Crosslinked Poly(Vinyl Alcohol) Supports for the Immobilization of a Lipolytic Enzyme

KATYA CARBONE, MAURIZIO CASARCI, MAURIZIO VARRONE

ENEA CR Casaccia, Innovation Department (sp 061), Via Anguillarese, 301-S. Maria di Galeria, 00060 Rome, Italy

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ABSTRACT: Different esters of crosslinked poly(vinyl alcohol) (PVA) were synthetized. They were developed for protein fractionation and immobilization. PVA was crosslinked with epichlorohydrin (CL-PVA) and esterified with linear fatty acids of different length (C*n*-CL-PVA). A characterization of the obtained products was made. The swelling behavior, the solubility, and the percentage of esterification were examined. Values of equilibrium water content of about 81% were reached for CL-PVA samples. The polymers' stability and morpholgy were also investigated. Thermal analysis showed an increase in matrices stability, while SEM data showed the superficial development due to crosslinking and esterification reactions. Moreover, evident morphological inhomogeneities, mainly in the commercial and crosslinked products rather than in the final polymer, were present. Finally, immobilization experiments with a commercial crude of *Candida rugosa* were performed. Experiments showed a greater affinity of the protein for carbon chain length ranging from 8 to 12. Data indicated that compared to Celite 545, C8-CL-PVA was a better support for enzyme immobilization by physical adsorption, confirming the fact that microbial lipases prefer hydrophobic supports. © 1999 John Wiley & Sons, Inc. J Appl Polym Sci 74: 1881–1889, 1999

Key words: poly(vinyl alcohol) fatty acids; *Candida rugosa*; hydrophobic interaction chromatography; enzyme immobilization

INTRODUCTION

Several studies have been reported on the use of poly (vinyl alcohol) (PVA) as support for biotechnological applications.[1] It is used in various crosslinked forms giving hydrogels or membranes to employ in protein purification and immobilization studies.[2–4] It is well suitable for these purposes as it is hydrophilic, it can be easily modified through its hydroxylic groups, and it is commercially available in a wide range of molecular weights at low price. In a previous work has been reported the use of a crosslinked PVA (CL-PVA), esterified with dodecanoic acid, as chromatographic stationary phase for a lipase purification.[5] A further development in the characterization of these kinds of matrices and protein immobilization experiments may offer an interesting tool to understand the mechanism of the interactions between the support and the enzyme. For this purpose we have chosen *Candida rugosa* lipase (CRL) for its structural characteristics and wide industrial applications.[6,8] Lipases (triacylglycerol ester hydrolase EC 3.1.1.3.) catalyze the hydrolysis of triglycerides and other carboxilic esters in presence of a water–oil interface. In the present work, the preparation of different esters of crosslinked PVA (C*n*-CL-PVA) is described. Obtained resins are characterized by IR analysis and swelling behavior. Their thermal characterization is studied by TG and DTA, while their morphology is evaluated through SEM micro-

Correspondence to: K. Carbone (kcarbone@iol.it).

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graphs. Then, these polymers are tested in the immobilization of CRL (EC 3.1.1.3.) and the obtained results compared with Celite 545 ones to evaluate the influence of support hydrophobicity on biocatalyst activity.

EXPERIMENTAL

Materials

PVA used in this study was a commercial product of Merck (Darmstadt, Germany), with a number average molecular weight, M_n, of 72000 and a saponification value $> 98\%$. Epichlorohydrin and acyl chlorides were purchased from Fluka (Switzerland). All solvents were purchased from C. Erba (Italy). Celite 545 was purchased from BDH Laboratory Supplies Poole (England), while tributyrin was supplied by Sigma-Aldrich (Italy). All products were used without further purification. A microbial lipase from CRL obtained from Sigma-Aldrich (Italy) was used as test enzyme (Type VII, lot. 12807 EQ), to produce the immobilized biocatalysts. The crude enzyme powder had a nominal specific lipolytic activity of 1438 units/mg, based on olive oil hydrolysis and 30% lactose as extender.

