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Immobilized keratinase and enrofloxacin loaded on pectin PVA cryogel patches for antimicrobial treatment

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HIGHLIGHTS

- Immobilized keratinase in pectin–PVA films.
- Pectin PVA-cryogels for wound healing.
- Release kinetics of enrofloxacin.
- Pectin with different ED are controlling molecular release.

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ABSTRACT

A keratinase isolated from *Paecilomyces lilacinus* (LPS #876) was tested against proteins present in the skin but the high enzyme activity was detected on collagen. Keratinase was physically immobilized onto PVA–pectin cryogels and enzyme release was 20.8 ± 2.1%, 63.8 ± 0.2%, 41.5 ± 3.5% and 26.0 ± 3.5% in cryogels containing pectins with esterification degrees (DE) 33.0%, 55.0%, 62.7% and 71.7% respectively at 37 °C after 3 h incubation. In presence of 0.75 M NaCl, the percentage of enzyme release changed to: 57.5 ± 1.5, 65.8 ± 3.8, 57.3 ± 0.2 and 34.0 ± 4.0 for the four pectins respectively.

In-vitro studies of enrofloxacin release from PVA–pectin cryogels at pH close to the human skin (pH = 5.5) showed 15.0% free antibiotic following first order kinetic at 37 °C after 5 h incubation. However, in the presence of keratinase only 6.9% of enrofloxacin was released under the same experimental conditions.

1. Introduction

Globally, burns are a serious public health problem. According to the World Health Organization, there are over 195,000 deaths each year from fires alone, with more deaths from scalds, electrical burns, and other forms of burns, for which global data are not available. Moderate to severe burn injuries requiring hospitalization account for approximately 100,000 cases per year, and about 5000 patients die each year from burn-related complications (World Health Organization, 2008).

Additionally, in some chronic pathologies like diabetes, ulcerating wounds are requiring strict surveillance, if not generally limb amputation is the consequence. Moreover, the treatment of diabetic foot complications is very expensive and accounts for 15–25% of diabetes healthcare resources in high-income countries (World Health Organization, 2005).

In general, dead tissue covering burn wounds provides an open door for microorganisms to grow and spread infection inside the body. Moreover, the antimicrobial activity of antibiotics is severely reduced in topical administration due to eschar formation (Wang et al., 2009). Consequently, increasing concentration of antibiotics are required for the medical treatment over passing the therapeutic window and producing undesirable side effects in many cases.

Third-degree burn wound eschar is avascular and frequently severe millimeters distant from microvasculature. Therefore, the administration of systemic antimicrobial agents may not achieve therapeutic levels by diffusion to the wound, where the amount of microorganisms is usually very high (Manafi et al., 2008). In the present scenario, enzymatic debridement of wound dead tissue is an interesting alternative for enhancing penetration of topical administered antibiotics, preserving the spontaneous epithelialisation potential (Krieger et al., 2012).

Some proteases that are able to catalyze the degradation of tissue proteins favor wound cleaning and also exhibit anti-inflammatory, fibrinolytic, and antiedemic effects (Vernikovskii and Stepanova, 2012).

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Polymeric cryogels are used in topic antimicrobial controlled release (Abdel-Mohsen et al., 2011). The synthesis made in aqueous media, the biocompatibility, and the lack of immunogenicity are the main advantages of PVA cryogels. Also, chemical crosslinkers are not required in their preparation. The main forces present in the polymeric network are hydrogen bonds leading to a stable gel formation (Hassan and Peppas, 2000). Additionally, PVA cryogels are reported to be more efficient for enzyme immobilization than other gels (Belokon et al., 2000). The PVA aqueous macroporous structure allows substrate diffusion into the matrix. Also, PVA cryogel matrices provide a friendly aqueous environment required for enzymes to catalyze reactions in aqueous and non-aqueous media (Belokon et al., 2000).

Enrofloxacin (EF) is a fluoroquinolone antibiotic used in veterinary against both Gram negative and positive bacteria. It is generally administered at high concentrations which can cause molecular stacking and crystal formation affecting many organs with undesirable side effects (Maurer et al., 1998).

