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ОЦЕНКА ВЛИЯНИЯ ПЕКТИН-ПАПАИНОВЫХ ВЗАИМОДЕЙСТВИЙ НА СТАБИЛЬНОСТЬ ФЕРМЕНТА, И МЕХАНИЧЕСКИЕ СВОЙСТВА ПЕКТИНОВЫХ ПЛЕНОК ИЗ МАРАКУЙИ, ИСПОЛЬЗУЕМЫХ ДЛЯ ЛЕЧЕНИЯ КОЖНЫХ РАН

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Пектин (биоразлагаемый водорастворимый полимер) получали из отходов производства сока маракуйи (пассифлоры) и использовали в качестве носителя для иммобилизации протеазы папаина при получении пленок, пригодных для лечения кожных ран. Исследовано влияние пектин-папаиновых взаимодействий на стабильность фермента и механические свойства пектиновых пленок в присутствии и в отсутствие пластификаторов, таких, как глицерин и поливиниловый спирт (ПВС). Показано, что добавки глицерина и ПВС улучшали механические свойства пектиновых пленок: глицерин увеличивал эластичность, а ПВА – прочность к растяжению. Применение пластификаторов также способствовало сохранению активности и стабильности папаина. Более того, исследования по лечению кожных ран, проведенные на добровольных пациентах, с применением папаин-пектиновых пленок, продемонстрировали ускорение заживления ран без каких-либо негативных побочных эффектов независимо от типа и глубины раны.

INTRODUCTION

Much effort has been focused in recent years to develop environmentally compatible bioplastic products from natural renewable materials as an alternative to synthetic polymers. As added advantages, renewable polymers are comparatively less expensive, environmentally friendly, nontoxic and naturally biodegradable. In these regard, materials such as renewable crops, agricultural waste and/or byproducts of food industry are a good source of low cost natural polymeric materials [1].

Passion fruit-maracuya (*Passiflora edulis*) processing produces large quantities of waste such as the shells and seeds, which create a disposal problem. Studies on conversion of passion fruit wastes to improve their use have been carried out in a number of different ways such as, candy manufacture, animal foodstuff, pectin liquid-extract and dietetic fiber [2–4].

Pectin, a biodegradable polysaccharide found in plant cell walls [4] is the one of the major structural biopolymer of higher plant cells. It is part of the family of heterogeneous branched polysaccharides consisting mostly of methylated galacturonan segments separated by rhamnose residues [2]. Pectin is used in a number of foods as gelling agent, thickener, texturizer, emulsifier and stabilizer, as well as a fat or sugar replacement in low-calorie foods [5, 6]. Previously, we reported that residues of passion fruit is useful for obtaining pectin, which has potential in the development of films for treatment of skin wounds, based on entrapped enzymes [3].

Films made from natural products are of increasing scientific and commercial interest. They are not only biodegradable but may be recyclable as well as acceptable for pharmaceutics applications [3]. Citric pectin films were first made and characterized [1, 7]. Previous work has shown that elasticized films made from high methoxy lime pectin and high amylase starch have very good mechanical properties. The use of glycerin or other suitable elasticizes (as polyvinyl alcohol, PVA) is necessary to make sufficiently flexible and no brittle films [1, 7]. The PVA is well suited for blending with natural polymeric materials since it is highly polar, can be manipulated in water solutions and is also nontoxic [1, 8].

Film-entrapped protease could be a promising drug for wound therapy [3, 9]. Furthermore, the interest in immobilized enzymes and their application to bioprocessing, analytical systems and enzyme therapy has steadily grown in the past decade [10, 11]. Gel entrapment method is attractive because is very simple, can be carried out under soft conditions (physiological, pH and temperature) and also can vary polymer matrix composition and structure [13]. Moreover, the enzyme interaction with polymeric system in presence and absence of plasticizers may change not only operative enzyme characteristics, but also their material properties. In present study, the attention was focused on papain as a hydrolytic enzyme entrapped on pectin films elaborated from maracuya waste, with and without glycerin and PVA.