Matrices Preparation

CL-PVA and C*n*-CL-PVA matrices were made according to Battinelli and colleagues[5], using linear fatty acids. In a typical preparation, 100 g of poly(vinyl alcohol) were suspended in 450 ml of 7 M NaOH in a three-neck flask. A 50 ml volume of epichlorohydrin was added to the mixture. The reaction mixture was heated under mechanical stirring for 1 h at 40°C and for 2 hs at 60°C, then it was neutralized with 3 M HCl. The obtained powder was washed in a Soxhlet extractor with acetone and then with water, till complete chloride ion disappearance. Then, the purified product was dried at 90°C overnight. CL-PVA was esterified by suspending it in pyridine, adding an excess of the appropriate acyl chloride, and refluxing for 4 hs at 60°C under mechanical stirring. The esterified product (C*n*-CL-PVA) was washed in Soxhlet with water and then with acetone. Finally, the polymer was dried at 90°C and ground until a granular powder was obtained.

Protein Immobilization Procedure

CRL was immobilized on Celite 545 and C*n*-CL-PVA polymers according to Munstranta and colleagues[9], with some modifications. In a typical experiment, a 20 mg/ml lipase solution (used buffer: Hepes 20mM and EDTA 2mM - pH 7.2) was centrifugated at 3000 g for 30 min in a thermostated centrifuge (4°C, Heraus Sepatech Biofuge 17 RS). After centrifugation, the supernatant was carefully removed and used for immobilization without further purification. Lipase solution (30 ml) and the support (5.0 g dry solid) were mixed together at room temperature for three hs, under mild conditions. Then, the suspension was filtrated, lyophilized for 48 hs, and stored at 4°C until use.

Methods

Water Uptake Measurements and IR Analysis

In order to measure the degree of swelling (DS) in distilled water, weighted amounts of dried CL-PVA samples were used. After equilibration in water at room temperature, they were weighted again and the equilibrium water content (EWC) was calculated as[10]

$$\mathrm{EWC} = \frac{100(m - m')}{m} \tag{1}$$

$$\mathrm{DS} = \frac{m - m'}{m'} \tag{2}$$

where m is the CL-PVA weight after water equilibration and m' was dried CL-PVA weight. IR spectrum was recorded to compare the PVA sample with the crosslinked one. The IR spectra were obtained on KBr disks using a 457 Perkin–Elmer spectrograph.

Determination of Esterification Percentages

C*n*-CL-PVA percentage of esterification has been calculated by means of indirect titration.[11] The procedure consisted of controlled base hydrolysis of a weighted amount of polymer with a known amount of alcoholic potassium hydroxide under reflux and titration of unreacted base. The same assay was performed with a sample of CL-PVA that was considered as a blank.

Morphology Analysis

A Stereoscan Cambridge 250 MKB was used as a scanning electron microscope. Samples for microscopy were sputter coated with Au (400 to 500 angstrom thickness).

Thermal Analysis

Thermal data were taken using a Netzsch STA–409 TG–DTA balance, flushed with nitrogen. The heating rates were 10 and 20°C/min.

Density and Surface Area Measurements

Density measures are taken with the stereopicnometry method. Surface areas are determined with a Quantasorb sorption system, using nitrogen (N_2 cross-sectional area: $16.2 \times 10^{-20} m^2$) as adsorbate and helium as a carrier. The B.E.T. equation used to determine the specific surface area (e.g., area per gram) of examined sample is

$$\frac{1}{X(P_0/P - 1)} = \frac{C - 1}{X_m C}\frac{P}{P_0} + \frac{1}{X_m C} \qquad (3)$$

where X is the weight of adsorbate adsorbed at relative pressure P/P_0, P is the partial pressure of adsorbate, P_0 is the saturated vapor pressure of adsorbate, X_m is the weight of adsorbate adsorbed at a coverage of one monolayer, C is a constant that is a function of the heat of the adsorbate condensation and heat of adsorption.

Immobilization Lipase Assay

Immobilized lipase activity was assayed by using tributyrin as substrate at 37°C and pH 7.2, according to Sigma Quality Test Procedure. The liberated fatty acids were titrated with 0.1 N NaOH solution with an automatic titrator (Metrohom AG 645 multidosimat) using phenolphtalein as an indicator. Triplicate experiments for each test were performed.