The aim of the present work is to develop a controlled release gel matrix for enrofloxacin and keratinase based on a cryogel of PolyVinyl Alcohol–Pectin (PVA–P) for the treatment of wounds and eschars. Pectins with different esterification degree (DE) and biopolymer concentrations were tested for optimum enzymatic and antibiotic release.

2. Methods

2.1. Preparation of aqueous solution polymers

Stock solutions of 30% PVA (MW = 13–23 kDa, 98–99% hydrolyzed, Sigma–Aldrich, Buenos Aires, Argentina) were made in distilled water as previously reported (Martinez et al., 2012). Pectins with 33.0%, 55.0%, 62.5% and 71.1% degree of esterification (DE) were kindly supplied by CPKelco (Buenos Aires, Argentina). Solutions of 2.0–4.0% (w/v) pectin were prepared in distilled water with slow stirring. Enrofloxacin (Sigma–Aldrich, Buenos Aires, Argentina) stock solution was prepared in 50 mM citrate buffer (pH = 3.5).

2.2. Preparation of cryogel film and enzyme immobilization

An aqueous solution of 30.0% (w/v) PVA was mixed with different amounts of 2.0–4.0% (w/v) aqueous pectin and diluted to produce solutions of 15.0% (w/v) PVA containing 0.50%, 0.75% and 1.00% (w/v) of pectin respectively. In typical experiments aqueous solution of 5.0 μg/ml EF and/or 0.163 enzyme units (EU) of keratinase were added while stirring to polymeric solution.

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 cycle of freezing/thawing was done three to five times in total. Later, the films were dried at room temperature under hood.

2.3. Release of enrofloxacin from PVA–P cryogels

Release experiments were performed by weighting a portion (25 ± 3.1 mg) of the cryogel and incubating the gel into 1.5 ml of 50 mM acetate buffer (pH = 5.5) at 37 °C. Aliquots of the solution were taken out every 15 min up to 1.5 h and then every 1 h of incubation and replenished with the same volume of fresh buffer. The antibiotic was measured by fluorescence at λ_{ex} 275 nm and λ_{em} 437 nm (PerkinElmer LS 50B spectrofluorimeter).

2.4. Enzymatic release from PVA–P Cryogels

Weighty portions of the cryogel were placed in individual glass tubes in contact with 300 μl of 50 mM of acetate buffer (pH = 5.5) under individual stirring at 37 °C. Supernatants samples were withdrawn at defined time, proteins and enzyme activity (azocasein as substrate) were measured as described below. Experiments were performed in duplicates.

2.5. Biochemical characterization of purified free keratinase

The enzyme capability to attack and degrade skin structure and related molecules was analyzed with a group of chromogenic substrates derived from skin components and determined by spectrophotometry. Chromogenic substrates tested were divided into insoluble substrates: keratin azure, hide powder azure, azocoll, azokeratin and elastin congo red, and soluble substrates: azoalbumin and azocasein.

2.5.1. Assay with azocasein and azoalbumin

Enzyme activity was determined by incubating an aliquot of free enzyme (100 μl), properly diluted, within 250 μl of 100 mM Tris–HCl buffer (pH 9.0) containing 1.0% (w/v) of the substrate (Sigma–Aldrich), incubated for 30 min at 37 °C and stopped by the addition of 1.0 ml of trichloroacetic acid (10.0%). The mixture was kept at room temperature for 15 min and then centrifuged at 10,000*g* for 10 min. One milliliter of 1.0 M NaOH was added to 1.0 ml of the supernatant and measured at 440 nm. A blank was performed using 100 μl of heat inactivated enzyme. One unit of protease activity (UE) was defined as the amount of enzyme that causes an increase of 0.10 units/min at 440 nm under the experimental conditions.