In this communication we report on: 1) preparation of pectin and pectin-papain films mixed with plasticizers as glycerin and PVA and their mechanical properties, 2) estimation of the effect of pectin-papain interactions on enzyme stability and activity in the biopolymer systems with or without plasticizers, 3) evaluation of pectin-papain films on the treatment of human skin lesions.

MATERIALS AND METHODS

Pectin was extracted from the shells of passion fruit (Pasiflora edulis) acquired from the juice factory in Puebla, Mexico by using the method reported previously [3]. The reagents: papain, casein, cysteine were purchased from Sigma-Aldrich (USA). Glycerin, PVA of molecular weight 128000–140000, phosphate salts were purchased from Jalmek (Mexico). All regents were of analytical grade.

Film Preparation and Papain immobilization

Films were prepared by mixing of 1% pectin solution with and without glycerin and PVA, as well as papain. The pectin was dissolved completely by adding it slowly to 20 mL of distilled water o plasticizer solution, using an agitation of 150 rpm for 1–2 h. The plasticizer (glycerin or PVA) was dissolved prior to pectin addition. The PVA solution was prepared by means of agitation (150 rpm) and by heating at 90°C. The final plasticizer concentrations were 0.25, 0.5 and 0.75% (*w*/*w*).

Papain immobilization by the entrapment method was performed in the presence or absence of 0.25 % of PVA (*w*/*v*) or 0.75% of glycerin (*v*/*v*). To immobilize, 20 mg of papain was dissolved on 20 ml of pectin solution with and without plasticizers. The final enzyme concentration was 1 mg/ml. The mixtures were distributed on a surface of a plastic plate. Twenty ml of solution completely covered the surface of plastic plate. The content was air-dried for 24 h at room temperature. The film was easily peeled from plastic plate. Reproducibility was attained by using fixed volumes for a uniform distributing environment.

Mechanical testing

Test samples were cut with razor blade to 10×70 mm dimensions. Sample thickness was measured on a micrometer of Instron Universal Testing System (USA). For each of the prepared films, the "% strain at break" related with the material elongation, "load at maximum load" (Kg) as resistance to tensile strength were measured in an Instron Universal Testing System (USA) according method 31, ASTM D 882. The crosshead speed was 50 mm/min. Data were collected at a rate of 10 points/s, at least five samples for each film were tested, and data were averaged.

Activity and stability measurements

The hydrolytic activity of free and immobilized papain were determined using spectrophotometrical technique for the caseinolytic measurements [9]. The reaction mixture consisted of 2 ml of 0.01 M phosphate buffer (PBS) at pH 7.0 and 1.0 ml of free enzyme solution or 4 mg of the immobilized enzyme suspended in 1 ml of 0.05 M PBS. The reaction was started by addition of 1.0 ml of 2.0% (*w*/*w*) casein solution. The reaction mixtures were stirred vigorously at 37°C for 30 min followed by addition of trichloroacetic acid to a final concentration of 3.0%. The absorbance of the centrifuged solution was measured at 280 nm. In all experiments, pectin without papain was used as control.

The activity was measured immediately after film preparations and after 30 and 180 days of their storage at 4°C.

Skin Lesion Treatment with Pectin-Papain films

Pectin-papain films were applied for the treatment of foot skin lesions of 22 neuropathic and angiopathic ulcers and one second degree burn. The 23 voluntary patients (17 diabetic patients and 6 old patients) were chosen randomly. They represented different profiles in age, gender, type-, deepness- of injury, and the time of lesion appearance with and without previous treatment. All of them were submitted to the same pectin/papain treatment for two times a week. However, the duration of treatment was variable depending on its clinical evaluation, related

to initial characteristics of lesion. All patients were submitted to antimicrobial treatment with antibiotics administered orally or intramuscularly. Moreover, all patients were submitted to a specific therapy of their chronic illness, *i.e.* hypoglycemic pellets, insulin injections, hemorheologic treatments, hypotensors, etc. The clinical evolution of wound healing was monitored by visual observation of healing processes to define the effect of applied treatment on different type of lesions and on repair process visualized by lesion characteristics. The photographs were taken to monitor and archive the evidence on the efficiency of the treatment.