RESULTS AND DISCUSSION

CL-PVA Synthesis and Characterization

PVA can be readily crosslinked in order to improve water resistance and mechanical properties, relating to its chromatographic applications.[12,13] All multifunctional compounds that react with the hydroxyl group can be used as crosslinking agents.[10,14,15] In this work we have used epichlorohydrine, according to the procedure reported in previous works.[5,10,16] The choice of this crosslinking agent is based on the fact that the epoxide ring reacts readily with the hydroxyl group in basic media and the reaction goes on rather completely.[10] (Fig. 1, step1). The CL-PVA networks obtained in this way are characterized by swelling properties analysis. Obtained products appear as light yellow or white powders, insoluble in cold and hot water and in common organic solvents, such as DMSO, DMF, acetone, ether, hexane, and so on, while PVA is soluble in water and DMSO near their boiling temperatures.[13] The swelling results are reported in Table I. As we can see, networks show a high degree of water content (DS $>$ 4 and EWC $>$ 80%). In Figure 2 we compare IR spectrum of PVA with that of CL-PVA. As expected, OH-stretching band (hydrogen-bonded OH groups) decreasing down to 3500 cm^{-1} is evident. The 1716 cm^{-1} band present in the PVA spectrum and considerably reduced in that of CL-PVA, shows the CO stretching. The disappearance of this band is due mainly to the hydrolysis of the acetate groups present in the starting PVA sample. Such groups are generally distributed in clusters along all of the PVA chains, produced by poly(vinyl acetate) (PVA precursor) hydrolysis.[13] They were hydrolyzed by 7N NaOH during CL-PVA synthesis.

C*n*-CL-PVA Synthesis and Characterization

C*n*-CL-PVA polymers have been synthetized from CL-PVA samples as reported by Battinelli and colleagues[5] (Fig. 1, step 2). Final polymers are identified and characterized by percentage of esterification and by the swelling behavior.

The modification extent of crosslinked PVA samples has been determined in a first moment by means of the alkaline hydrolysis using a known solution of potassium hydroxide and titrating the unreacted base with 0.1 N HCl. Results (Table II) showed a too low ester content, probably due to a poor matrix swelling. In fact, in water medium the C*n*-CL-PVA matrix tends to form a hydrophobic layer on the water surface. To avoid this problem we have chosen an alcoholic medium: KOH/2-propanol. This solvent has been chosen to increase the swelling of the polymer and in this way to facilitate the ester hydrolysis. Such hypothesis is supported by the percentage values, obtained with a hydrolysis reaction in a water medium, that are lower than those obtained using alcoholic potassium hydroxide (Table II). Data shown in Table III demonstrate that the esterification percentages along the homologue series of used acyl chlorides are higher than 70%, except for C6-CL-PVA.