2.5.2. Assay with keratine azure and azokeratin

Keratinase activity was determined using keratin azure (Sigma–Aldrich) and azokeratin, as previously described (Riffel and Brandelli, 2006). Five milligrams of keratin azure was placed into a test tube, then 900 μl of 100 mM Tris–HCl buffer (pH = 9.0) and 100 μl of appropriate diluted enzyme was added. The mixture was stirred at 37 °C for 90 min. and the reaction stopped by placing the tubes on ice-water for 30 min. Absorbance was measured at 595 nm. One keratinase unit was defined as the amount of the enzyme that causes an increase of 0.01 units at 595 nm/min under experimental conditions. The same methodology was used with azokeratin, but the amount of substrate used was 30 mg, incubation time was 25 min and absorbance of the supernatant was measured at 440 nm (Joshi et al., 2007).

2.5.3. Assay with hide powder azure, azocoll and elastin congo red

Hide powder azure (HPA) is a partially denatured collagen-rich preparation to which a blue dye is covalently bonded, azocoll is insoluble collagen attached to a brilliant red azo dye and elastin-congo red is a substrate used to obtain information on the elastinolytic activity were used to characterize the enzyme activity using the same protocol of keratin azure. The reaction mixture was incubated 20 min at 37 °C and the absorbance of the supernatant was measured at 595, 520 and 495 nm for HPA, azocoll and elastin congo red, respectively.

2.6. Enzyme activity in the presence of enrofloxacin

In order to study the effect of the enrofloxacin (EF) on enzyme activity, free enzyme was incubated with 5 μg/ml of EF at 37 °C for 3 h. After incubation, residual activity was determined using azocasein as substrate. Enzyme activity without EF was taken as 100%. Experiments were performed in triplicate.

2.7. Protein quantification

Protein quantification was assayed by Fluorescamine reactive protocol (Udefriend et al., 1972) using BSA (fraction V) as protein standard. Fifty microliters of proteins were mixed with 325 μl of 12 mM borate buffer (pH = 9.0), then 250 μl of 300 μg/ml of fluorescamine reactive was added and homogenized. Fluorescence was measured at λ_{ex} = 390 nm and λ_{em} = 478 nm (PerkinElmer LS 50B spectrofluorimeter).

2.8. Enrofloxacin (EF) antibacterial activity in the presence of enzyme

EF antibacterial activity was tested against *Pseudomonas aeruginosa* ATCC 15492 (non-fermentative Gram-negative bacteria) using modified Disc Diffusion Method (DDM) according to CLSI/NCCLS. The DDM was modified by using sterile glass cylinders of 8 mm (external diameter), 6 mm (internal diameter) and 10 mm (length) instead of filter paper discs. The glass cylinders were placed on the surface of the inoculated Mueller–Hinton agar plate. After their placement, 25 μl of each 100 μg/ml EF solutions containing different amounts of keratinase (5.5, 11.0, 22.0 and 44.0 μg/ml) were placed inside the cylinders and the plates were incubated at 37 °C for 24 h. Then, inhibition zones were determined. The assays were performed in duplicate.

3. Results and discussion

Enzymatic activity was measured against substrates derived from skin components like albumin, collagen, elastin and keratin. *Paecilomyces lilacinus* keratinase has broad substrate specificity and it can hydrolyze not only keratin, but also a large variety of soluble and insoluble proteins. The present study showed that among all the substrates tested, partially denaturalized collagen displayed the highest degree of hydrolysis (Table 1). The broad range of substrates hydrolyzed by *P. lilacinus* keratinase implies a big potential industrial use in leather and detergent industries, textiles waste bioconversion, medicine and cosmetics for drug delivery through nails and degradation of keratinized skin as previously suggested (Najafi et al., 2006). For example, topical therapies of nail diseases posses the limitation of drug low permeability through the nail plate which can be enhanced by the keratinase administration. Keratinolytic enzymes might decrease the barrier properties of the nail plate by hydrolyzing the nail keratins. Therefore, the enzymatic disruption of nail plate can be associated in an enhanced drug penetration (Gupta and Ramnani, 2006).

Additionally, keratinase (8.3 UE/ml) assays in presence or absence of EF (5.0 μg/ml) did not show any significant difference on hydrolytic activity ($p < 0.05$).

