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Stability and activity of bovine prostaglandin H synthase immobilized on *Opuntia imbricata* (coyonoxtle)

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Abstract

An immobilization method for prostaglandin H synthase (PGH-synthase, EC 1.14.99.1) from microsomes of bovine vesicular glands on *Opuntia imbricata* was developed. Study of sorption kinetics showed that the protein sorbed on periodate activated and non-activated support was 45% and 38%, respectively, after 1 h, while after 24 h, it was 68% and 71% of applied protein. The immobilized enzymes retained around 30–40% of initial PGH-synthase activity. The effect of support on enzyme ability to catalyze the synthesis of prostaglandin E_2 was observed and compared with cyclooxygenase and cyclooxygenase plus peroxidase reaction, which was detected using electrochemical method and spectrophotometry. Immobilized microsomes were able to catalyze several cycles of arachidonic acid transformation and they were more stable than free enzyme solution upon storage at 4 °C. The activation of the support by means of treatment with periodate showed positive effects on the activity and storage stability of immobilized enzyme. Further, the enzyme immobilization on *Opuntia imbricata* can be performed by physical adsorption as well as by the chemical attachment on carrier activated by periodate. The differences in the structure of periodate activated and non-activated supports after microsome immobilization were studied using electron microscope.

Keywords: Prostaglandin H synthase; Cactus *Opuntia imbricata*; Covalent and non-covalent immobilization; Enzyme stability

1. Introduction

Opuntia imbricata is a cactus, also known as coyonoxtle and is considered as invasive plant in the North of Mexico (Fig. 1). The dry coyonoxtle trunk is composed of (wt.%): hemicellulose (28.7 ± 6.3), cellulose (34.0 ± 5.0) and (the most abundant fraction) lignin (37.6 ± 6.3) [1]. In our earlier study, we demonstrated fungal penicillin acylase immobilization on coyonoxtle particles with high yield and activity [1]. This support was also tested to develop biofilm reactor systems useful for the treatment of different wastewaters [2–4]. The operational and chemical stability, resistance to hydraulic pressure as well as to microbial attacks have been demonstrated [2–4]. Contrary to some synthetic organic or inorganic polymer materials such as resins, gels and fibers conventionally used for the enzymes immobilization, the coyonoxtle is renewable, natural, solid carrier [1–4] with high grade of reusability and without disposal problems [3,4]. These characters were considered important to test this natural material as a support for immobilization of prostaglandin H synthase, an enzyme from animal source used for prostaglandin biosynthesis.

The prostaglandin H synthase (PGH-synthase, EC 1.14.99.1) is a multisubstrate enzyme exhibiting high cyclooxygenase and peroxidase activities, which can be measured together or independently. It is a key enzyme in the biosynthesis of prostanoids (physiological active substances) from polyunsaturated fatty acids [5–7]. It catalyses the first step in biosynthesis of various prostaglandins, thromboxanes and prostacyclines by converting arachidonic acid to prostaglandin H_2 (PGH_2) in the cells of almost all mammalian organs. Despite considerable progress in gene engineering of enzymes of prostaglandin (PG) synthesis, the biosynthesis of PG by means of natural enzymes remains the main method for the preparation of these compounds.

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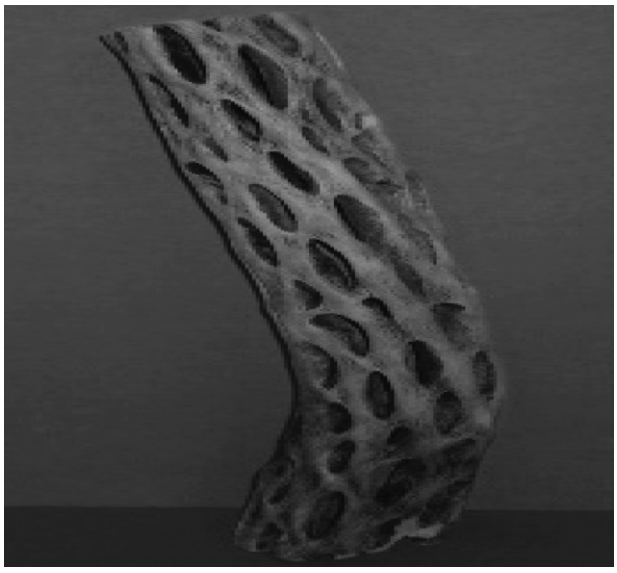

Fig. 1. Dry *Opuntia imbricata* trunk before its treatment as a support for enzyme immobilization.

Prostaglandin H synthase is easily inactivated both during enzyme reaction and upon storage [5,8]. The most extensive data on the mechanism of PGH-synthase action and substrate-induced inactivation during the course of the reaction had been described in the monograph by Varfolomeyev and Mevkh [9], some reviews [5,10] and other publications [8,11,12].

It had been reported that the enzyme can be partially stabilized by its immobilization on DEAE-Sephadex [13], *n*-alkyl and aryl amino-agar gels [14] and silica gel [15–17] by adsorption, as well as by entrapment on pectin gel films [18]. The most excellent results of enzyme stabilization on various cycles of PGE_2 synthesis and upon its storage at 4 °C were obtained with silica gel [16,17]. In this system, the effect of adrenaline addition applied to stabilize the enzyme during its storage at 4 °C also was reported [16].

