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ENTRAPMENT OF ENZYMES IN NATURAL POLYMER EXTRACTED FROM RESIDUE OF FOOD INDUSTRY: PREPARATION METHODS, PARTIAL CHARACTERISATION AND POSSIBLE APPLICATION

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Method for pectin extraction from residue of passion fruit (*Passiflora edulis*, maracuya) from juice industry was developed. By means of viscosity measurements molecular weight of obtained biopolymer was determined. Using 1 and 2% solutions, this natural polymer was applied for preparation of films. The mechanical test was done to characterize the resistance of these membranes to puncture. The measured force was 101 g/mm. The tests performed on CD1 mice demonstrated that subcutaneous application of 1 and 2 % pectin solution and application of obtained films in linear surgical wounds did not cause inflammation or other negative secondary effects. Therefore, it was proposed to use this polymer like support for papain immobilization. The enzymes were active after their immobilization and after more than 6 months of storage at 4°C.

Biopolymers are increasingly being studied and used for applications in which synthetic polymers have traditionally been the materials of choice. The impetus for the heightened interest in this new technology is the need to increase the use of biodegradable and recyclable materials to limit the volume of material sent to landfills, and the desire to use renewable raw materials sources instead of nonrenewable petroleum sources [1].

Pectin is the one of the major cell wall structural polysaccharides of higher plant cells, belongs to a family of heterogeneous branched polysaccharides consisting mostly of variably methylated galacturonan segments separated by rhamnose residues [2]. Polysaccharide pectin found in plant cell walls is biodegradable [3]. Isolation of pectin from plant cell walls is achieved by breaking up the gel structure, usually stabilized by calcium cations, to solubilize large aggregates of pectin [3]. Pectin has always been a natural constituent of human foods, its use is allowed in all countries of the world; pectin is used in a number of foods as gelling agent, thickener, texturizer, emulsifier and stabilizer. In recent years, pectin has been used as a fat or sugar replaced in low-calorie foods [4].

Accurate determination of molecular weights is difficult, partly because of the extreme heterogeneity of commercial pectin samples, and partly because of the tendency of pectin molecules in solution to aggregate. Pectin molecular weight can be expressed either as a weight average or a number average value. Owens et al. [5], using viscometry and osmometry, carefully and systematically studied molecular weights and molecular weight distribution of pectins. They reported molecular weights varying from 20,000 to 300,000, depending on the preparation procedure [5–7]. More interestingly, they always found a substantial difference between M_n (from osmometry) and M_w (from viscometry), indicating a high degree of polydispersity. Berth et al. [8] studied a series of pectin preparations made from partial depolymerization by methanolysis of original pectin.

Passion fruit-maracuya (*Passiflora edulis*) processing produces large quantities of waste in the form of rind and seed, that creates a disposal problem. Studies on transformation 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, dietetic fiber, pectolytic enzyme production and pectin extraction [3, 9, 10].

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. Citric pectin films were first made and characterized [1, 11]. These were made with low methoxy pectin and required the use of calcium or other multivalent cations as crosslinking agents. These materials exhibited fair mechanical properties, but had poor folding endurance. Previous work has shown that elasticized films made from high methoxy lime pectin and high amylose starch have very good mechanical properties. The use of glycerin or other suitable elasticizer is necessary to make sufficiently flexible and nonbrittle film [1, 11].

Since the recovery yield and the reusability of free enzymes as industrial catalysts are quite limited, attention has been paid to enzyme immobilization, that offers advantages over free enzymes in choice of batch or continuous

processes, rapid termination of reactions, controlled product formation, ease of enzyme removal from the reaction mixture and adaptability to various engineering design. Furthermore, the interest in immobilized enzymes and their application to bioprocessing, analytical systems and enzyme therapy has steadily grown in the past decade [3, 12]. Gel entrapment method is attractive because it is very simple, can be carried out under soft conditions (physiological, pH and temperature) and also allows variation in polymer matrix composition and structure [13]. In this study, papin is selected as a hydrolytic enzyme and the support employed is porous pectin.

In this paper we report on: 1) development of method for pectin extraction from residue of passion fruit (*Passiflora edulis*, maracuya); 2) partial characterization of obtained pectin; 3) preparation of pectin membranes and determination of their mechanical properties; 4) studying of effect of pectin membrane application on wound healing in a mouse model.