From the results presented here and with the knowledge that some proteases have been used in wound cleaning preparations, in the enhancement of nail treatment, etc. it can be concluded that this enzyme may be suitable for eschar debridement using a sustainable release system. However, when native proteases are directly applied on a wound undergo fast deactivation, making the enzyme therapy inappropriate. For example, the necrolytic effect of proteolytic enzymes on a wound lasts as little as 15–30 min. (Abdel-Mohsen et al., 2011). Immobilization of the enzyme allows the effect of proteases to be localized in a specific region of the body and enhances their stability under the conditions of the wound healing process. Previous results showed the effective controlled release of EF from PVA–pectin cryogels (Martinez et al., 2012). In order to extend enzyme activity, co-immobilization of the keratinase in PVA–pectin biopolymeric matrix containing EF is studied.

In a first attempt to study the hydrogel immobilization system, keratinase was immobilized in PVA–pectin matrix using highly methoxylated pectin (71.1% DE) at two different concentrations (0.5% and 1.0% w/v). Controlled release of enzyme was measured at different times in the supernatant using azocasein as substrate. Only 19.6% and 25.4% of enzyme were released from the PVA matrix containing 1.0% and 0.5% (w/v) of pectin after 3 h of incubation respectively. These results may indicate that the tight polymeric network prevents the enzyme diffusion from the film to the solution. From these first results, the pectin concentration in the matrix was fixed to 0.5% (w/v) in order to study the effect of pectins esterification (methoxylation) degree.

Fig. 1 shows the enzyme kinetic release from pectins with different degrees of methoxylation. Keratinase release was 63.8 ± 0.2% from the PVA cryogel containing 55.0% DE pectin in 180 min. Meanwhile, the amount of enzyme released was 37.3% and 26.0% in PVA cryogels having 62.0% and 71.7% DE pectins respectively in 3 h. On the other side, PVA gels containing 33.0% DE pectin showed an intermediate enzyme release, 29.1%, in between of high methoxylated pectins. These results could be explained in terms of interactions between the enzyme and the biopolymers with different methoxylation degree and considering the pectin p*K*a is in the range of 3.5–4.0 (Plaschina et al., 1978).

The swelling degree of PVA cryogels can change drastically in response to external stimuli such as temperature, pH, ionic strength and the presence of certain chemicals. The swelling ratio is mainly related to the characteristics of the external solution, such as ion charge and ionic strength (Zhu et al., 2012). Particularly, the swelling ratio increased concomitantly with the salt concentration. High swelling ratios allows more amount of molecules to get out from the hydrogels. In order to test the effect of ionic interactions between the pectins and the enzyme, keratinase

Table 1
Hydrolysis of complex substrates by *P .lilacinus* keratinase (8.3 units/ml measured with azocasein as substrate).

Substrate	Absorbance (nm)	Hydrolysis rate (ΔAbs/min) × 10^3
Azoalbumin	440	177.3 ± 0.6
Azocasein		120.3 ± 2.3
Azokeratin		52.5 ± 7.2
Azocoll	520	240.3 ± 17.6
Elastin congo red	495	240.0 ± 7.1
HPA	595	3502.3 ± 186.4
Keratin azure		2.4 ± 0.1

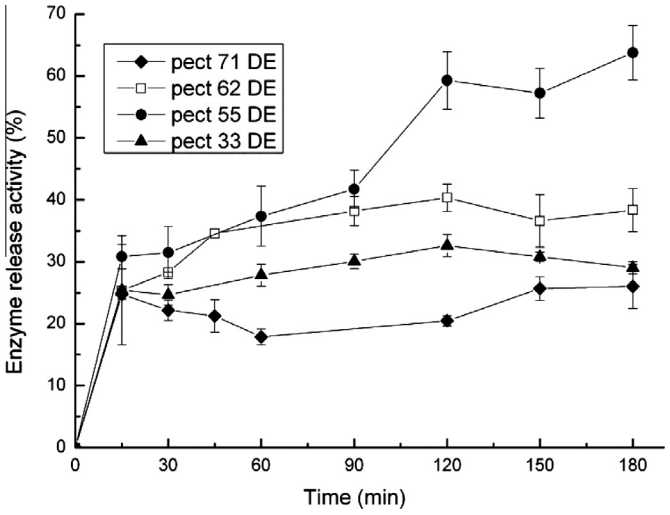

Fig. 1. Kinetic release of keratinase from PVA–pectin systems with different degrees of esterification (DE) at pH 5.5.

