Colloids and Surfaces B: Biointerfaces 117 (2014) 284–289

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

Studies on PVA pectin cryogels containing crosslinked enzyme aggregates of keratinase

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ARTICLE INFO

Article history:
Received 30 November 2013
Received in revised form 22 February 2014
Accepted 27 February 2014

Keywords:
CLEA
Keratinase
Controlled release
PVA
Cryogel
Autolysis

ABSTRACT

Polyvinyl alcohol-pectin (PVA-P) films containing enrofloxacin and keratinase were developed to treat wounds and scars produced by burns and skin injuries. However, in order to prevent enzyme inactivation at the interface between the patch and the scars, crosslinked enzyme aggregates (CLEAs) from a crude extract of keratinase produced by *Paecilomyces lilacinus* (LPSC#876) were synthesized by precipitation with acetone and crosslinking with glutaraldehyde. Soluble vs. CLEA keratinase (K-CLEA) activities were tested in 59% (v/v) hydrophobic (isobutanol and n-hexane) and hydrophilic (acetone and dimethylsulfoxide) solvents mixtures. K-CLEA activity was 1.4, 1.7 and 6.6 times higher in acetone, n-hexane and isobutanol than the soluble enzyme at 37 °C after 1 h of incubation, respectively. K-CLEA showed at least 45% of enzyme residual activity in the 40–65 °C range, meanwhile the soluble biocatalyst was fully inactivated at 65 °C after 1 h incubation. Also, the soluble enzyme was completely inactivated after 12 h at pH 7.4 and 45 °C, even though K-CLEA retained full activity. The soluble keratinase was completely inactivated at 37 °C after storage in buffer solution (pH 7.4) for 2 months, meanwhile K-CLEAs kept 51% of their activity.

K-CLEA loaded into polyvinyl alcohol (PVA) and PVA-P cryogels showed six times lower release rate compared to the soluble keratinase at skin pH (5.5). Small angle X-ray scattering (SAXS) analysis showed that K-CLEA bound to pectin rather than to PVA in the PVA-P matrix.

1. Introduction

As previously reported, keratinase isolated from *Paecilomyces lilacinus* strain LPSC#876 was immobilized into polyvinyl alcohol-pectin (PVA-P) cryogel showing a controlled release profile without loss of enzymatic activity [1]. The use of PVA-P cryogel in wound healing, however, exposes the biocatalyst to harsh environmental conditions at the interface and over the skin wound area. The keratinase activity can be influenced by changes of CO_2 partial pressure, autolysis–proteolysis, ionic strength, temperature and pH gradients, and desolvation–solvation processes. Partial pressure of CO_2 (pCO_2) is enhanced in a wound microenvironment because it is insoluble at hyperoxial conditions during the healing process. This phenomena has been observed in animals were ambient pCO_2 was increased up to 5% producing respiratory acidosis. The damaged tissue tends to metabolically compensate this effect by alkalosis response reducing collagen deposition [2]. Also, autolysis is a common inactivation phenomenon reported for many proteases [3]. Either combined or individual factors can be a serious drawback during shelf storage of therapeutic devices containing keratinase. Therefore, the main challenge is to keep the enzyme active inside the cryogel, at interfacial conditions and in the wound microenvironment.

Crosslinked enzyme aggregates (CLEAs) are a promising alternative to enhance protein structural stability and to extend the half-life of the biocatalyst even at harsh operational conditions. CLEAs can be produced by a simple method of enzyme precipitation under non-denaturing conditions followed by cross linking with bifunctional reagents, such as glutaraldehyde [4]. Enzyme precipitation can be produced by the addition of many compounds such

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http://dx.doi.org/10.1016/j.colsurfb.2014.02.049
0927-7765/

as salts, organic solvents, non-ionic polymers or acids [5]. In addition, hyperactivation has been observed in the synthesis of CLEAs of some enzymes, such as lipase and penicillin acylase [6,7].

In order to study enzyme stability at harsh environmental conditions such as interface and solvation changes, the use of organic solvents was proposed as a model system. Solvents partition coefficient (Log *P*) values regards to solvent's ability to replace water molecules bound to enzymes. Also, dielectric constant (ε) values of the different solvents used was taken into account to study the replacement of water molecules bound to the enzyme and how this phenomenon affects enzyme stability [8].