RESULTS AND DISCUSSION

The enzyme application for wound treatment requires alternative immobilization techniques. Films made from natural polymers may be acceptable for pharmaceutical applications [3]. The film obtained from pectin can easily be applied to a skin wound and can be removed by washing (due to pectin solubility in water) without alteration of tissue that sometimes take place in the case of solid materials, for example gauze. However, the mechanical properties of pectin films may be one of the problems for their commercial application.

In the present study pectin-papain films were prepared according to the previously described method [3]. The films elaborated using 1% pectin solution with and without glycerin were transparent, while the films containing papain and PVA were opaque.

The mechanical tests demonstrated that the presence of papain influenced the mechanical properties of the films. There was no significant change in the thickness of pectin film with and without papain. In both cases, it was approximately of 0.04 mm. However, the measured values obtained on pectin/papain film were more variable in comparison with films with papain alone (Fig. 1). It indicated that the papain addition led to the change on homogeneity of the obtained films and there was greater variability in the mechanical properties.

The results observed in the Fig. 2 demonstrated that the pectin/papain films showed a decrease in the mechanical properties. Pectin films recorded a resistance of approximately 0.45 Kg, while the papain addition decreased it to 0.27 Kg. It meant that the pectin/papain films were more fragile than pectin films, and also was difficult to manipulate. Nevertheless, the elongation of films to break expressed in "% strain at break" (Fig. 3) was not affected by the papain addition. The per cent strain at break of the pectin and pectin/papain films were 9.03 and 8.32%, respectively, and the difference was not statistically significant. The results showed that the papain interaction with the

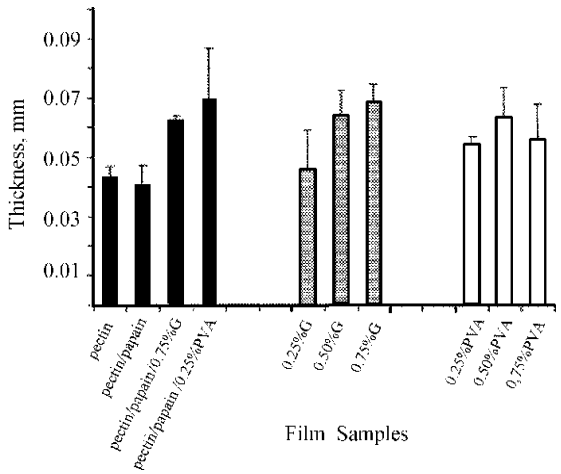

Fig. 1. Thickness of different pectin films: pectin – films prepared with pectin alone; pectin/papain – pectin films containing papain; pectin/papain/0.75%G – pectin films with papain prepared with 0.75% glycerin (v/v); pectin/papain/0.25%PVA – pectin films with papain prepared with 0.25% PVA (w/v); 0.25%G – pectin films prepared with 0.25% glycerin (v/v); 0.50%G – pectin films prepared with 0.50% glycerin (v/v); 0.75%G – pectin films prepared with 0.75% glycerin (v/v); 0.25%PVA – pectin films prepared with 0.25% PVA (w/v); 0.50%PVA – pectin films prepared with 0.50% PVA (w/v); 0.75% PVA – pectin films prepared with 0.75% PVA (w/v)