Table 2
Effect of ionic strength in the enzyme release from PVA–P gels with different esterification degrees (DE).

Pectin (%, DE)	Keratinase release (%) in 180 min	
	With NaCl	Without NaCl
71.7	34.0 ± 4.0	26.0 ± 3.5
62.7	57.3 ± 0.2	41.7 ± 3.5
55.0	65.8 ± 3.8	63.8 ± 0.2
33.0	57.5 ± 1.5	29.1 ± 1.1

release from the PVA cryogel matrix doped with four methoxylated pectins was observed in all cases in presence of 50 mM acetate buffer (pH = 5.5) with and without 750 mM NaCl (Table 2). Also in this process of salting-in, the enzyme is more soluble and in consequence, the release is enhanced such as in the case of 33% and 62.5% DE pectin ($p < 0.05$). However, 71.7% DE pectin posses strong hydrophobic environment inside the matrix due the presence of high amount of methoxylated groups (–$ROCH_3$). In this case, no statistically significant differences were found in the percentage of enzyme release with and without NaCl. It is shown that hydrophobic interactions between the matrix and the enzyme delayed the release compared with the others matrices because the presence of NaCl outside, favoring hydrophobic interactions between the enzyme and the matrix.

On the other side, the percentage of enzyme released in 55.0% pectin, is high and constant (without significant differences, $p < 0.05$) with or without NaCl. Ionic interactions between positive charged enzyme amino acid residues and free carboxylate groups of pectin are probably taking place. This hypothesis is supported based on the predominant polar interactions in the aqueous PVA matrix, such as the case of very low DE pectins, in where the amount of enzyme released is lower compared to high and medium methoxylated pectins.

Fig. 2 shows the kinetic of keratinase release. The constant profile of enzyme specific activity indicates that the matrix does not affect the enzyme stability.

Based on the results, the PVA matrix containing 55.0% DE pectin was selected for further experiments because the enzyme release

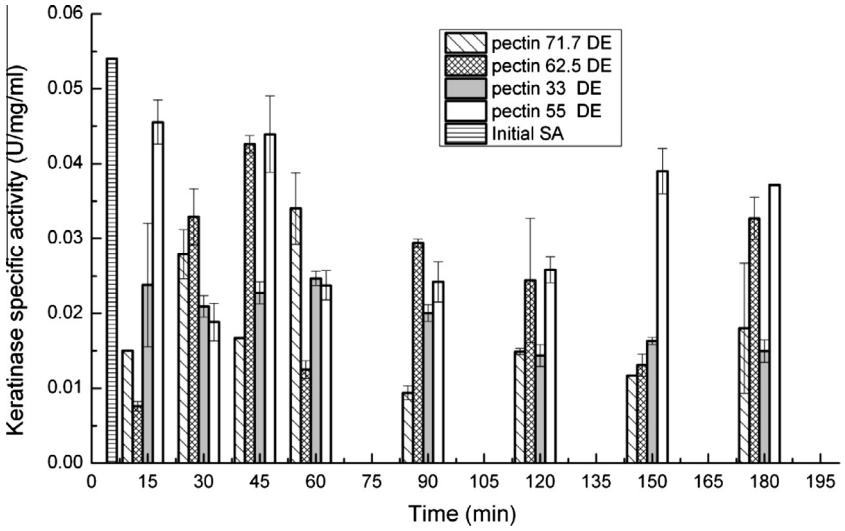

Fig. 2. Effect of pectin esterification degree on keratinase specific activity kinetic.