The aim of the present work is to develop and study CLEAs of keratinase (K-CLEAs) from crude extracts of *P. lilacinus* LPSC#876. Effects of polar and non-polar solvents, kinetic constants, temperature and ionic strength are compared between soluble keratinase and K-CLEA. K-CLEAs were analyzed by electron microscopy. Inclusion of K-CLEAs into PVA and PVA-P cryogels was analyzed by small angle X-ray scattering spectrometry (SAXS). Finally, K-CLEA controlled release was monitored under simulated skin conditions.

2. Materials and methods

2.1. Materials

Dimethyl sulfoxide (DMSO), ethanol and isobutanol were of analytical grade and provided by Biopack (Buenos Aires, Argentina). Acetone and buffers were provided from Anedra S.A. (Buenos Aires, Argentina). Azocasein, bovine serum albumin (BSA, fraction V), polyvinyl alcohol (PVA, MW_{av} = 13–23 kDa, 98–99% hydrolyzed) and serine protease (from *Streptomyces griseus*) were purchased from Sigma–Aldrich (Buenos Aires, Argentina). Pectin of 55% methoxylation degree was kindly supplied by CPKelco (Buenos Aires, Argentina). All remaining reagents were of analytical grade or higher quality.

2.2. Keratinase extract

P. lilacinus strain LPSC #876 was used as source of keratinase [1]. The strain was maintained in potato-dextrose agar slants under mineral oil at 4 °C. *P. lilacin*us LPSC#876 was cultivated in 500 ml Erlenmeyer flasks with 100 ml of medium containing (per liter): 10.0 g hair waste, 0.50 g NaH_2PO_4, 2.49 g K_2HPO_4, 0.16 mg $FeCl_3{\cdot}6H_2O$, 0.13 mg $ZnCl_2$, 0.10 mg $MgCl_2$, and 0.011 mg $CaCl_2$, 2 g yeast extract and 5.0 g of glucose adjusted to pH 6.0. The culture flasks were sterilized at 121 °C for 15 min and then inoculated after cooling with 2×10^7 conidia per ml. The cultures were incubated in a shaker at 200 rpm and 28 °C for about 5 days. Later, the culture was centrifuged at 5000 × *g* and 4 °C for 20 min and the biomass discarded. The cell-free supernatant was used as a source of keratinase.

Keratinase crude extract (2.0 mg/ml) was precipitated with one volume of cold acetone on ice bath. The resulting suspension was centrifuged at 10,000 × *g* for 10 min. The supernatant was collected and the enzymatic activity was determined as described before. The pellet was redissolved in 500 μl of 100 mM Tris–HCl buffer (pH 7.4) and enzymatic activity was measured.

2.3. Enzymatic assays

Enzymatic activity of soluble keratinase was assayed using azocasein as substrate as reported previously [1]. In the case of CLEA, 10 mg of enzyme were used. One unit of keratinase activity (EU) was defined as the amount of enzyme that causes an increase of 0.10 units of absorbance/min at 440 nm under the experimental conditions.

2.4. Preparation of CLEAs

Firstly, 100 ml of acetone were added under agitation to 10 ml of 2 mg/ml of crude extract to precipitate the enzyme. After 30 min, 4.36 ml of glutaraldehyde solution (25%, v/v) were added to crosslink the enzyme precipitate and the mixture was kept under stirring for 1 h. The resulting K-CLEA was washed four times with 5 ml of 100 mM Tris–HCl buffer (pH 7.4) and centrifuged at 10,000 × *g* for 10 min. All the steps were performed at 0 °C.

2.5. Determination of kinetic parameters

Kinetic parameters of soluble keratinase and K-CLEA were determined by measuring initial reaction rates at different azocasein concentrations in the range from 0.153 to 30 mg/ml in 100 mM Tris–HCl buffer pH 9.0 at 37 °C. The K_m and V_{max} values of soluble keratinase and K-CLEA were calculated from Hanes-Hultin nonlinear regression fitting of the initial reaction rates corresponding to different azocasein concentrations.

$$\frac{S_0}{V_0} = \frac{K_m}{V_{max}} + \frac{S_0}{V_{max}} \quad (1)$$

where S_0 is the initial substrate concentration (time 0), V_0 is the initial reaction rate, K_m is the Michaelis-Menten constant, and V_{max} is the maximum enzyme rate.

2.6. Preparation of aqueous solution of polymers

Stock solutions of 30% (w/v) PVA were prepared in distilled water. The mixture was heated at 80 °C for 90 min under slow stirring until total dissolution. Solutions of 2.0–4.0% (w/v) pectin were prepared in distilled water with gentle stirring.