The immobilization of microsomes by entrapment on polyacrylamide gel or covalent binding by glutaraldehyde method [13] as well as on solid supports like alumina G and controlled pore glass [15] were not effective.

So far very few reports have been published about successful PGH-synthase immobilization [13–18]. Moreover, only with gel carriers good results were obtained, while the solid supports are practically not mentioned and rejected. In fact, previous to the present research the use of solid natural support as *Opuntia imbricata* with interesting operational properties [1–4] had been demonstrated for various biotechnological systems, but not for PGH-synthase immobilization.

The goals of this work were: (1) to develop an immobilization method for PGH-synthase from bovine vesicular glands on solid natural support *Opuntia imbricata*; (2) to evaluate the storage stability and reusability of immobilized enzyme by means of activity measurement using different methods; (3) to evaluate the effect of adrenaline addition on enzyme stability upon storage at 4 °C.

2. Experimental

2.1. Pretreatment of the support

The coyonoxtle trunk was cut to pieces of approximate 0.5 cm × 0.5 cm × 0.5 cm dimensions. Eighty grams of coyonoxtle pieces were hydrolyzed with 250 cm^3 of 2% HCl (ALQUIME, Mexico) aqueous solution under reflux condition for 3 h to eliminate hemicellulose and other soluble fractions, washed three times in boiling distilled water for 15 min and then dried at 60 °C for 24 h [1].

Samples of treated and untreated support were analyzed by scanning electron microscopy (SEM, Philips XL30 ESEM operated at 20 kV) to characterize the structure of both samples.

2.2. Support activation

For activation of the support, 2 g of coyonoxtle was treated with 10 cm^3 of 0.1 M $NaIO_4$ (Sigma–Aldrich, USA) aqueous solution at pH 2 [1]. The activation was carried out under agitation at 250 rpm for 1 h in the dark. Finally, the activated support was washed three times with 20 cm^3 of distilled water and than with 0.05 M phosphate buffer (PBS) at pH 8, and immediately used for enzyme immobilization.

2.3. Enzyme preparation and immobilization

Microsomal fraction containing PGH-synthase was isolated from bovine vesicular glands according to the method described previously [8,12,19], using calcium ions to precipitate microsomes and subsequently by centrifugation at 10,000 × *g*. The reagents used in this study were of analytical grade: Trizma, diethyldithiocarbamate sodium salt (DEDTC), Tween-20, $CaCl_2$, HCl (all of Sigma–Aldrich, USA) and EDTA disodium salt (Baker Analyzed, USA). Precipitated microsomes were suspended in 0.05 M PBS (aqueous solution, pH 8) and used for immobilization as well as for evaluation of enzyme activity and storage stability by means of three techniques.

Enzyme was immobilized on both periodate activated and non-activated coyonoxtle supports in order to compare the physical sorption and chemical attachment.

Kinetics of the protein sorption on activated and non-activated supports were defined using microsome solution containing 0.93 mg/cm^3 of protein. Approximately 2 g of *Opuntia imbricata* was put in 10 cm^3 of microsome aqueous suspension and incubated for 24 h under agitation at 250 rpm at 4 °C. The samples (0.1 cm^3) were drawn from the flasks at every 2 h, after an initial sampling at 10 min. The protein concentration was determined by employing Bradford method. As a control, the same procedure was carried out with microsome aqueous suspension in the absence of *Opuntia imbricata*.

The amount of protein bound after 1 and 24 h of incubation was determined by calculating the protein balance. Microsome aqueous suspensions (10 cm^3) containing 1.4–5.8 mg and 2.1–9.9 mg of protein were added to activated or non-activated *Opuntia imbricata* (approximately 2 g in each flask) and the supports were incubated for 1 h or 24 h at 4 °C, respectively. At

the end of incubation period, the supports were separated, twice washed with 10 cm^3 of 0.05 M PBS (aqueous solution, pH 8). The initial protein concentration, as well in the supernatant and in two samples obtained after washing of the supports with PBS, was determined by Bradford method, using BSA as a standard. Protein balance was quantified to define the amount of bound protein. The fraction of sorbed protein was estimated as the tangent of the slope obtained in the coordinates: bound protein vs. added protein values.

For immobilization of the enzyme, 10 cm^3 of microsome suspensions with a protein concentration at 0.24 and 0.48 mg/cm^3 were added to 2 g of activated and non-activated support, and incubated at 4 °C for 24 h. The enzyme activity was assayed in the initial samples, after immobilization for various cycles and after storage in PBS (0.05 M aqueous solution, pH 8) at 4 °C for 3 weeks in the presence or absence of electron donor (0.15 cm^3 of 100 mM adrenaline aqueous solution in 10 cm^3 of PBS).

Samples of enzyme immobilized on activated and non-activated supports were studied by scanning electron microscopy in order to compare their structures.

2.4. PGH-synthase activity assays

The activity of soluble and immobilized PGH-synthase was determined by arachidonic acid transformation, using three different techniques, *viz*., (1) initial rate of oxygen consumption in cyclooxygenase reaction [18]; (2) initial accumulation rate of the oxidized form of electron donor (adrenaline) in the cyclooxygenase plus peroxidase reaction [8,12,19]; (3) the yield of PGE_2, which is formed during spontaneous decomposition of PGH_2 (45 min of reaction [16,20]). The initial rates of reaction were determined as the tangent of the slope of the initial part of the corresponding kinetic curves. The phosphate salts, arachidonic acid, L-adrenaline, hemine and ethanol used in the assay were of analytical grade and purchased from Sigma–Aldrich (USA).