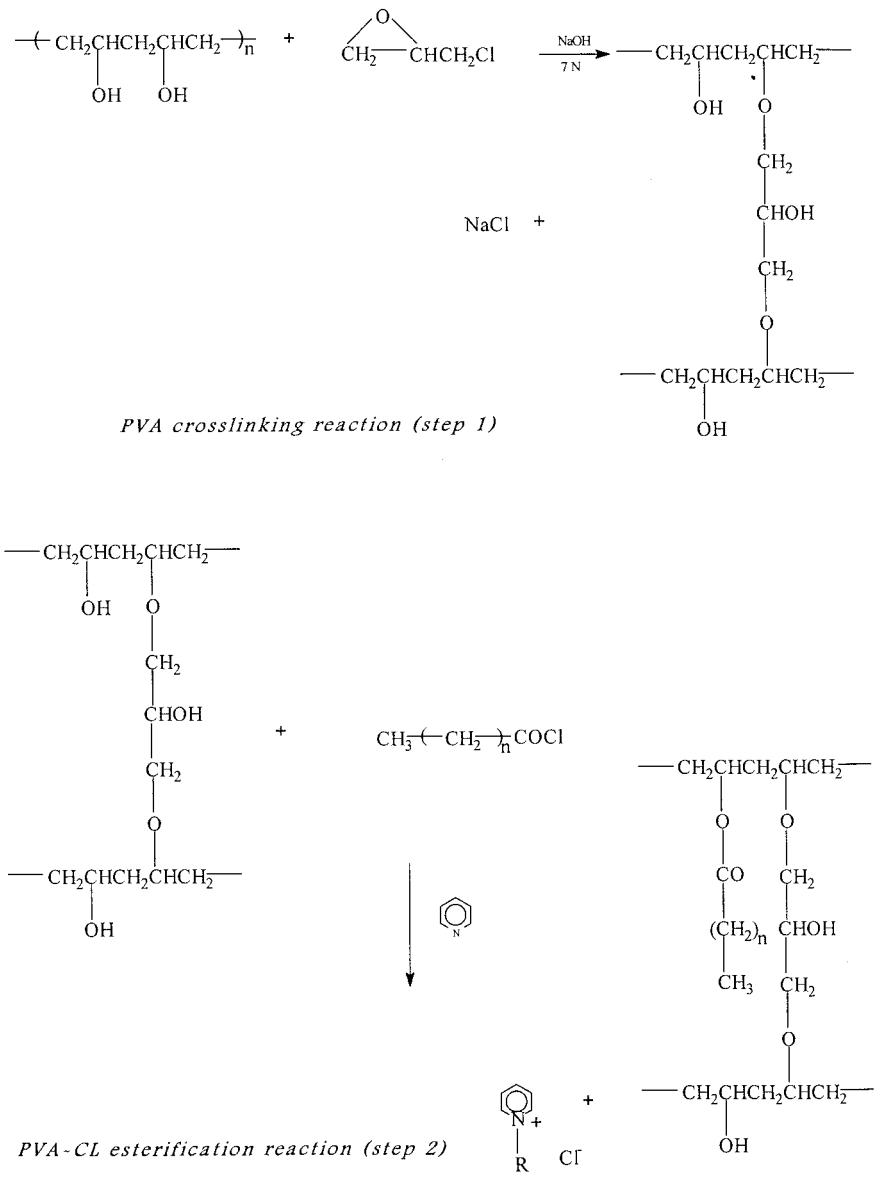

Figure 1 Two-step reaction for the synthesis of esters of (C*n*-CL-PVA).

A Comparison between PVA, CL-PVA, and C*n*-CL-PVA Polymers

We try to evaluate the effects of crosslinking and esterification reactions on PVA chemical and physical characteristics. In this context, we have tested PVA, CL-PVA, and C*n*-CL-PVA matrices to know their thermal stability and morphology. Thermal data for the PVA samples (Fig. 3) show two different critical temperatures. At about 200°C (T_i), the sample has the main loss of water corresponding to the formation of an unsaturated macromolecule. At about 350°C, polymer decomposition occurs. The effect of the crosslinking re-

Table I CL-PVA Swelling Degree and Equilibrium Water Content

CL-PVA Sample[a]	Equilibrium Water Content (EWC %)	Degree of Swelling (DS)
a	80.53	4.14
b	80.97	4.26
c	81.16	4.31
d	81.17	4.31
e	80.40	4.15

[a] They represent CL-PVA samples coming from different batches.

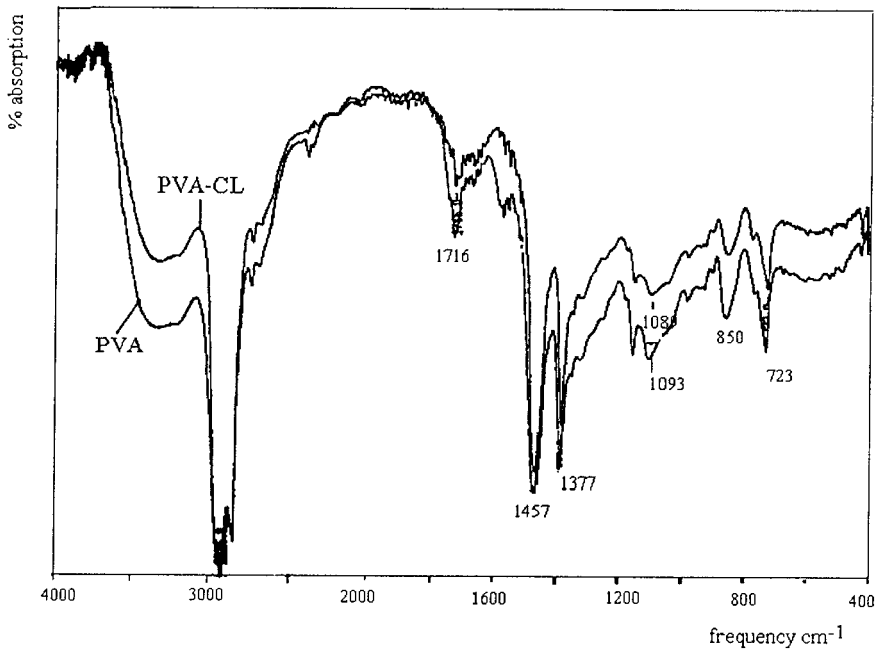

Figure 2 Infrared spectrum of PVA and CL-PVA (KBr disk).

