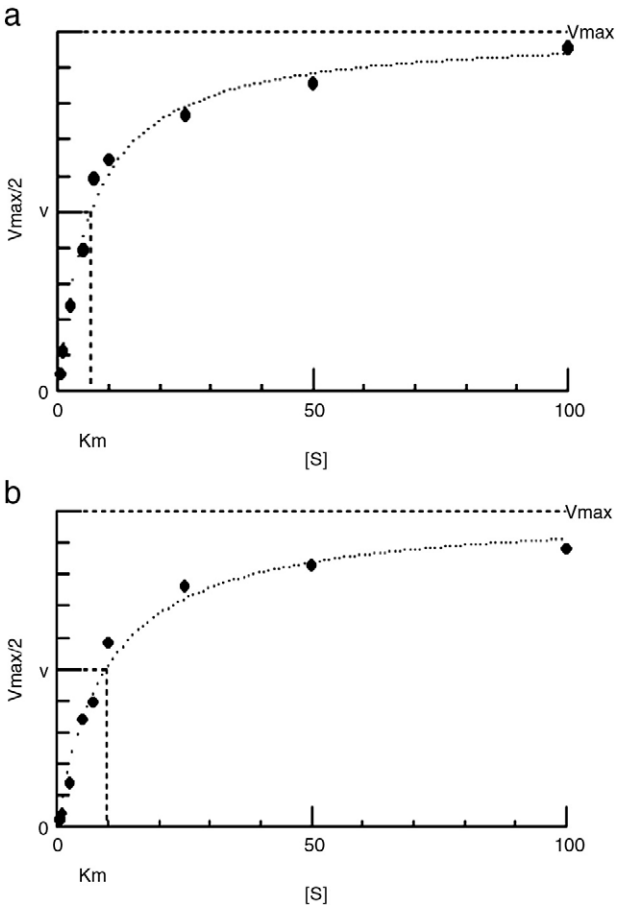

Fig. 4. Hyperbolic plots for free (a) and encapsulated fungal protease (b).

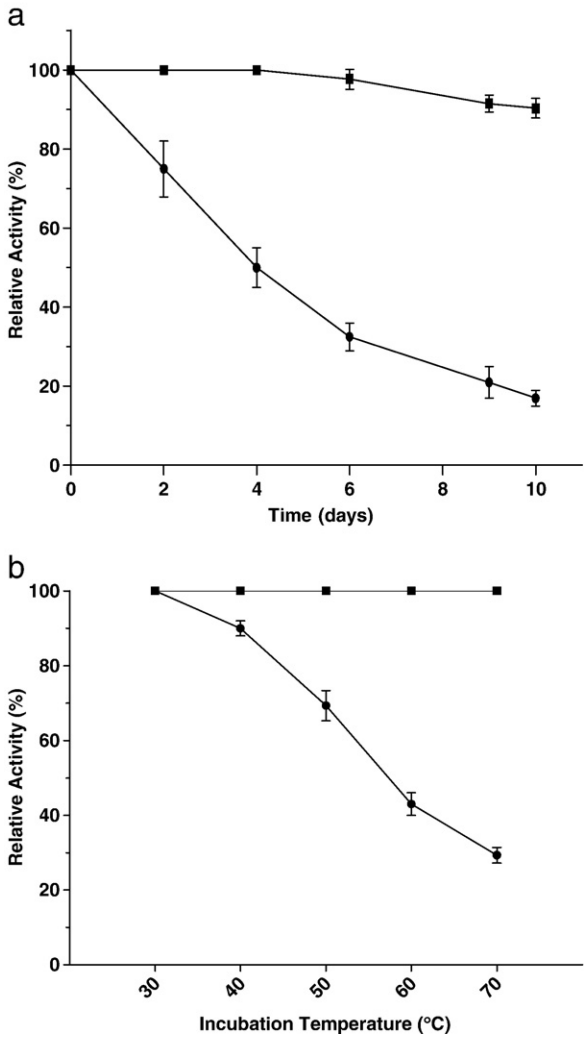

Fig. 5. Stability of free (-•-) and encapsulated (-■-) protease from *A. Oryzae* at room temperature (a) and incubated at different temperatures for 5 min (b).

The encapsulated enzyme retained more than 90% of its initial activity when stored at room temperature for 10 days. The free enzyme however, progressively lost its activity (Fig. 5a). The thermostability of free and encapsulated enzymes was investigated to find out if the silica nanospheres could protect the encapsulated enzyme molecules from thermal denaturation or not. The activity of free enzyme was progressively decreased with the increasing temperature of incubation. Free enzyme lost ~70% of its initial activity when incubated at 70 °C, indicating a profound sensitivity to heat. The encapsulated enzyme however, retained 100% of its initial activity at any incubation temperature (Fig. 5b). Immobilization of enzyme molecules onto beads [10] and nanoparticles [14] has a quite poor effect on thermal stability of enzymes and the immobilized enzymes exhibit very similar stability to their free counterparts. In agreement to the results obtained at the present study Luckarift et al. [10] reported that encapsulation of butyrylcholinesterase within nanospheres generated via biomimetic silicification of R5 peptide caused a 100% retention of its activity upon incubation at 65 °C for 1 h. The significantly enhanced thermostability of encapsulated protease is due most probably to the fixation of enzyme molecules in silica matrix of nanospheres that hinders the thermal fluctuations [22] and