The enzyme activity was measured at 25 °C by the three techniques at the same time for the same reaction mixture that contained 10 cm^3 of 50 mM Tris–HCl buffer (aqueous solution, pH 8), 0.15 cm^3 of 100 mM adrenaline aqueous solution, 0.15 cm^3 of 0.02 mM hemine aqueous solution, 0.15 cm^3 of 15 mM arachidonic acid ethanol solution. Enzymatic reaction was initiated by arachidonic acid addition. In the case of soluble enzyme, the reaction mixture contained 0.15 cm^3 of microsome suspension, while 2 g of support with immobilized enzyme was used for determining the activity of immobilized preparations.

Oxygen consumption was continuously monitored [18] using a portable oxygen meter (Model HI 964400, Hanna Instruments, USA). Optical absorbance of oxidized form of adrenaline ($\varepsilon_{480} = 4200\ M^{-1}\ cm^{-1}$) was measured spectrophotometrically using fiber optic probe of CARY-50 (Varian Instruments, USA).

To define the activity from the yield of PGE_2, the same reaction mixture was stirred for 45 min at 25 °C [13,16,20]. Then, it was decanted and the support was washed with the buffer and utilized again in the next experiment. The liquid phase was acidified, extracted with ethyl acetate, and the extracts were dried over Na_2SO_4. The obtained residue was dissolved in 3 cm^3 of ethanol, and 4 M KOH solution in ethanol (0.1 M) was added to this solution to isomerize PGE_2, which was formed upon spontaneous decomposition PGH_2, into PGB_2 [13,16,17]. The mixture was kept at 25 °C for 60 min, and the PGB_2 concentration was determined at 278 nm ($\varepsilon_{278} = 23000\ M^{-1}\ cm^{-1}$).

The enzyme activity was detected after immobilization and at different time of enzyme storage, as well as after repeated use of immobilized enzyme preparations. All measurements were carried out in three replications.

The conditions applied for microsome extraction, kinetic evaluations and activity measurements were the same as that reported in various works carried out with PGH-synthase from sheep vesicular glands [16,17] and with silica gel as a carrier, in order to have the possibility of results comparison. Contrary to the referred works, the present study was realized with three different techniques applied at the same time for enzyme activity assays, while in the other studies [16–18] only one of them was used, for example, O_2 consumption or PGE_2 measurements.

3. Results and discussion

The image of dry *Opuntia imbricata* trunk is presented in Fig. 1. The trunk is characterized with porous structure contrary to the common wood samples and this facilitates an increase of the surface area and thereby biofilm formation [2–4]. The microstructure of the support was also studied by means of electronic microscopy (Fig. 2). The structure of the *Opuntia imbricata* dried trunks is formed by multiply repeated layers of cylindrical pores (diameter of 5–20 μm, length of 10–100 μm) which are oriented in perpendicular direction to that of previous layer. The main part of these pores is padded with low-molecular hydrocarbons. The treatment of this support with HCl promoted formation of continuous system of pores and a slight increase in their diameter (Fig. 2; bottom). Further an approximate weight loss of 26.4% was also recorded after this pretreatment. This weight loss could be attributed to the elimination of soluble fractions including hemicellulose [1].

Based on the results obtained from microscopy studies, it can be supposed that microsomes may be immobilized on *Opuntia imbricata* either passively through hydrophobic or ionic interactions or covalently by attachment to activated surface groups. Generally, non-covalent surfaces are effective for many applications; and passive adsorption has failed in many cases [1,15–17]. Covalent immobilization is often necessary for binding molecules that do not adsorb, adsorb very weakly, or adsorb with improper orientation and conformation to non-covalent surfaces. Covalent immobilization results in greater stability and reduces non-specific adsorption, however, sometimes leads to the loss of enzymatic activity [13].

In the present work both types of immobilizations were performed. There are a number of ways to modify solid support *Opuntia imbricata* for the covalent immobilization of microsomes. This article focuses on preactivation of support by sodium periodate to produce aldehyde groups [21]. The aldehyde groups of the activated carrier were able to react with amino groups of microsomes to form covalent bonds and result in the immobilization of the enzyme preparation. In our previous study [1,22], the decrease in periodate concentration after support