action on PVA chains, causing an increase in the matrix thermal stability compared to that of the parent polymer, is also shown (Fig. 3). We can note (for CL-PVA networks) the presence of two endothermic peaks between 300 and 400°C and the modification of the sharp peak at 450°C, which is now broad to indicate lack of a transition phase (DTA curves). Figure 4 shows thermogravimetric curves (TG analysis) for the esterified CL-PVA samples (C4-CL-PVA and C10-CL-PVA). The esterification reaction produces a further increase in thermal stability of the polymeric matrix (T_i = 300°C). The carbon chain length of acyl chlorides used during the reaction seems to affect TG shape mainly between 350 and 500°C. It is possible to note from micrograph analysis a morphological change of the initial polymer (PVA) as a consequence of the reactions. In fact, we can observe a superficial development of the polymer. The presence of nonhomogeneous zones in the starting sample is nevertheless evident. This phenomenon increases during the crosslink reaction (Fig. 5). As we can see, there are strongly compact zones near porous ones. This may cause kinetic problems related to the enzyme adsorption process. With respect to PVA samples, it is possible to note the presence of a highly branched structure with pores of about 10 mm (Fig. 6). Such morphology depends on the high cohesion existing between PVA chains.[13] Micrographs on CL-PVA sample show a morphological heterogeneity, with the presence of compact, lamellar, and highly fibrous zones (Figs. 7–9). On comparing the two polymers, a morphological change due to

Table II Influence of Different Carbon Chain Length on the Esterification Percentage of C*n*-CL-PVA Matrices

Matrix	% Esterification[a]
C4-CL-PVA	80.56
C6-CL-PVA	57.63
C8-CL-PVA	70.08
C10-CL-PVA	81.14
C12-CL-PVA	94.13

[a] 100% is CL-PVA completely esterified. The esterification percentages are calculated using KOH/2-propanol as hydrolysis medium.

Table III Influence of Different Solvents Used in the Hydrolysis Reaction on the Esterification Percentage Determination of C*n*-CL-PVA Samples

Solvent	% Esterification
Water	3.58
2-propanol	80.56

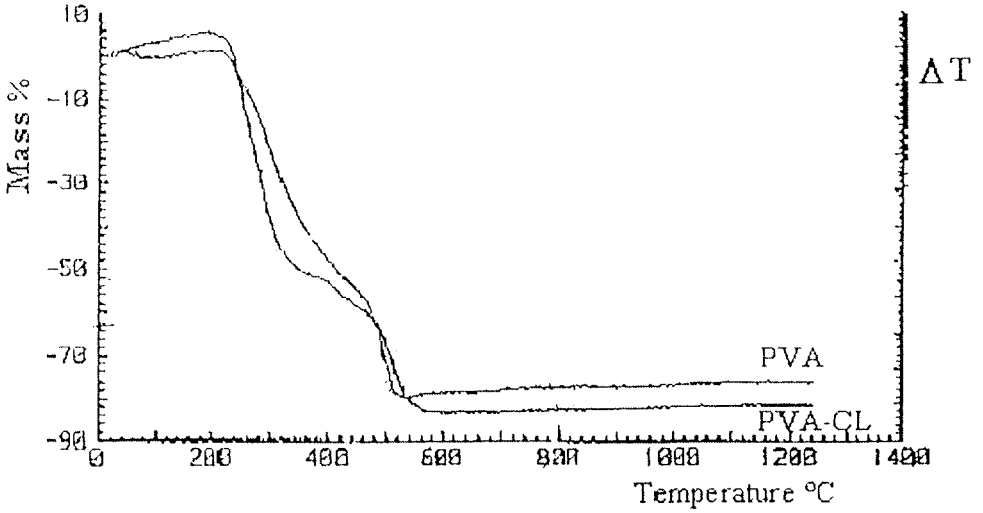

Figure 3 TG-DTA plot of PVA (7.61 mg) and CL-PVA (6.95 mg) matrices. The heating rate was 10°C/min under nitrogen atmosphere.

the chemical reaction is evident. Figures 10 and 11 show the esterified CL-PVA sample. The beads have a wide range of pore diameters and appear more uniform and compact than previous samples (PVA and CL-PVA). From a more general point of view, we suggest that the esterification reaction may promote the "breakdown" of fibrous-type structures of PVA in systems with spherical crystal structure. This would also explain the decrease of the surface area at the following synthesis steps (Table IV).