2.7. Preparation of cryogel films and soluble keratinase and K-CLEAs immobilization in the cryogels

Aqueous stock solutions of 30% (w/v) PVA were mixed with 2–4% (w/v) pectin and properly diluted to make 15/0.5% (w/v) PVA/pectin solutions. In typical experiments 3.5 EU of soluble keratinase and K-CLEA (100 mg) were added to the gels. Each solution was cast into Petri plates, frozen at −18 °C for 20 h and then thawed at room temperature (25 °C) for 8 h. This freezing/thawing cycle was repeated three times. The films were then dried at room temperature.

2.8. Protein quantification

Protein quantification was assayed by the Coomasie blue technique [9]. Calibration curve was performed with BSA (fraction *V*) as protein standard.

2.9. Enzymatic activity under harsh environmental conditions

2.9.1. Effect of solvents and NaCl in soluble enzyme and K-CLEA stability

K-CLEA (10 mg) and keratinase (3.5 EU) were incubated in 2.0 ml vials containing 100 mM Tris–HCl buffer (pH 7.4) and properly diluted with acetone, ethanol and DMSO to obtain concentrations of 20, 39, 60 and 79% (v/v) at 37 °C for 1 h.

K-CLEA (10 mg) and keratinase (3.5 EU) were incubated in 2.0 ml 50 mM buffer acetate (pH 5.5) in the range from 250 to 1000 mM NaCl at 37 °C for 1 h.

At defined time intervals samples were withdrawn to analyze the enzyme activity. The enzymatic activity was referred to the keratinase activity in aqueous medium without solvents and NaCl under the same experimental conditions. All experiments were

performed at least by two independent experiments run in duplicates.

2.9.2. Effect of temperature on enzymatic stability

K-CLEA (10 mg) and keratinase (3.5 EU) were incubated in 100 mM Tris–HCl buffer (pH 7.4.) in the range from 37 to 65 °C for 1 h and residual enzymatic activity was assayed as previously described.

Inactivation kinetics of soluble enzyme and K-CLEA were determined in 100 mM Tris–HCl buffer (pH 7.4) at 45 °C at times ranging from 30 min to 12 h, residual enzyme activity being analyzed as mentioned before.

2.9.3. Stability and storage of soluble keratinase and K-CLEA

K-CLEA (100 mg) and soluble keratinase (3.5 EU) were incubated in 100 mM Tris–HCl buffer (pH 7.4) at 37 °C. Aliquots of 100 μl of soluble enzyme were taken at 1, 12, 24 h and 2 months. K-CLEA samples were centrifuged 10,000 × *g* for 10 min and 10.0 mg were removed at each time interval. Enzyme activity was assayed as mentioned before.

2.10. Release of soluble keratinase and keratinase CLEAs from PVA-P cryogels

K-CLEA (100 mg) and soluble keratinase (3.5 EU) were incubated with 1.5 ml of 15% and 30% (w/v) PVA solutions mixed with 1.5 ml of 1% (w/v) pectin. The cryogels were prepared as described before.

PVA cryogels (40 mg) were placed in 2-ml glass vials containing 300 μl of 50 mM acetate buffer (pH 5.5) and incubated at 37 °C. At defined time intervals, the whole volume of the vial was withdrawn to determine protein and enzyme activity. Release experiments were performed at least by two independent experiments run in duplicates.

2.11. Characterization of K-CLEAs

2.11.1. Scanning electron microscopy

The morphology of K-CLEAs was analyzed by SEM Philips SEM 505 (Rochester, USA).

2.11.2. Small angle X ray scattering (SAXS)

SAXS measurements were carried out with an incident beam of 8 KeV and λ = 1.55 Å at D1B-SAXS beamline using a Pilatus 3000K detector (National Laboratory of Synchrotron Light, Campinas, Brazil). Solid samples were placed into stainless steel sample holders and covered in both sides with Kapton tape. All samples were measured at room temperature. Silver behenate powder was used to calibrate the distance between samples, detector and beam direction. Samples were measured at a distance of 0.8 m between sample and detector. Scattering vector (q) was analyzed vs. intensity where:

$$q = \left(\frac{4\Pi}{\lambda}\right) \operatorname{sen}\theta \quad (2)$$

where 2θ is the dispersion angle and λ the wavelength.

The scattering vector (q) is reaching a maximum (q_{max}) when the intensity is the highest.