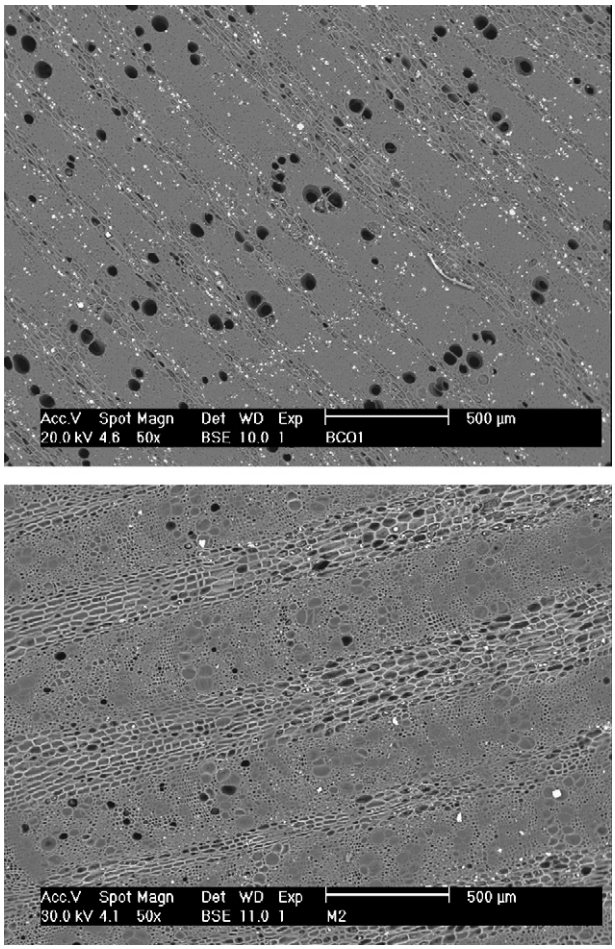

Fig. 2. Microphotographs of *Opuntia imbricata* before (top) and after (bottom) treatment with HCl.

activation was demonstrated by means of common analytical technique [23]. It could be considered as an indirect evidence for aldehyde group formation [1].

Valuable data on the nature of the functional groups, involved in the catalytic activity of PGH-synthase, were reported previously [5,9–11]. It had been shown that Ser-530 was responsible for the interaction between the enzyme active center and the substrate, but was not directly involved in catalysis. The presence of catalytically competent tyrosine residues (Tyr-335, Tyr-385 and 417) was reported in the papers [5,9–11]. Tyr-385 was assigned the role of an initiator of the cyclooxygenase transformation of arachidonic acid. The importance of His-207, His-309 and His-388 for catalysis had also been supposed [5,9–11]. PGH-synthase is a haemoprotein. It has been observed that the use of non-ionic detergent for enzyme extraction leads to almost total loss of heme and obtaining apo-PGH-synthase. Heme enters a narrow slit between polypeptide chains and interacts with Tyr and His groups. So, due to the fact that catalytically important amino acids do not include the amino groups, it was suggested that the interaction of the enzyme with aldehyde groups of periodate activated support does not have to modify the enzyme activity. Hence, this type of support activation was chosen for this study.

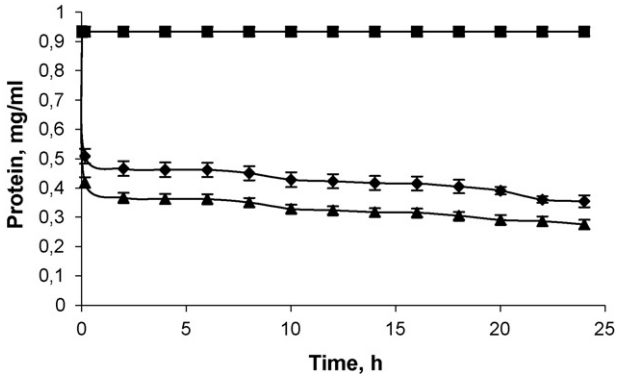

Fig. 3. Kinetic data on protein concentration in microsome suspension in the absence (■) and in the presence of *Opuntia imbricata* activated with periodate 0.1 M (▲) and non-activated (♦).

Kinetic data of the protein removal from microsome suspension in the presence of activated and non-activated supports are presented in Fig. 3. About 55% and 45% of microsome protein were quickly removed within 10–15 min after addition of activated and non-activated supports. The adsorption equilibrium reached after more than 24 h (Fig. 3). The amount of bound protein (Fig. 4) was estimated after 1 h and 24 h of incubation as the difference between the amount of protein applied to the support at wide range of concentrations and the amount of protein recovered in the supernatants and washings. The fraction of immobilized protein was higher after 24 h of reaction (68–71%) than after 1 h of reaction (38–45%) (Fig. 4). How-

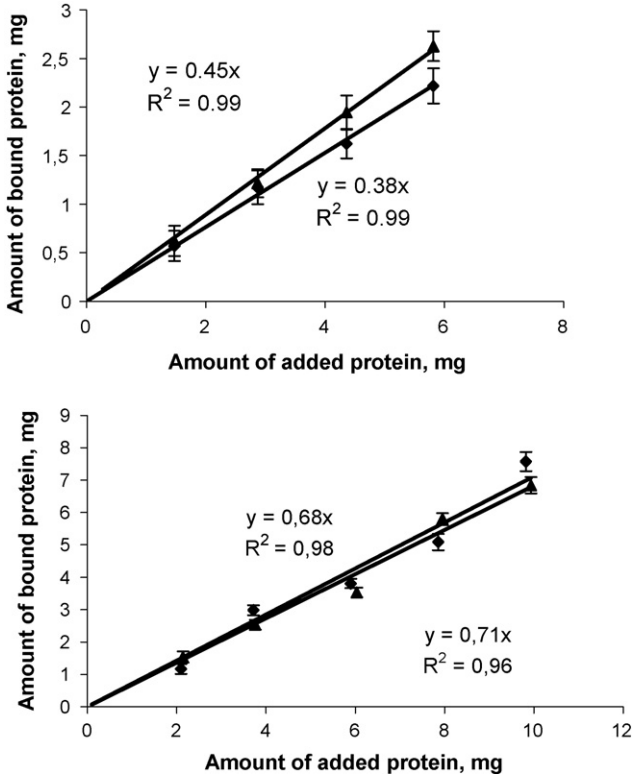

Fig. 4. Sorption of microsome protein on *Opuntia imbricata* activated with periodate 0.1 M (▲) and non-activated (♦): top, after 1 h of incubation; bottom, after 24 h of incubation.