Table IV shows surface area and density data of CL-PVA and C*n*-CL-PVA matrices. As we can see, both decrease as a consequence of the reactions. If we assume that the closed pores fraction present in matrix structures is negligible, we can

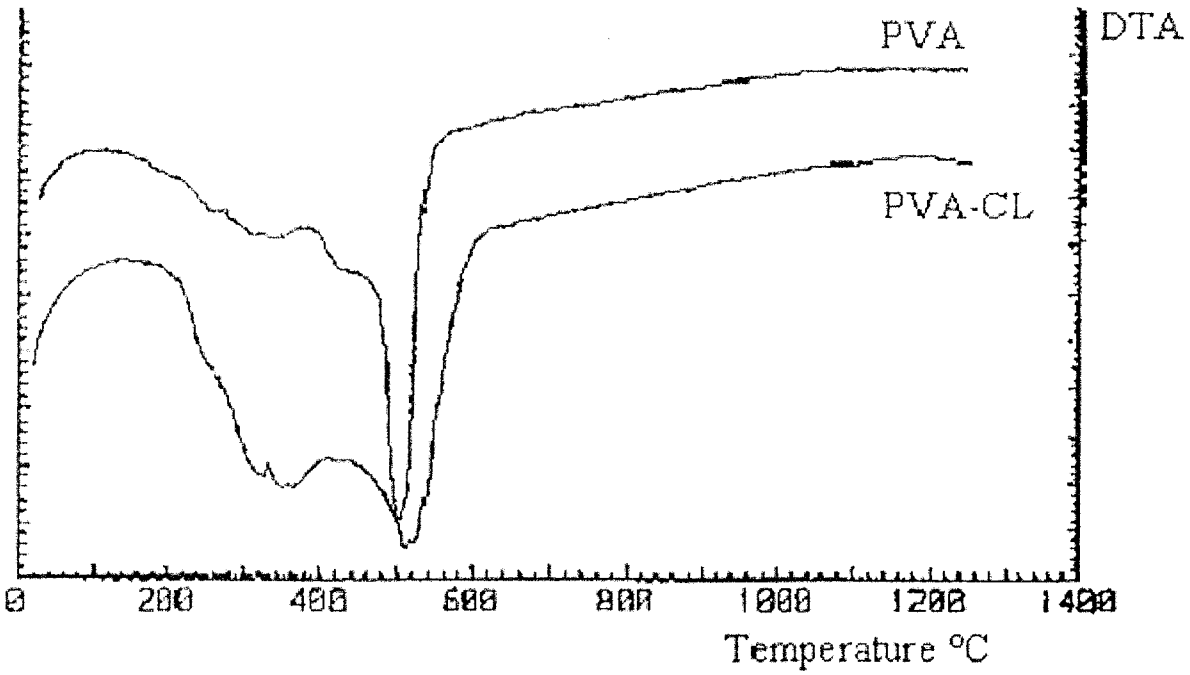

Figure 4 Effect of different chain length on the thermal stability of C*n*-CL-PVA matrices. The heating rate was 20°C/min under nitrogen atmosphere.

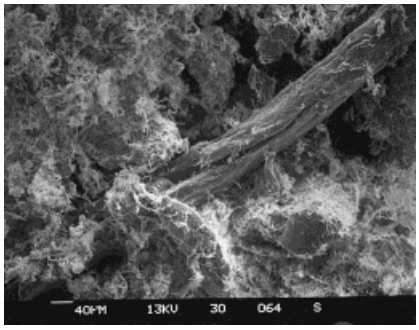

Figure 5 SEM micrograph of commercial PVA sample, 150×.

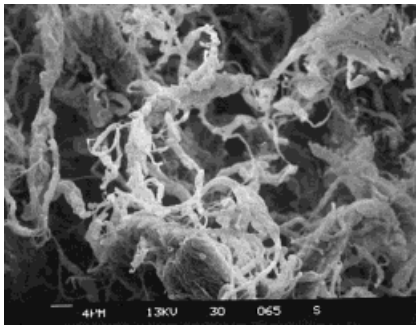

Figure 6 SEM micrograph of commercial PVA sample, 1000×.

notice a volume increase due to the esterification reaction. Moreover, as we can see from Tables IV and V, while we have a strong variation passing from the crosslinked polymer to the esterified one, we do not have large differences between the homologous series of acyl chlorydes used.

Different particle size fractions for C*n*-CL-PVA samples were obtained by wet sieving. Data obtained for different matrices are reported in Table VI. All of them show mainly 16-and 16- to 30-mesh granulometric fractions. After the esterification reaction we ground our polymers in order to obtain beads suitable for chromatographic columns filling. The effect of mechanical grinding of polymers is visible in the SEM scans where we observe fragile fracture zones that are normally found in steel samples.