3. Results and discussion

3.1. Optimization of CLEA production

Keratinase with specific activity of 18.35 EU/mg was precipitated with increasing concentrations of acetone. Full enzyme precipitation was detected in 75% (v/v) acetone without loss of keratinase activity (Fig. 1 of additional material). Keratinase activity was fully recovered up to 90% (v/v) acetone after solvent evaporation and redissolution in buffer (Fig. 2 of additional material). However, the recovery of keratinase was only 60% when acetone concentrations were higher than 90%. Over 90% acetone concentration, essential water molecules involved in enzyme structure and catalysis were sequestered by the solvent molecule rigidifying the protein structure. Hence irreversible changes in the tridimensional structure of the biocatalyst are probably the cause of keratinase inactivation.

K-CLEAs expressed 15% of the starting soluble keratinase activity. The reduction of enzyme activity could be attributed to protein structure rigidification and/or diffusional restrictions of substrate and products. Despite the reduced activity, the advantages of using keratinase CLEA over the soluble enzyme are described below.

Table 1
Kinetic parameters of free keratinase and K-CLEA.

Keratinase	V_{max} (EU)	K_m (mg/ml)
Soluble	62.5	2.43
K-CLEA	50.0	5.50

3.2. Kinetic parameters of soluble and CLEA keratinase

There is a significant difference between the K_m and V_{max} values of soluble keratinase and K-CLEA (Table 1), being the apparent K_m of K-CLEA twice the value of the intrinsic K_m of the soluble keratinase. Such difference can be explained in terms of enzyme rigidity and substrate diffusional restrictions.

3.3. Effect of temperature, organic solvents and ionic strength on soluble keratinase and keratinase CLEAs stability

The choice in using polar and non-polar solvents to study their effect on keratinase activity was based on their physicochemical properties (see Table 1 of additional material). The low solubility of isobutanol and n-hexane in water was overcome by using ethanol as "solvent helper" leading to a monophasic solvent reaction condition.

The effect of solvents on the enzymatic activity was studied at increasing concentrations of solvents in order to determine the minimum concentration needed to reduce the enzyme activity after 1 h of incubation at 37 °C. Once this concentration was determined, it was used to study and compare the effect of organic solvents between soluble keratinase and K-CLEA.

Keratinase showed high stability in the tested range of polar solvents such as acetone and DMSO. No inactivation of soluble enzyme was observed up to 79% (v/v) acetone, meanwhile keratinase showed enhanced activity in presence of 20–30% DMSO, and kept 100% activity at 60% (v/v) DMSO, but the enzyme was fully inactivated at 79% (v/v) DMSO. This is an unusual high DMSO concentration for enzyme inactivation because DMSO is regarded as strong chaotropic solvent, so inactivation at lower solvent concentrations was expected as in most of the enzymes reported [10]. When comparing the ε values for both solvents, the ε value for DMSO is 2.2 times higher than the one for acetone (see additional material). At 79% (v/v) DMSO, it is probable that most of the enzyme molecules are interacting with the solvent rather than with water. In this way, DMSO has more tendency than acetone to interact with the residual electric charges of the protein, establishing stronger interactions with enzyme polar residues and so rigidifying the enzyme structure (Fig. 1).

The ε values of polar organic solvents are lower than that of water; consequently, strong ionic interactions between enzyme and solvent are favored, rigidifying the enzyme structure. Also, water molecules intimately linked to the protein molecular