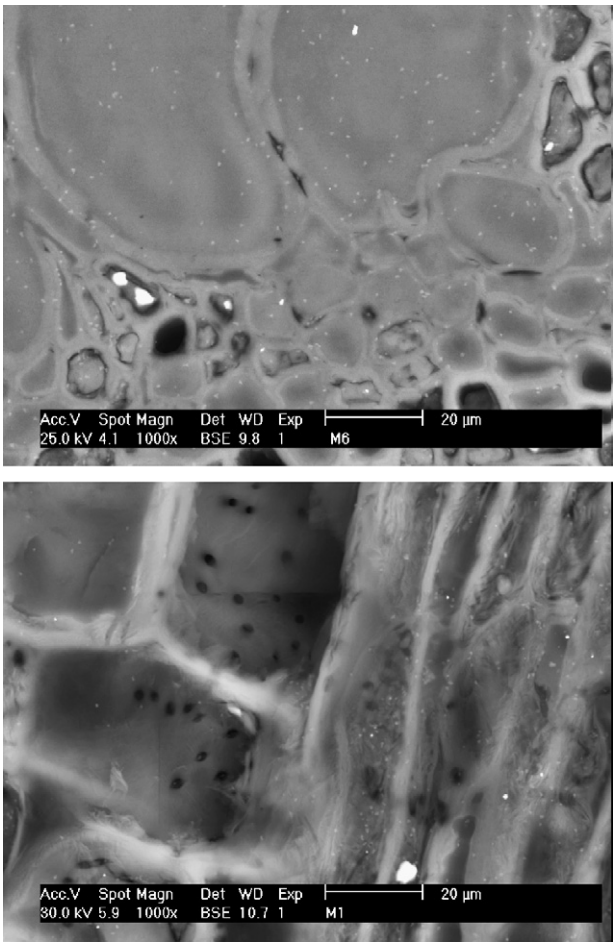

Fig. 5. Microphotographs of *Opuntia imbricata* after microsome immobilization, periodate activated (top) and without periodate activation (bottom).

ever, a very small difference between activated and non-activated supports was observed only after 1 h of incubation (Fig. 4, top). Based on these results, the time for support/microsomes reaction was chosen as 24 h for other experiments in this study. The obtained results showed that the protein immobilization on *Opuntia imbricata* performed by means of a passive adsorption mechanism also recorded same yield as that of covalent mechanism in periodate activated support.

In the case of structural analysis using electron microscopy, it was observed that the nano- and micropores were clearly visible inside the pores of major size in non-activated support, before and after immobilization (Fig. 5, top and bottom). However, these small pores were scarce and difficult to distinguish in the electron micrography of periodate activated support after enzyme immobilization (Fig. 5, top).

As mentioned earlier, PGH-synthase activity was quantified by means of three different assays. The activity and storage stability of free microsomal fraction applied for enzyme immobilization are presented in Tables 1 and 2. In general, only one method is used for determining PGH-synthase activity [13,16,18]. It was observed that the three applied techniques gave a good correlation between them (Table 1), as well as with the reaction scheme. With 1 mol of arachidonic acid, 2 mol of oxygen was consumed in cyclooxygenase reaction, resulting in the formation of 1 mol of PGG_2, which is transformed to PGH_2 in peroxidase reaction with 1 mol of adrenaline as donor of two electrons, and during spontaneous decomposition of 1 mol of PGH_2, 1 mol of PGE_2 is obtained [5–7]. The ratio of oxygen and adrenaline (2:1) led to the detection of higher activity (twice) in electrochemical method than with spectrophotometric assay. The decrease in the protein concentration applied for enzymatic reaction led to a proportional decrease in activity, while the specific activity was not changed (Table 1).

The PGH-synthase from bovine vesicular glands is easily inactivated upon storage (Table 2). Only 36–48% of initial activity was maintained after 24 h of storage at 4 °C (temperature applied in immobilization assay). Approximately 90–95% of enzyme activity was lost after 2 days and it was completely inactivated after 4 days of storage. The difference in the sensibility of techniques applied for activity detection led to a difference on quantified values, more specifically when the enzyme activity was low. It is known that spectrophotometric assay is more sensitive than electrochemical detection [13], while the extraction of low concentrations of PGE_2 can result in low estimation [16,17]. These could be the reason for the major deviation observed in the experimental data on the second day of enzyme storage and for the zero activity recorded in PGE_2 synthesis. The low storage stability of PGH-synthase demonstrated in this work was in accordance with earlier reports [8,13,16,18,20].

The cyclooxygenase activity, as well as cyclooxygenase plus peroxidase activity, and ability to catalyze the synthesis of prostaglandin E_2 were studied with activated and non-activated immobilized supports using two different contents of microsomes (Table 3). An increase in the protein concentration of microsomes led to increase in the amount of bound protein; however, there was no corresponding increase in the activity. The total activity, expressed in μmol/min, did not change significantly, but the specific activity calculated for 1 mg of bound protein showed a decrease (Table 3). It is likely that the accumu-

Table 1
PGH-synthase activity of soluble microsomal fractions applied for immobilization