C*n*-CL-PVA for Lipase Immobilization

CRL has been immobilized on various hydrophobic supports by physical adsorption.[17] In Figure 12 is shown the effect of these different matrices on the specific activity of the biocatalyst. Data reveal that the enzyme reached its highest activity when it was immobilized onto crosslinked PVA esterified with carbon chain length ranging from 8 to 12. This trend is consistent with the hydrolysis results on p-nitrophenol esters. In fact, CRL seems to prefer these kind of linear esters.[5] Moreover, these results are also consistent with those already observed during the purification process.[5] We have also compared the ability of C*n*-CL-PVA resins to adsorb the enzyme with that of Celite 545, a more hydrophilic support. Under the same

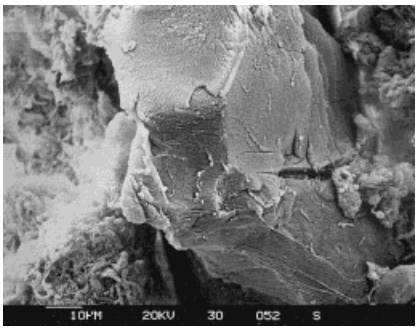

Figure 7 Morphological state of CL-PVA sample, 800×.

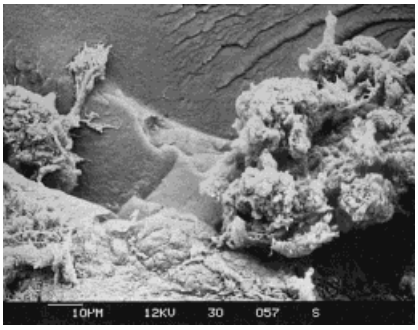

Figure 8 Morphological state of CL-PVA sample, 800 ×.

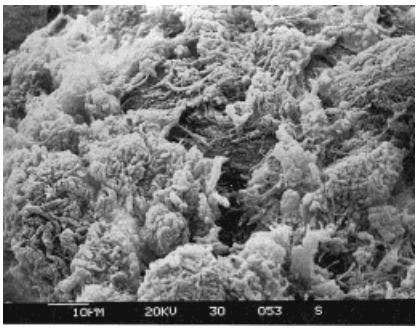

Figure 9 Morphological state of CL-PVA sample, 1000×.

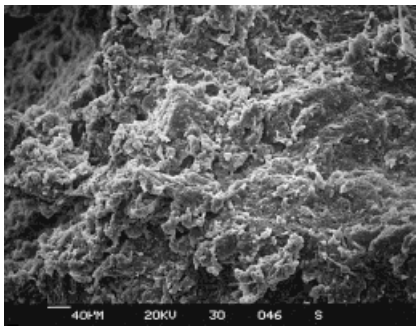

Figure 10 Representative scanning electron micrograph of C10-CL-PVA matrix, 150×.

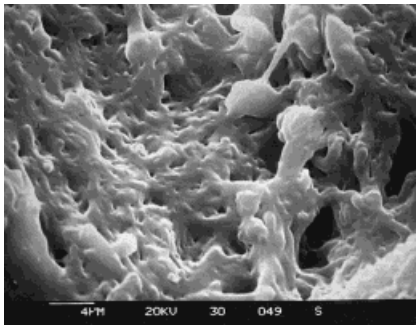

Figure 11 Representative scanning electron micrograph of C10-CL-PVA matrix, 3000×.

quantity of immobilized protein, CRL onto Celite 545 shows the lowest activity using tributyrin as standard substrate.

CONCLUSIONS

This work investigates the feasibility of using the reported functionalized CL-PVA matrices to give supports for lipase immobilization via physical adsorption. Results point out that the polymers are able not only to adsorb the enzyme selectively, but also to give a better biocatalyst than Celite ones. We also describe the preparation and characterization of crosslink and subsequently modified PVA granules. Results point out an increase in the polymer thermal stability due to the reactions, while a little porosity is reached after the esterification reaction. This fact is consistent with the surface area values and with their behavior at the water interface. In fact, C*n*-CL-PVA tends to form a quite hydrophobic layer on the water surface. As a consequence, the adsorption of the enzyme on these matrices will probably take place on the polymer surface and the mass-transfer resistance will be mainly in the external film. This fact is particularly relevant as the immobilization takes place in water medium. The increased ther-