MATERIALS AND METHODS

Method of pectin extraction. Pectin was obtained from passion fruit (*Pasiflora edulis*) acquired from the city of Puebla (Mexico). They were washed and latern cut manually in progressive form, separating first the flavedo and then the albedo. The albedo was treated with steam during 15 minutes, after the process, the remainders were washed separately with distilled water twice in a ratio of 1:2 (m/v). The remainders were dehydrated first by ethanol (2x) and then by acetone (1x). The obtained dehydrated material was powdered for 3 minutes, then it was packed in plastic containers.

Determination of the molecular weight of pectin. About 90 g of a 1% sodium hexameta phosphate (MHP) solution (pH 4.5) in a 250 ml breaker was warmed to 40–45°C and added with 100 mg of pectin. Pectin was completely dissolved by stirring, cooled and MHP was added to complete 100 g. This solution was filtered through 0.45 micrometer membrane (Swinnex-25). The viscosity was measured with Oswald tube at 25°C. The molecular weight (M.W.) was calculated as described previously, using the following equation [14]:

$$\text{M.W.} = 1.277\times10^{6}(\text{ur}^{1/6} - 1)$$

The distribution of particle size and galacturonic acid content was determined as described early [14].

Film Preparation. Pectin (1–2 g) was added to 10 ml of water with rapid stirring until it was dissolved completely. Then, the viscous solution was poured directly into a plastic petri dish and air-dried for 24 h at room temperature.

Each film was easily peeled from plastic dish, a 10 ml volume of polysaccharide solution was finally taken to completely cover the plastic dish, good reproducibility was attained by using fixed volumes for a uniform casting environment.

Mechanical testing. Dynamics tests were performed with Instron (Model 4301) fitted with cylinder (diameter = 3.25 mm, height = 35 mm). The conditions of measurement were carried out at a speed of pre-test 25.4 mm/min Method 31. Filmstrips were excised from the center of the circular films with a razor blade. Sample thickness was measured with a Model 3 Dial Comp. Micrometer.

Immobilization of papain. Papain was immobilized on the pectin by the entrapment method. Pectin (1–2 g) was added to rapidly stirred water (10 ml) and to the resultant solution, 2 ml of ice-cold 0.05 M PBS containing 3 mg/ml of papain was added. The mixture was shaken gently. Then, the procedures described above were performed. The activity of immobilized papain was determined by UV measurement (280 nm) after hydrolysis of casein solution. In all experiments, pectin without immobilized papain was used as control in determination of the activity of the immobilized papain.

Activity measurements. The hydrolytic activity of free and immobilized papain were determined using the caseinolytic determinations [15]. The reaction mixture consisted of 2 ml 0.01 M PBS at pH 8.0 and 1.0 ml free enzyme solution or the immobilized enzyme suspension in 0.05 M PBS, which contained 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 concentration of 3.0 %. The absorbance of the centrifuged solution was detected at 280 nm.

Effect of pectin on wound healing in a mouse model. Subcutaneous injections of pectin solution (1% and 2%) were performed on CD1 mice to observe the organism reaction on pectin introduction. Moreover, the effect of pectin and immobilized papain on tissue repair was tested in mice. All experiments were carried out using aseptic technique with regard to anesthesia and surgery [13]. Pectin films (1% and 2%) with and without papain were put on back lineal wound (1 cm or 2 cm) of experimental mice just after wounding. Control wound was performed in the same mouse and had no films (on the assay with pectin), or had pectin film as blank polymer (without papain) in assay with immobilized preparations. The healing of the wounds was determined by periodical measurement of the linear size of the wounds.

RESULTS AND DISCUSSION

The fiber yield of pectin of passion fruit obtained by the developed method, was of 68 g dry base/kg of fruit. The color of the fiber was pale yellow. The distribution of size of particle showed a greater frequency of retention in 25 mesh and the galacturonic acid content as pectin index was 29.6. The obtained pectin was soluble in water. The pectins obtained by other methods, for example by the treatment with acids, are only partially soluble in water [14].

Molecular weight of pectin was determined by the

method of viscometry described by Grassin [14]. Pectin obtained in this study has a molecular weight of $1{,}07\times10^5$ Da, which is within the average of the values described by Owens [5, 6]. He reported molecular weights between 20.000 and 300.000 Da determined by the methods of viscometry and osmometry for pectin obtained by different methods from other types of fruit.

The pectin membranes were elaborated, according to the previously described method and some mechanical tests were made. Due to their rigid structure, it was not possible to use the obtained membranes in the determination of resistance to mechanical force by stretching. Hence a test of puncture was performed. The results of puncture test in membranes using 1 and 2% pectin solution and membranes of pectin-papain are presented in the Table 1. It was observed that papain decreased the mechanical resistance of membranes (Table 1). Hence, glycerol at 0.2%, 0.5% and 0.7% was used as a plasticizer [1] for pectin films to improve the mechanical properties of membranes. Using 0.7% of glycerol, more flexible membranes were obtained and their brittleness were effectively reduced.