[Protein] (mg/cm^3)	Detection method	μmol/min	μmol/(min × mg)
0.48 mg/cm^3 (0.072 mg of protein in reaction)	1. O_2 consumption	0.026 ± 0.003	0.358 ± 0.039
	2. Absorbance detection	0.013 ± 0.001	0.185 ± 0.015
	3. PGE_2 detection	0.012 ± 0.0	0.166 ± 0.002
0.24 mg/cm^3 (0.036 mg of protein in reaction)	1. O_2 consumption	0.014 ± 0.001	0.374 ± 0.020
	2. Absorbance	0.006 ± 0.0	0.157 ± 0.003
	3. PGE_2 detection	0.006 ± 0.0	0.165 ± 0.006

Table 2
PGH-synthase activity of microsomes (0.24 mg of protein/cm^3) during storage at 4 °C

Storage time (h)	Detection method	μmol/min	μmol/(min × mg)	Relative activity (%)
0	1. O_2 consumption	0.0135	0.375	
	2. Absorbance detection	0.0056	0.156	100
	3. PGE_2 detection	0.0059	0.164	
24	1. O_2 consumption	0.0049	0.136	36
	2. Absorbance detection	0.0027	0.075	48
	3. PGE_2 detection	0.0023	0.064	39
48	1. O_2 consumption	0.0007	0.019	5
	2. Absorbance detection	0.0006	0.017	11
	3. PGE_2 detection	0	0	0
96	All applied techniques	0	0	0

To calculate the relative activity the initial specific activity was used as 100%.

lation of the protein on the support gave rise to regions where microsomes are sorbed in several layers, and the active site of the enzyme became inaccessible. Partial enzyme inactivation due to the formation of bi- and tri-layers on the carriers was also reported for other enzymes, e.g., henokinase and PGH-synthase of sheep vesicular glands upon immobilization on silica gel, penicillin acylase immobilized on *Opuntia imbricata* and other systems of immobilized enzymes [1,16,21].

The specific activity of the immobilized enzyme on periodate activated carrier was slightly higher than enzyme immobilized on non-activated support (Table 3). This effect was more evident for cyclooxygenase activity, which was measured by means of oxygen consumption.

The stoichiometric relation between activities detected by the three techniques for free enzyme preparations (Table 1) was not the same as that of the immobilized system (Table 3). It may be considered as an evidence of the effect of support on the enzymatic conformation as well as on the conditions of performed reactions. The ability of the immobilized enzyme to catalyze the synthesis of prostaglandin E_2 was decreased considerably in comparison with cyclooxygenase and both cyclooxygenase plus peroxidase reactions, detected electrochemically and spectrophotometrically, respectively. This could be as a result of difficulties in the diffusion process of the product or stabilization of PGH_2 as the precursor in the synthesis of PGE_2. However, the mechanism is not clear, as similar studies have not been carried out previously. In general, for heterogeneous immobilized systems, oxygen consumption and the PGE_2 extraction methods were used for activity measurements [13,16,18]. The results obtained with fiber optic probe in this study led to the observation of the effect of support on the measurements of immobilized enzyme activity. The use of fiber optic probe was possible as the enzyme reaction mixture did not contain any suspended particles, and also the supernatant was optically transparent.

The relative remaining activities of the enzyme immobilized on periodate activated carrier, using microsomes with 0.24 mg of protein/cm^3, measured by oxygen consumption and absorbance (Table 3) was of 46.5% and 37.6%, respectively, while in the PGE_2 detection it was only 2% in comparison with the initial activity of free enzyme (Table 1). In the case of non-activated support the relative activities were 31.6%, 34.4% and 2%, respectively. However, if the partial inactivation of enzyme is considered due to the conditions applied for immobilization (Table 2), the immobilized preparations obtained with

Table 3
Activity of PGH-synthase immobilized on periodate activated and non-activated *Opuntia imbricata*, detected by means of three different techniques[a]

Support treatment/applied protein concentration (mg/cm^3) and bound protein (mg)	Assay	μmol/min	μmol/(min × mg of protein)	μmol/(min × g of support)
Non-activated support/0.24 mg/cm^3/1.24 ± 0.07 mg	1	0.146 ± 0.018	0.118 ± 0.021	0.073 ± 0.009
	2	0.067 ± 0.002	0.055 ± 0.004	0.027 ± 0.010
	3	0.005 ± 0.001	0.003 ± 0.001	0.002 ± 0.0
Periodate activated support/0.24 mg/cm^3/1.27 ± 0.11 mg	1	0.230 ± 0.011	0.174 ± 0.004	0.115 ± 0.006
	2	0.076 ± 0	0.059 ± 0.004	0.038 ± 0
	3	0.004 ± 0	0.003 ± 0	0.002 ± 0
Non-activated support/0. 48 mg/cm^3/2.62 ± 0.13 mg	1	0.177 ± 0.010	0.070 ± 0.005	0.088 ± 0.005
	2	0.052 ± 0.017	0.020 ± 0.007	0.026 ± 0.008
	3	0.006 ± 0.003	0.002 ± 0.001	0.003 ± 0.001
Periodate activated support/0.48 mg/cm^3/2.65 ± 0.09 mg	1	0.25 ± 0.042	0.094 ± 0.016	0.124 ± 0.021
	2	0.055 ± 0.012	0.021 ± 0.005	0.027 ± 0.006
	3	0.005 ± 0.002	0.002 ± 0.001	0.003 ± 0.001

[a] (1) Assay by O_2 consumption measurements, (2) assay by measurements of absorbance corresponded to donor electron product, (3) assay by measurements of PGE_2.

the non-activated support maintained more than 70–80% of cyclooxygenase and cyclooxygenase plus peroxidase activity after storage of enzyme at 4 °C for 24 h. While the interaction between periodate activated support and microsome helped to prevent the inactivation of free enzyme, its cyclooxygenase activity was measured as one of the free enzymes stored at the same conditions.