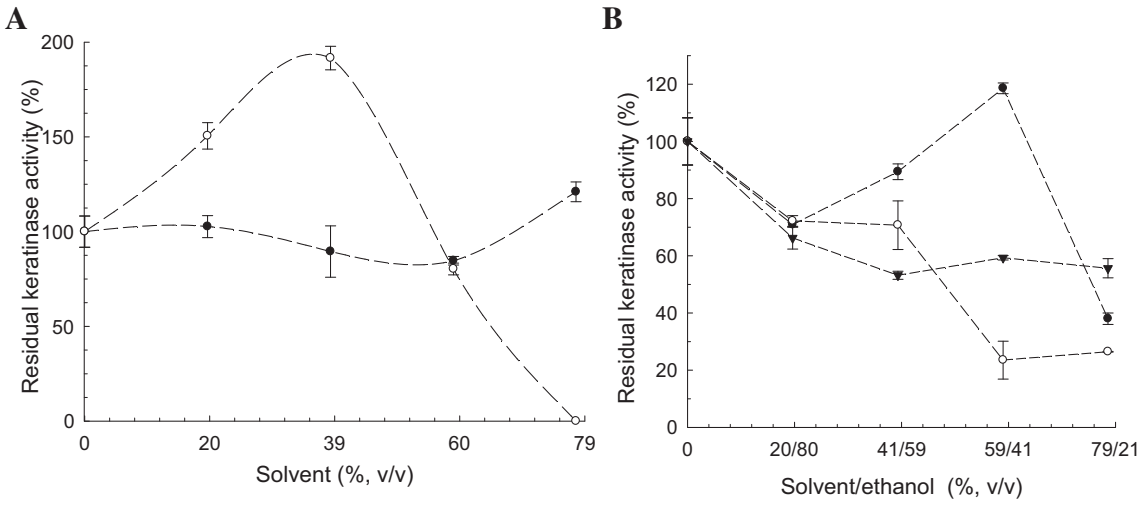

Fig. 1. (A) Effect of polar solvents on soluble keratinase incubated at 37 °C for 1 h (SD ≤ 10%, $n = 2$). Symbols: ●, acetone; ○, DMSO. (B) Effect of non-polar solvents on soluble keratinase stability incubated at 37 °C for 1 h (SD ≤ 12%, $n = 2$). Symbols: ○, n-hexane; ●, Isobutanol; ▼, ethanol.

structure are displaced by the polar solvents and consequently the catalysis is strongly affected [11].

On the other hand, at 79% (v/v) non-polar solvent concentration the enzyme is not completely inactivated, but the keratinase activity was reduced to 60–30% depending on the solvent (Fig. 1B). In such case, hydrophobic patches of the enzyme are interacting with non-polar solvents; meanwhile keratinase, a serine protease with polar microenvironment in the active center was not strongly inhibited. Similar results were found studying the stability of several proteases in organic solvents [12,13].

The experiments in organic solvents showed that keratinase stability is generally higher in polar solvents than in non-polar solvents, that is to say solvents with negative log P values and ε values higher than the ones of non-polar solvents (see Table 1 of additional material). Because of the low solubility in aqueous media of non-polar solvents, like isobutanol and n-hexane, and also in order to avoid interfacial problems, experiments were done solubilizing previously the non-polar solvent in ethanol. Control activity assays were performed in ethanol as well (Fig. 2).

On the other side, the residual activity of K-CLEAs was 1.4, 1.7 and 6.6 times higher than the soluble enzyme ($P \leq 0.05$) in presence of 59/41 water–solvent mixtures of acetone, n-hexane and isobutanol respectively incubated at 37 °C for 1 h (Fig. 2). The higher stability of K-CLEA with respect to the soluble keratinase could be attributed to the structure stabilization promoted by crosslinking and/or to solvent partition in the CLEA microenvironment. The high stabilizing effect of CLEA in organic solvents has been also observed in subtilisin (a serine protease from *Bacillus* sp.) [14].

Effect of temperature on K-CLEA stability showed 45% of residual enzyme activity at 65 °C after 1 h of incubation; meanwhile the free biocatalyst was completely inactivated under the same experimental conditions (Fig. 3). Based on the previous results, inactivation kinetics of soluble keratinase and K-CLEA were studied at 45 °C. After 12 h of incubation at such condition, K-CLEA retained 51% of the enzymatic activity while the soluble keratinase was completely inactivated (Fig. 5).

Effect of ion strength in keratinase catalytic activity is considerable. For instance, it has been reported that enzyme activation occurs after enzyme liophilization in the presence of salts. This phenomenon is related to possible coordination effects of water molecules on enzyme biomolecule which leads to a more flexible enzyme structure and higher activity [15]. Kosmotropic ions are in

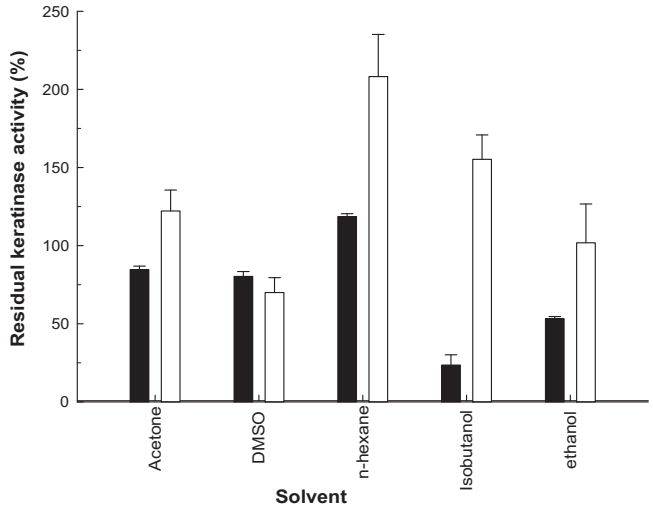

Fig. 2. Stability of soluble keratinase and K-CLEA in 59% solvents incubated at 37 °C for 1 h (SD ≤ 13%, $n = 2$). Symbols: ■, keratinase; □, K-CLEA.