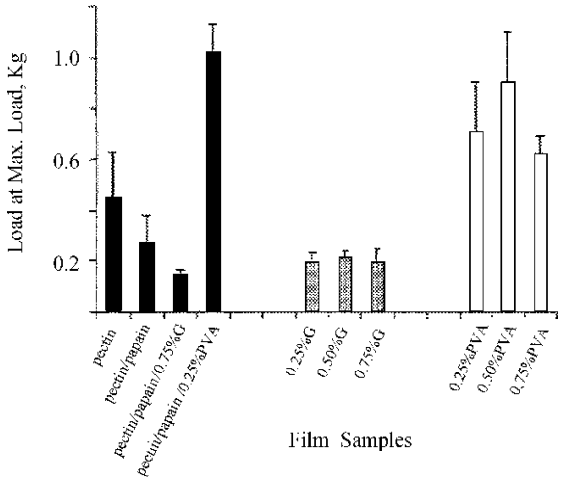

Fig. 2. Tensile strength measured for different pectin films: pectin – films prepared with pectin alone; pectin/papain – pectin films containing papain; pectin/papain/0.75%G – pectin films with papain prepared with 0.75% glycerin (v/v); pectin/papain/0.25%PVA – pectin films with papain prepared with 0.25% PVA (w/v); 0.25%G – pectin films prepared with 0.25% glycerin (v/v); 0.50%G – pectin films prepared with 0.50% glycerin (v/v); 0.75%G – pectin films prepared with 0.75% glycerin (v/v); 0.25%PVA – pectin films prepared with 0.25% PVA (w/v); 0.50%PVA – pectin films prepared with 0.50% PVA (w/v); 0.75%PVA – pectin films prepared with 0.75% PVA (w/v)

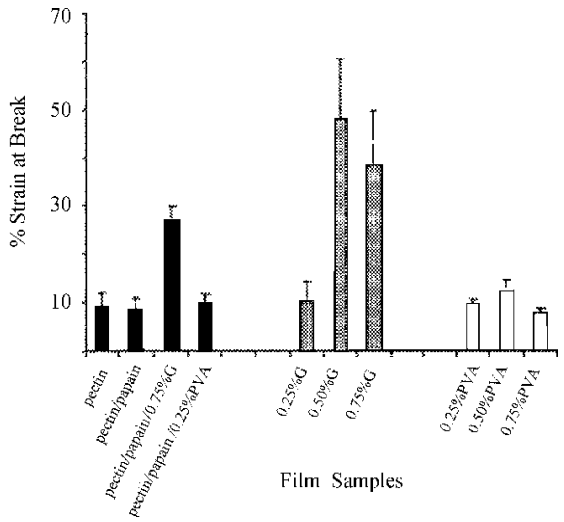

Fig. 3. Per cent of strain at break measured for different pectin films: pectin – films prepared with pectin alone; pectin/papain -pectin films containing papain; pectin/papain/0.75%G - pectin films with papain prepared with 0.75% glycerin (v/v); pectin/papain/0.25%PVA - pectin films with papain prepared with 0.25% PVA (w/v); 0.25%G- pectin films prepared with 0.25% glycerin (v/v); 0.50%G- pectin films prepared with 0.50% glycerin (v/v); 0.75%G- pectin films prepared with 0.75% glycerin (v/v); 0.25%PVA- pectin films prepared with 0.25% PVA (w/v); 0.50%PVA- pectin films prepared with 0.50% PVA (w/v); 0.75%PVA- pectin films prepared with 0.75% PVA (w/v)

pectin matrix led to the change on tensile strength of films but not their elongation.

Due to the fact that the presence of papain diminished the mechanical properties of the films, some tests were realized using glycerin and PVA as plasticizers [6, 14].

Initially, the effect of different concentrations of plasticizers on mechanical properties of maracuya pectin films was defined in order to demonstrate that these compounds may improve the properties of obtained films.

The addition of more than 0.25% glycerin increased the film thickness from 0.04 mm to 0.06 mm (Fig. 1). Moreover, more flexible pectin films were obtained. Increasing the glycerin from 0.25 to 0.75% considerably increased the % of elongation at break, from 9 or 10% of elongation in pectin films and pectin/0.25% glycerin increased it up to 38–47%. A difference in % elongation at break of pectin/0.5% glycerin and pectin/0.075% glycerin was observed (Fig. 3) but was not significant statistically.

Nevertheless, the tensile strange of pectin films was decreased by the glycerin addition. In the presence of all glycerin concentrations the resistance of pectin/glycerin films was about 0. 2 Kg, which was at 2 fold lower than pectin films alone (Fig. 2).