Previously, it has been reported that the enzymatic systems from sheep vesicular glands can be stabilized to some extent by immobilization on DEAE-Sephadex by adsorption [13]. They have reported that microsomal PGH-synthase retained 40% of the initial PGH-synthase activity after storage for 1 day. Similar results were obtained in the present study. Immobilization of sheep PGH-synthase into the matrix of polyacrylamide led to a complete inactivation of the enzyme [13]. However, the immobilization conditions applied for enzyme sorption on silica gel helped to obtain a sorbed preparation retaining 80–90% of the initial PGH-synthase activity and 90–97% of the microsomal protein. In this case the employed time of incubation was 20–25 min and enzyme was not inactivated considerably [16,17].

Stabilization of enzyme also was carried out using an electron donor (Table 4). Based on the available literature data [16], adrenaline was used as an electron donor in this study. It has been reported that the adrenaline addition significantly increased the storage stability of PGH-synthase immobilized on silica gel [16], and the electron donors decreased the inactivation of free enzyme [16]. In the case of PGH-synthase immobilized on *Opuntia imbricata*, the addition of electron donor did not increase the enzyme stability. It could be due to the problem in the adrenalin diffusion on the support used in this study or because of the influence of the support structure on mechanism of enzyme—donor interaction (Table 4). However, higher stability was detected in the non-covalently immobilized enzyme preparations, which retained, after 1 week of storage at 4 °C, approximately 65–67% of the initial enzymatic activity. The decrease in activity for both immobilized enzyme systems might be related to the slow detachment of non-covalently bound microsomes stored in solution. The difference in the structure of activated and non-activated supports (Fig. 5) might be related to partial degradation of the support reflected in the storage stability of covalently bounded microsomes. Distortions of structural correspondences between the size of protein cavity of active center and the prosthetic group and substrates prevent formation of the catalytically active complex. Such distortions can possibly occur during storage of immobilized enzyme and that could also be a reason for loss of the enzyme activity. The preparation of PGH-synthase from sheep vesicular glands immobilized on silica gel retained 55% of the initial PGH-synthase activity after 6 days of storage [16], while the adrenaline addition helped to maintain 80% of activity for 65 days. The preparations obtained by sheep microsome immobilized on DEAE-Sephadex were active only for a storage period of one week [13]. In both cases PGE_2 assays were carried out to detect the enzyme activity. The enzyme of bovine vesicular glands immobilized on pectin films retained up to 88% of its activity at −15 °C during first 5 days and 41% after 60 days of storage, and was determined

Table 4
Storage stability of PGH-synthase immobilized on periodate activated and non-activated *Opuntia imbricata*, detected by means of three different techniques[a]

Days	Assay	Preparations obtained without periodate				Preparations obtained with periodate			
		Storage in absence of electron donor		Storage in presence of electron donor		Storage in absence of electron donor		Storage in presence of electron donor	
		μmol/(min × mg of protein)	Relative activity (%)	μmol/(min × mg of protein)	Relative activity (%)	μmol/(min × mg of protein)	Relative activity (%)	μmol/(min × mg of protein)	Relative activity (%)
0	1	0.132 ± 0.001	100	0.103 ± 0.001	100	0.171 ± 0.001	100	0.176 ± 0.001	100
	2	0.057 ± 0.001	100	0.051 ± 0.001	100	0.061 ± 0.001	100	0.056 ± 0.001	100
	3	0.002 ± 0.001	100	0.004 ± 0.001	100	0.0032 ± 0.001	100	0.003 ± 0.001	100
3	1	0.096 ± 0.002	72.7	0.071 ± 0.002	69.3	0.075 ± 0.001	43.9	0.067 ± 0.002	39.6
	2	0.036 ± 0.003	63.1	0.036 ± 0.001	70.6	0.0289 ± 0.001	47.4	0.0261 ± 0.001	46.7
	3	0.002 ± 0.0003	100	0.0038 ± 0.001	95	0.003 ± 0.001	93.7	0.0028 ± 0.001	94.2
7	1	0.091 ± 0.001	68.9	0.069 ± 0.001	67.0	0.055 ± 0.001	32.2	0.039 ± 0.001	22.2
	2	0.032 ± 0.001	56.1	0.033 ± 0.001	64.7	0.023 ± 0.001	37.7	0.0216 ± 0.001	38.6
	3	0.0007 ± 0.0001	35.0	0.0027 ± 0.001	67.5	0.0009 ± 0.0001	28.1	0.0017 ± 0.001	56.7
14	1	0.0058 ± 0.001	4.4	0.001 ± 0.00	1.0	0.0	0	0.0	0
	2	0.0086 ± 0.001	15.1	0.009 ± 0.001	17.6	0.0078 ± 0.001	12.8	0.0058 ± 0.001	10.4
	3	0.0	0	0.0	0	0.0	0	0.0	0

[a] (1) Assay by O_2 consumption measurements, (2) assay by measurements of absorbance corresponded to donor electron product, (3) assay by measurements of PGE_2.