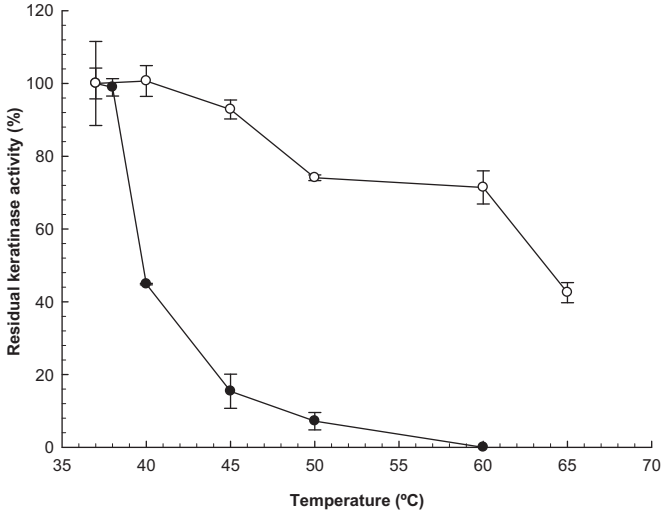

Fig. 3. Thermal stability of free keratinase and K-CLEA incubated at 37 °C for 1 h (SD ≤ 10%, $n = 2$). Symbols: ●, keratinase; ○, CLEA.

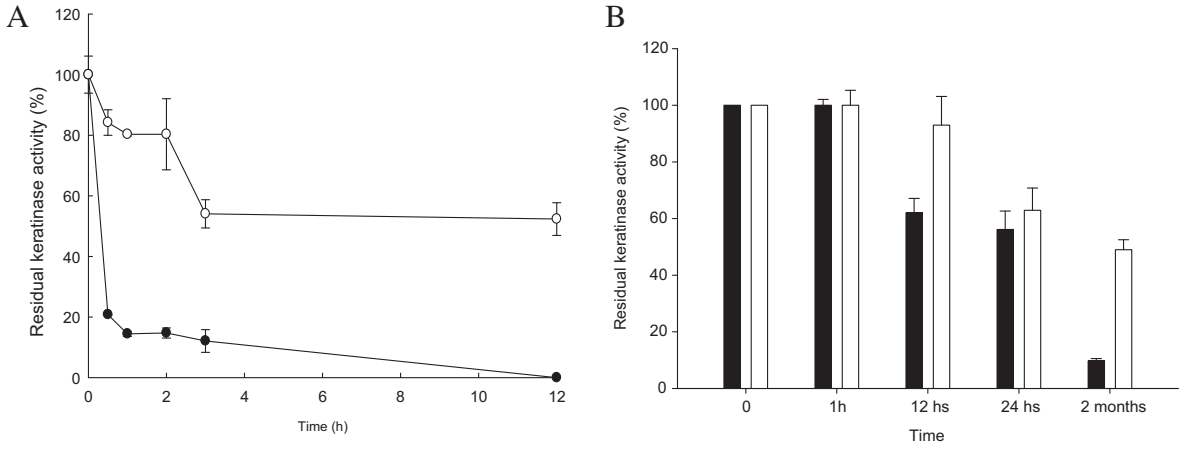

Fig. 4. (A) Thermal stability of free keratinase and K-CLEA incubated at 45 °C (SD ≤ 10%, $n = 7$). Symbols: ●, keratinase; ○, CLEA. (B) Stability of soluble keratinase and K-CLEA at 37 °C (SD ≤ 10%, $n = 7$). Symbols: ■, keratinase; □, K-CLEA.

general those with a high charge density and, consequently, they are able to order water molecules and make stronger interactions than water molecules among themselves. The entropy value resulting of the breaking of H bonds between water molecules is lower because water molecules are better organized around ions [16]. Soluble keratinase and K-CLEA were activated after incubation in the presence of 0.75 and 1 M NaCl (Fig. 3 additional material).