Due to the increase of the % elongation at break favored the film storage and its application in the wounds, 0.75% glycerin was selected for the preparation of films with papain.

The response of pectin/papain films to the presence of glycerin was similar to results observed with glycerin and pectin alone. The thickness and flexibility of films was increased (Figs 1 and 3), whereas the tensile strength was decreased in comparison with pectin and pectin/papain films. This showed that while the presence of glycerin improved one of the mechanical properties, worsened the other. The glycerin increased the flexibility by two probable modes of action [14]. It probably dissociated stiff networks of pectin held together by polymer-polymer interactions, which aided the movement of dissociated pectin chains by lubricating the passage between chains under stress and that could be the reason for the decrease in tensile strength. The papain interactions with pectin matrix influenced mechanism of action of glycerin, since the flexibility in the presence of enzyme is lower than in its absence.

When PVA was applied as plasticizer, the thickness (Fig. 1) and tensile strength of pectin films (Fig. 2) increased without considerable effect on elongation at break (Fig. 3). The tensile strength for films obtained by using of 0.25% and 0.50% PVA were similar (Fig. 2), but decreased for higher PVA concentrations. A similar response was observed for % elongation at break with application of different concentrations of PVA.

The addition of 0.25% PVA on papain/pectin blend increased the tensile strength and thickness of obtained films without an effect on elongation. The tensile resistance of pectin/papain/0.25% PVA films was increased by 2.26 and 3.78 fold in comparison with pectin and pectin/papain films, respectively. It was slightly higher than the PVA/pectin samples. The PVA chains were more mobile than the unplasticized pectin chains, and allowed easy molecular rearrangement [6]. The papain interaction with pectin matrix improved the PVA effect.

Besides the mechanical properties of the pectin/papain films formed with and without plasticizers, for enzyme application to the treatment of skin wounds, the enzyme activity and storage stability is also important. Table 1 showed that free papain is very unstable and totally lost its activity after 7 days of storage at 4°C. The storage stability under the same conditions in presence and absence of plasticizers were higher and variable

Table 1

Activity and storage stability of different papain preparations

Preparation	Storage time, days	Activity	Relative activity, %
Papain solution		Abs/min	
	0	0.022+/–0.004	100
	3	0.0022+/–0.001	9.9
	7	0.000+/–0.000	0.0
Papain/pectin films		Abs/ (min x g of film)	
	0	6.409+/–0.628	100
	30	6.345+/–0.32	99
	180	6.28+/–1.696	98
Papain/pectin/0.75%		Abs/ (min x g of film)	
glycerin films	0	3.974+/–0.168	100
	30	3.925+/–0.175	98
	180	3.568+/–1.013	89
Papain/pectin/0.25% PVA		Abs/ (min x g of film)	
films	0	6.585+/–0.657	100
	30	1.904+/–0.13	29
	180	1.097+/–0.411	16

(Table 1) for different preparations. The enzyme immobilized in the pectin film preserved its activity up to 98% after 30 and 180 days of storage. Papain immobilized in pectin/glycerin films lost 38% of activity after its immobilization in comparison with the enzyme on pectin films. However, it was stable during its storage at 4°C and maintaining an activity of 98 and 89% after 30 and 180 days of storage, respectively. In contrast, papain in PVA/pectin films did not loss the activity after immobilization in comparison with papain/pectin samples but it was more unstable during its storage at 4°C. After 30 days of storage, the enzyme activity was only 29% and after 180 days it lost 84% of its activity.

It can be assumed that the improvement of mechanical properties by plasticizers application conduced to the change on papain activity and stability. The PVA application increased the tensile resistance of films but led to the lower stability. The glycerin improved the film flexibility at the cost of enzyme activity. The interactions with the added compounds are

Table 2

Characteristics of skin lesion until and after 1 week of treatment with papain/pectin films applied on different types of wounds

Wound characteristic	Edema	Erythema	Fibrin	Phlebitis / Infection	Necrosis	Granulation tissue forming
Initial	++	++	+	++	–	–
After 1 week of treatment	–	–	–	–	–	+++

"+" – the presence of property in qualitative increasing scale, "–" – its absence.