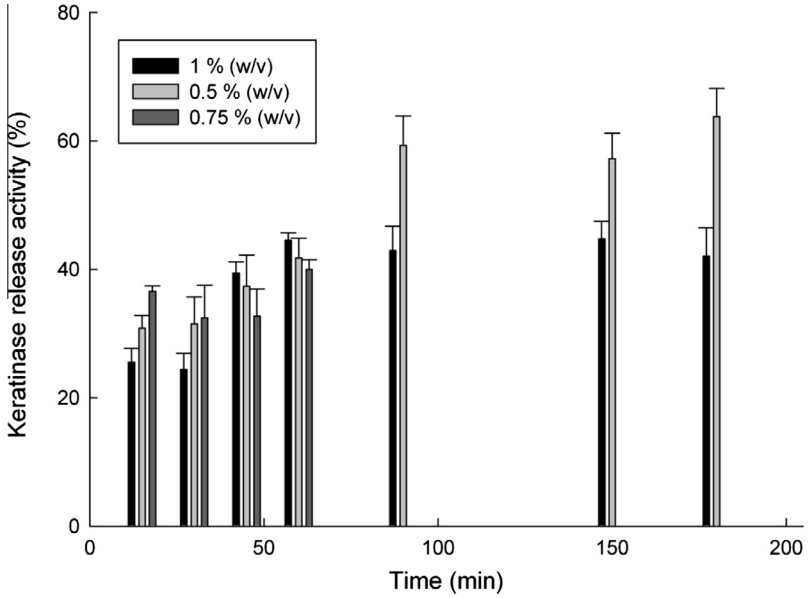

Fig. 3. Effect of pectin concentration in the hydrogel expressed as percentage of keratinase release at different times.

was the highest. In order to adjust the concentration of pectin in the system a series of experiments were performed.

Kinetic of enzyme release in 55.0% DE pectin–PVA was tested under three different pectin concentrations 0.50%, 0.75% and 1.00% (w/v). PVA gels containing pectin concentrations below 0.5% do not shown reproducible results under our experimental conditions (data not shown). Fig. 3 displays that the most suitable system is the gel composed of 0.50% (w/v) pectin because it reached the highest percentage of enzyme release after 3 h. of incubation.

In order to determine potential interactions between the keratinase and EF in PVA–pectin cryogels, agar diffusion tests were performed. EF antibacterial activity assayed using modified disc diffusion method showed no differences between control halo (EF) and halos formed by EF in the presence of different enzyme concentrations. The results showed no interference between keratinase and enrofloxacin which allows the dual immobilization of the antibiotic and the enzyme in the film for the controlled release purposes (data not shown). Similar results of no interaction between a growth factor, and the corticosteroid dexametasone were previously reported using similar methodology for the differentiation of stem cells into chondrocytes (Park et al., 2009).

Finally, the simultaneous release of the EF and the keratinase was studied when both of them were entrapped in the hydrogel, using as control a system without enzyme. In this study, the optimum hydrogel matrix composed of PVA and 0.50% (w/v) pectin 55.0% DE was used. The EF release profile increasing along time displays a first order kinetic behavior. The release of EF in absence of the enzyme is faster (15.4 ± 0.6% after 5 h of incubation) than the hydrogel containing both EF and enzyme (6.9 ± 0.7% after 5 h of incubation). Probably, both the EF and the enzyme may have the same "pathway out" of the matrix, so when the enzyme is present in the film, the antibiotic release is delayed. According to the gel use, the amount of EF release could be modified by changing the thickness of the gel.

4. Conclusions

In the present work, none of the four formulations studied seems to be a hostile environment for the enzyme because it retained 100% of it enzymatic activity. This may be due to PVA macroporous structure and aqueous environment, where solute diffusion inside the matrix is possible as well as enzyme release. These results show a versatile system for enzyme immobilization where proteins with more or less hydrophobic zones can be adsorbed keeping the biological activity.

Finally, the controlled release of EF and keratinase from PVA films can be tailored changing the amount and type of pectin by the methoxylation degree.

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