Storage of proteolytic enzymes, like keratinase, in aqueous solutions is a disadvantage because of autolysis [17]. Hence, soluble keratinase and K-CLEA stability was studied, measuring residual activity after 1, 12, 24 h and 2 months at 25 °C in buffer solution at (pH 7.4). Soluble keratinase retained around 60% and 10% of the initial enzyme activity after 24 h and 2 months respectively (Fig. 4B). On the other hand, K-CLEA retained 51% of its enzymatic activity after 2 months under the same experimental conditions.

These results altogether show the protective effect against adverse conditions of temperature, solvents and prolonged storage conferred to the enzyme by aggregation and crosslinking.

3.4. CLEA and soluble enzyme release kinetics from the cryogel matrices

Immobilization and release experiments of keratinase were carried out onto PVA and PVA-P cryogels as previously reported [1].

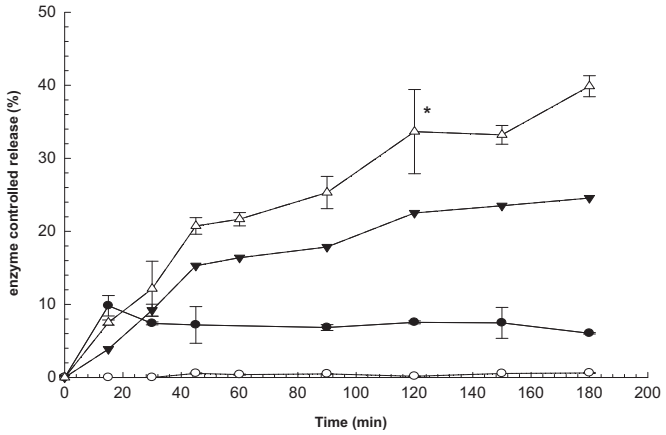

Fig. 5. Controlled release of soluble keratinase and CLEA from PVA and PVA-P cryogels (SD ≤ 10%, $n = 2$; *SD ≤ 20%, $n = 2$). Symbols: ●, PVA K-CLEA; ○, PVA-P K-CLEA; ▼, PVA-K; △, PVA-P K. *Abbreviation*: K, keratinase.

The presence of pectin in the PVA cryogel formulation reduced K-CLEA release rate from the cryogel matrix to the solution six times with respect to the release rate from the PVA cryogel alone. Besides PVA and PVA-P K-CLEA release kinetics were slower than the one of soluble keratinase from the same cryogel type after 3 h of incubation (Fig. 5). This fact can be explained by the dimension of K-CLEAs, whose particle size is much bigger than the molecular size of soluble keratinase (37.0 kDa, approximately 8.7 nm) [18]. It was expected then, that CLEAs were retained through the polymeric chain network impairing their release from the polymeric matrix.

3.5. CLEA characterization

K-CLEAs were characterized by scanning electron microscopy (SEM, Fig. 6). Two K-CLEA size distribution populations were found with average diameters (d) of 3.15 ± 0.25 and 2.13 ± 0.24 μm.

X-ray diffraction patterns were obtained for all the K-CLEA samples in order to gain insight about the polymer pattern changes after incorporation into the cryogels. Amorphous SAXS scattering pattern of K-CLEA and a semi-crystalline scattering pattern of PVA and PVA-P samples with $q_{max} = 0.7\ nm^{-1}$ were detected (Fig. 7). Incorporation of K-CLEA into PVA cryogels showed an amorphous scattering of the cryogel, leading to a wider crystalline size distribution.

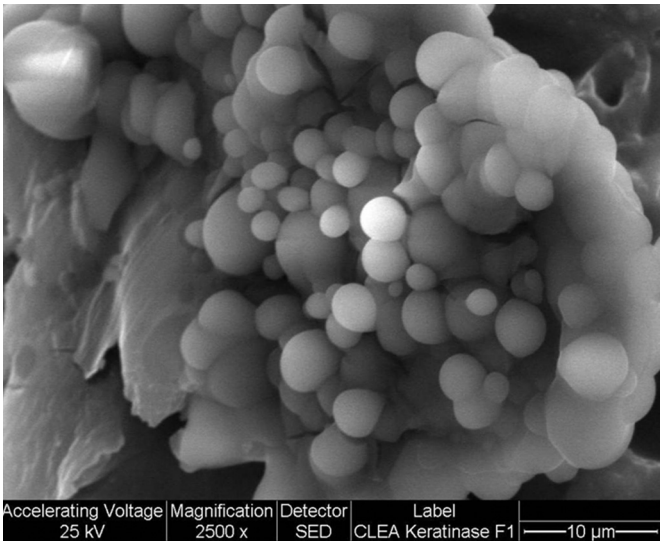

Fig. 6. Scanning electron microscopy of keratinase on CLEA.