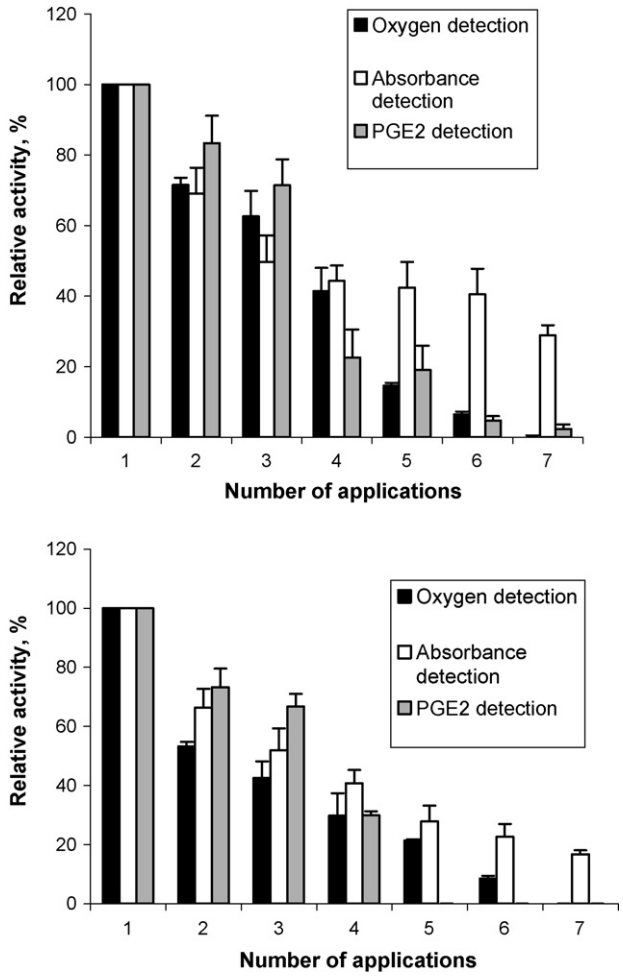

Fig. 6. Relative activities of PGH-synthase immobilized on *Opuntia imbricata* activated with periodate 0.1 M (top) and non-activated (bottom) in different subsequent catalytic cycles, detected by means of three techniques: O_2 consumption (black columns); measurements of absorbance, corresponded to donor electron product (white columns); measurements of PGE_2 (grey columns).

using O_2 monitoring assay; however, in this case the storage temperature of 4 °C, used in the present study, was not tested [18].

The PGH-synthase immobilized on the *Opuntia imbricata* lost its activity after 2 week of storage at 4 °C. Thereafter, the effect of the immobilized enzyme stabilization is evident and comparable with some other supports. However, the positive effect of additives, observed in the silica gel system, was not evident in the case of *Opuntia imbricata*.

PGH-synthase displays a phenomenon of fast and irreversible inactivation through catalysis that proceeds due to the formation of intermediate enzyme–substrate complexes. It was considered as the mechanism to control the production of physiologically active substances in live systems [5,12], however, present an immense problem for reuse of enzymes in biotechnological applications. The nature of this irreversible inactivation is related to the chemical reaction of active intermediates (radicals) followed by changes in protein structure and activity loss (inactivation). The important role of tyrosyl radicals (Tyr-385), intermediates of hemoprotein radicals, arachidonic acid, protein radicals and histidine groups had been reported in some previous publications [5,9–11]. Enzyme immobilization on support leads to partial control of modification and distortion of protein structure by means of decreasing degree of freedom. The resultant loss of movement of absorbed protein is related to the properties and sizes of protein active centers as well as enzyme stabilization.

The microsomal PGH-synthase immobilized on DEAE-Sephadex retained only 15% of activity after four cycles of use [13]. Whereas, the microsomes immobilized on silica gel in the presence of calcium ions retained about 50% of their activity after three cycles [16]. The addition of adrenaline into the system aided to maintain 96% and 66% of the initial activity after three and eight cycles, respectively [16].

In the present study it was not possible to use adrenaline addition and hence it was applied as a substrate in peroxidase reaction and its oxidation product was measured by spectrophometric assay. Immobilization of the enzyme on *Opuntia imbricata* improved the repeated use of enzyme (Fig. 6). Higher relative remaining activities were observed in enzyme immobilized on periodate activated support than non-activated support. All assays tested with regard to enzyme activity measurements demonstrated reliable results for six cycles, and the cyclooxygenase plus peroxidase activity and the ability to synthesize the PGE_2 were quantified in seventh cycle too (Fig. 6, top). The values of relative activity were less in enzyme immobilized on non-activated support, and as well as PGE_2 synthesis was detectable only up to four cycles (Fig. 4, bottom). The relative activities observed in this study are in accordance with the earlier results reported for other immobilized systems without additives.

4. Conclusions

The results demonstrated the development of a method for immobilization of PGH-synthase from bovine vesicular glands on *Opuntia imbricata*. The immobilization of PGH-synthase on *Opuntia imbricata* increased the enzyme stability during storage and permitted repeated use of the enzyme preparations for various cycles. It was demonstrated that the enzyme immobilization on *Opuntia imbricata* can be performed by physical adsorption, and as well as by the chemical attachment on periodate activate support. The difference in the structure of activated and non-activated supports was demonstrated by electron microscopy technique. Although immobilization of PGH-synthase from bovine vesicular glands did not show high yields on PGE_2 synthesis, dried *Opuntia imbricata* system could be useful not only for PGH-synthase but also for other enzymes. It is possible to consider this new kind of natural support material as a promising support for enzyme immobilization.

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