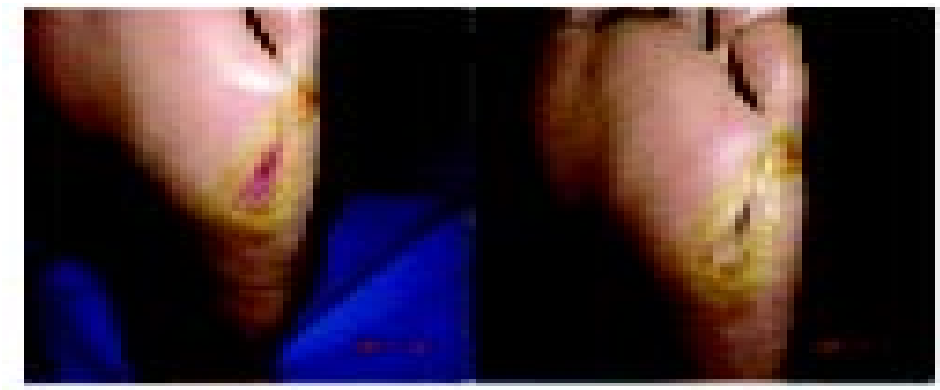

Fig. 4. The effect of pectin/papain films on the treatment of neuropathic lesion of human skin: left – after first film application; right – after 1 week of 2 applications

responsible to the observed effect, which must be taken in to account for the preparation and application of immobilized enzyme samples. Based on the results so far, the next assays were carried out with the papain/pectin preparations.

Previous tests on laboratory animals [3], demonstrated that the pectin/papain film application decreased the time needed for repair of surgical wound by approximately 2-fold in comparison with wounds treated with pectin film alone without enzyme and control (untreated). In the present study the enzymatic treatment was performed on voluntary patients.

The films easily adhered to lesions and were eliminated with water washing. The first observation was that the pectin/papain treatment did not show any negative secondary effects, such as allergic and inflammatory symptoms, microbiological contamination, *etc* on any patients.

Table 2 shows significant positive changes characterized wound repair process after 1 week of treatment application, independent of type of wound and patient profile (Fig. 4). Moreover, the healing time was reduced significantly from 6–8 weeks without treatment to 1–3 weeks for all studied cases. Thus, it was demonstrated that independent of the type and profundity of skin wound the treatment with papain/pectin films led to an accelerated repair of skin wounds without any negative secondary effects.

The results of the present study demonstrated that pectin of passion fruit can be used for the development of new biopolymer materials, which can be used for immobilization of enzymes for treatment of human skin wounds. The mechanical properties of pectin film material were improved by addition of plasticizers such as glycerin and PVA. However, the activity and stability of the immobilized enzymes were modified due to the interaction of pectin matrix with added plasticizers.

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EVALUATION OF THE EFFECT OF PECTIN-PAPAIN INTERACTIONS ON THE ENZYME STABILITY AND MECHANICAL PROPERTIES OF MARACUYA'S PECTIN FILMS FOR THE TREATMENT OF SKIN WOUNDS

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Pectin is a biodegradable and water-soluble polymer. In this work, the pectin was obtained from residues of the maracuya (passion fruit) juice industry. It was used as support for protease (papain) immobilization to obtain the films suitable for treatment of skin wounds. In the present study the effects of pectin-papain interactions on enzyme stability and mechanical properties in the presence and absence of plasticizers such as glycerin and polyvinyl alcohol (PVA) were evaluated. It was demonstrated that the addition of glycerin and polyvinyl alcohol improved the mechanical properties of the films, glycerin increased the flexibility and the PVA increased the tensile strength of pectin films. Further, the plasticizers application conduced to the change on papain activity and stability. Moreover, in assays performed on the voluntary patients with the treatment of skin wounds with papain/pectin films it was demonstrated that there was acclerated healing of the wound with out any negative secondary effects, independent of the type and profundity of the wound.