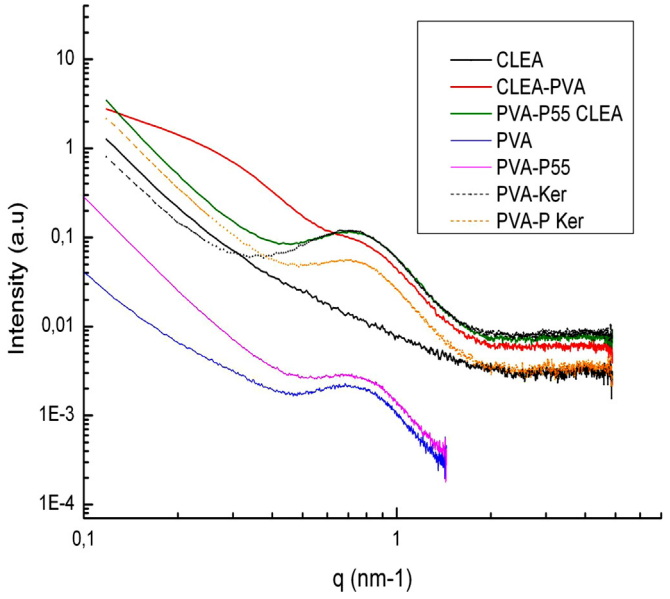

Fig. 7. Small angle X ray scattering of dried samples. Symbols: (—), K-CLEA; (▬ ▬ ▬) K-CLEA-PVA; (• • •), PVA-P55 K-CLEA; (▬ • • •), PVA; (—•—), PVA-P55. Abbreviation: P55, pectin 55% methoxylation.

That is, in the presence of CLEA the distance between PVA nanodomains is wider due to q_{max} shifting to lower values so the polymeric network is more relaxed than PVA-P CLEA network where q_{max} value is the same as PVA and PVA-P. In the presence of pectin, PVA semi-crystallinity is not affected because the q_{max} vector value does not change in any of the samples ($q_{max} = 0.7\ nm^{-1}$). It can be concluded that K-CLEA aggregates preferably bind to pectin and not to the PVA chain network in the PVA-P cryogel matrix leading to intermolecular chain interactions in PVA structure preserving its semi-crystalline structure. The polymeric network of PVA-P K-CLEA cryogel is tighter than PVA-K-CLEA network. So it is expected that release of K-CLEA is retarded. In the presence of soluble keratinase, PVA and PVA-P crystalline size distribution is not affected ($q_{max} = 0.7\ nm^{-1}$) (Fig. 7). The faster release of keratinase from PVA-P matrix may be explained in terms of pectin solubilization leading to the fast diffusion of the enzyme.

4. Conclusions

The development of CLEAs for keratinase has many advantages such as higher enzyme stability compared to soluble keratinase under harsh environmental conditions, e.g. in the presence of organic solvents, high temperatures and ionic strength, and prolonged storage. In addition, the procedure for the production of K-CLEAs developed is simple, reproducible and scalable.

Immobilization of CLEA in PVA and PVA-P cryogels was tested in kinetic release experiments. In the presence of pectin, K-CLEA release is retarded six times compared with PVA K-CLEA. As observed after SAXS experiments, K-CLEAs bind preferably to pectin in the PVA-P cryogel matrix. In this way is possible to tailor cryogel matrices in order to regulate K-CLEAs release from the cryogel which can be significantly slower than soluble enzyme release.

Further studies will be conducted in skin models in order to test the results found in the present work and apply the developed system in the treatment of skin injuries.

Acknowledgments

The present work was supported by Argentinian grants from CONICET (National Council for Science and Technology, PIP 0214), The National Agency of Scientific and Technological Promotion (ANPCyT) and UNLP (National University of La Plata, 11/X522, 11/545 and PRH 5.2). We want to thank Mr. Erick Araya Garmendia (*Escuela de IngenieríaBioquímica*, *Universidad Católica de Valparaíso*, Chile) for his technical help on CLEA synthesis, to Dr. Mateus Cardoso (National Laboratory of Synchrotron Light, Campinas, Brazil) and to Ms. Fiona M. Britto for the synchrotron SAXS measurements.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.colsurfb.2014.02.049.

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