Table IV Influence of Two-Step Reaction on Density and Surface Area Values for Studied Matrices

Sample	Density (g/ml)	Surface Area (m^2/g)
CL-PVA	1.3512	2.34
C4-CL-PVA	1.1256	0.8
C8-CL-PVA	1.0427	0.73
C10-CL-PVA	1.0352	0.67
C12-CL-PVA	0.9874	notdetected
C14-CL-PVA	1.199	notdetected

Table V Influence of Carbon Chain Length on the Determination of Equivalent Diameter Sphere for Studied Matrices

Sample	Diameter Equivalent Sphere (ϕ μm)[a]
CL-PVA	1.9
C4-CL-PVA	6.7
C8-CL-PVA	7.9
C10-CL-PVA	8.6

[a] Diameter equivalent sphere: 6/density∗specific surface area.

Table VI Distribution of Different C*n*-CL-PVA Particle Size Fractions

Matrix	Granulometric Fractions (mesh)	Quantity[a] (g)
CL-PVA-C4	>16	18.48
	16–30	56.25
	>40	25.27
CL-PVA-C6	>16	63.18
	16–30	36.80
CL-PVA-C8	>16	49.84
	16–30	32.13
	>40	10.62
CL-PVA-C10	>16	50.91
	16–30	49.05
CL-PVA-C12	>16	64.23
	16–30	27.20
	30–40	7.72
CL-PVA-C14	>16	37.44
	30–40	32.75
	>40	28.96

[a] Size distribution on samples of 100 grams.

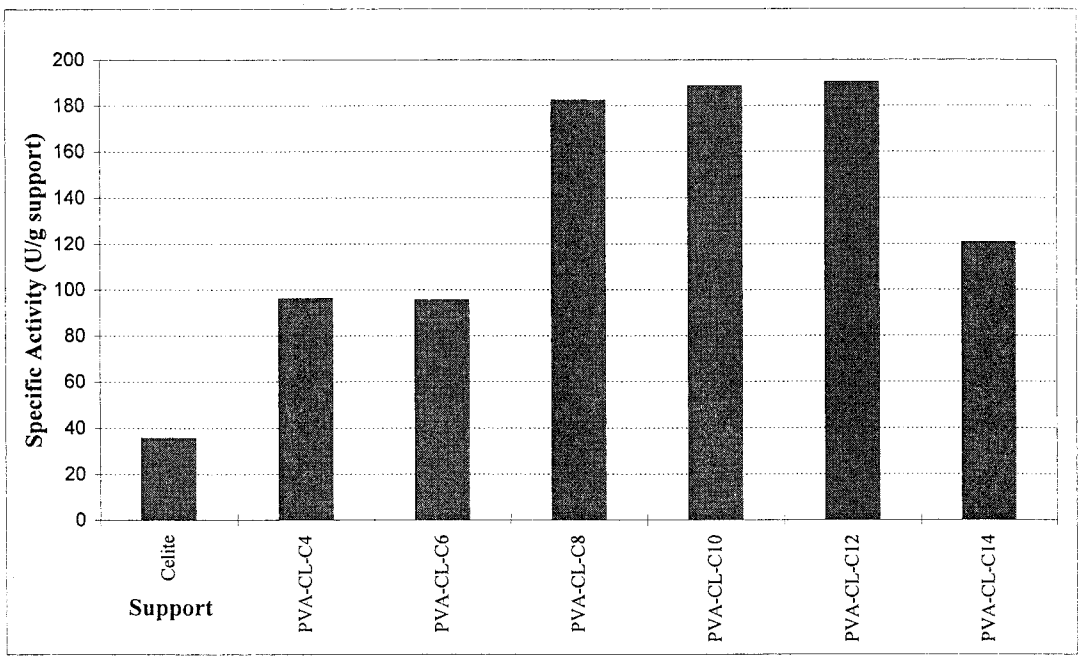

Figure 12 CRL activity immobilized on different supports. Tributyrin was used as standard substrate

mal stability gives matrices that may be employed in other fields than in proteins recovery where temperatures never exceed 100%C. In fact, in the present work, measurements of polymer thermal degradation are reported only to prove the effect of chemical manipulation on the starting sample (PVA) and on the intermediate one (CL-PVA). In conclusion, the matrices described in this work represent, to our opinion, a good system for a more detailed study on CRL structure–function relationship, giving the possibility to clarify protein–polymer interactions and relate them to the enzyme performances. Further work in this area is being done in our laboratory.

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