Taking into account that the objective of the study is to obtain new materials from wastes of the juice industry, to propose the possible application of these materials, the experiments with mice were performed. In the assay with subcutaneous injection of pectin solutions, it was observed that there was no injurious effect on the mice.

Table 1

Tensile properties of pectin films

Film type	Thickness (μm)	Maximum load (g/ μm)
Pectin 1% /papain	54	108
Pectin 2%/papain	89	135.8
Pectin 1%	54	212.1
Pectin 2%	74	214.8

Table 2

The effect of pectin films on healing of skin incisional wounds in a mouse model

Films	The 5th day	The 10th day	The 15th day
	Relative wound size,%	Relative wound size,%	Relative wound size,%
Control (without the film)	95	90	90
Pectin 1%	70	60	40
Pectin 2%	85	60	45

*An initial wound size was taken as 100%.

Table 3

The effect of papain entrapped in pectin films on healing of skin incisional wounds in a mouse model

Films	The 2nd day	The 4th day	The 6th day
	Relative wound size,%	Relative wound size, %	Relative wound size, %
Pectin 1%	65	60	50
Pectin - papain	50	45	40

* An initial wound size was taken as 100%.

Table 4

The relative activity (RA) of papain during its immobilization and storage

Enzyme preparation	Abs/ μg . min	Relative activity %
Papain	17	100
Pectin 1%/papain initial	14.5	85
Pectin 1%/ papain/glycerol initial	15	88
Pectin 1% / papain after 6 month of storage	15	88
Pectin 1% / papain/ glycerol after 6 month of storage	13.5	79

The membranes of pectin 1% and 2% were applied on back lineal wound followed by the process of healing for a period of 15 days. As the control the wound in the same animal but without film was used. It was observed that the membrane application did not present any secondary indirect effect on the wound healing. Further, the healing was partially improved in the treatment carried out with the membranes then in the control without membrane. The obtained results are presented in the Table 2. It was observed that the wounds with pectin membranes showed quicker healing process than the control without membrane (Table 2).

It was also observed that pectin does not have a negative effect on the mice.

The similar experiment was performed with papain immobilized in pectin membranes using as control the pectin films (blank polymer without papain). In this assay the wound was healing in 10 days. The Table 3 demonstrates that immobilized papain contributed in accelerating of wound healing in the mice.

The Table 4 summarizes the experimental results, which shows that the immobilization of the papain by entrapment method was not affected the enzymatic activity.

The storage stability of immobilized preparations of papain was also evaluated. It was observed (Table 4) that

papain entrapped in pectin could be stored for six months without loss of activity.

The results of this study demonstrate that residues of passion fruit is useful for obtaining the natural polymer that can has potential in the development of new materials for skin injury treatment base on entrapped enzymes.

New technology will be 100% Mexican since it is based on the use of the regional and national materials, taking advantage of the wastes generated by the nutritional industry.

Apart from the use of waste as a resource, this also forms mechanism for waste management.

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ВКЛЮЧЕНИЕ ФЕРМЕНТОВ В ПРИРОДНЫЙ ПОЛИМЕР, ПОЛУЧЕННЫЙ ИЗ ОТХОДОВ ПИЩЕВОЙ ПРОМЫШЛЕННОСТИ: МЕТОДЫ ПОЛУЧЕНИЯ, НЕКОТОРЫЕ СВОЙСТВА И ВОЗМОЖНОЕ ИСПОЛЬЗОВАНИЕ

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Разработан метод выделения пектина из отходов, получаемых после промышленной экстракции сока из плодов маракуйа (*Passiflora edulis*). По вязкости определен молекулярный вес выделенного биополимера. Растворы пектина (1 и 2%) использованы для получения мембран. Прочность мембран измерена с помощью механического теста. Разрыв мембран наблюдали при силе 101 г/мм. В экспериментах на мышах линии СД1 показано, что подкожное введение растворов пектина (1 и 2%) и наложение мембран на линейные хирургические раны не вызывало воспалительных процессов и каких-либо других отрицательных побочных эффектов. Предложено использовать полученный полимер в качестве носителя для иммобилизации папаина, иммобилизация которого не сопровождалась потерей его активности. Иммобилизованная протеаза сохраняла активность в течение более 6 мес. хранения препарата при 4°С.