prevents from conformational changes upon heating. The significant thermostability of encapsulated protease provides a number of processing advantages such as reduced microbial risk, lower viscosity, improved mass transfer rates and substrate solubility [10]. Stability of free and encapsulated enzymes to sonication was investigated to find out if silica nanospheres could protect the encapsulated enzyme molecules against ultrasonic treatment or not. Free enzyme lost ~25% and 55% of its initial activity due to sonication at 4.5 W and 27 W for 15 and 1 min, respectively (Fig. 6). This is attributed to a series of phenomena originated from cavitation. Bubbles generated by ultrasound undergo a number of oscillations during their existence. Diffusion of gases into and out of bubbles during oscillations can create microcurrents around them [23] which may lead in conjunction with microstreaming to turbulence and eventually to intermolecular collisions. The capability of microstreaming to disrupt DNA or disaggregate bacteria has been reported [24]. Inactivation of enzymes at interfaces is also of note. It proceeds via destabilization of electrostatic, hydrophobic and hydrogen bonds of proteins, leading to the irreversible denaturation of enzyme molecules [14]. Besides, the oscillating bubbles generated by low frequency ultrasound have significant pounding force because of their large resonance and maximum expansion radii [25] during their long life [26]. As well, the shearing force of imploding bubbles can extensively disintegrate the enzyme molecules in a solution. Finally, highly reactive chemical species such as hydroxyl, hydrogen and organic radicals generated by thermolysis of water vapor molecules and volatiles inside the cavities [25,27] can extensively degrade the proteins [28]. It is worthy to note however, that at low frequencies as 23 kHz applied in the present study, number of generated radicals is low and therefore played a minor role in the inactivation of enzyme molecules. The encapsulated fungal protease not only retained 100% of its initial activity upon sonication at 4.5 W for 15 min but interestingly its relative activity increased up to ~112% due to ultrasonic treatment at 27 W (Fig. 6). It is clear that encapsulation within silica matrix protected the enzyme molecules from inactivation at the air-liquid interface and preserved them against pounding and shearing forces of microbubbles. An enzyme activity corresponding to the increased relative activity of encapsulated enzyme due to sonication at 27 W was detected in supernatant. This indicates that a proportion of silica nanospheres were disrupted near to the end of harsh sonoprocessing, leading to release of some enzyme molecules into the surrounding medium. A prolonged sonication of encapsulated enzyme solution at such a high acoustic power could logically disrupt a higher number of silica spheres and subsequently inactivate the released enzyme molecules.

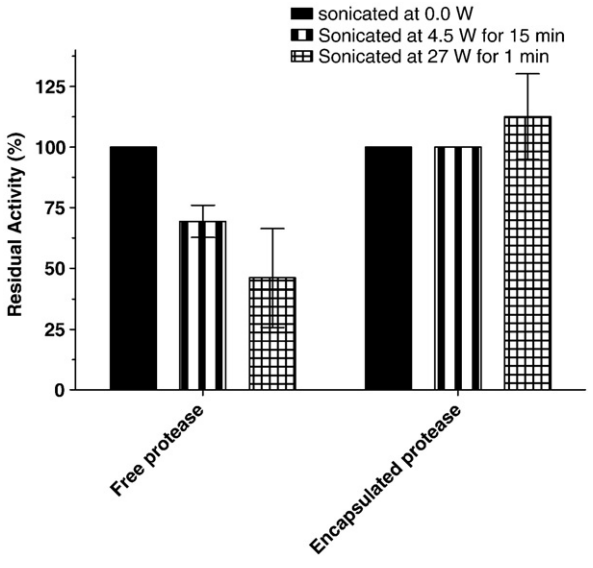

Fig. 6. Stability of free and encapsulated *A. Oryzae* protease sonicated at different conditions.

4. Conclusion

In the current communication, capability of PAMAM dendrimer generation 0 for effective and rapid encapsulation of a fungal protease from *Aspegilus Oryzae* was represented. We are optimistic that other enzymes preferentially with acidic *pI* may also be immobilized effectively within nanospheres originated via biomimetic silicification of dendrimers. The encapsulated protease could be easily separated from the reaction solution by a magnet or via centrifugation, terminating the proteolysis without need to thermal or acidic denaturation of enzyme molecules. This also provides the possibility to purify the nutraceuticals produced through enzymatic hydrolysis of proteins such as bioactive peptides. The encapsulated protease possessed a significant stability during storage at room temperature and against heat treatment making it an ideal choice for proteolysis at conditions when high temperatures are required. As well, the significantly improved ultrasonic stability of encapsulated protease expands the range of its applications for example to ultrasound-assisted proteolysis in proteomics.

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