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New class of poly(vinyl alcohol) polymers as column-chromatography stationary phases for *Candida rugosa* lipase isoforms separation

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Abstract

The preparation of new stationary phases of cross-linked polyvinyl alcohol esterified with various linear fatty acids is described. The physico-chemical properties of these polymers are reported, including electron microscopy and swelling measurements. Batch adsorption experiments were performed in order to characterize the basic separative properties of these phases. Cross-linked polyvinyl alcohol esterified with dodecanoic acid was used for hydrophobic interaction chromatography of a commercial crude preparation of *Candida rugosa* lipase. Characterization of the purified fractions was carried out via native electrophoresis and sodium dodecyl sulfate–polyacrylamide electrophoresis.

Keywords: *Candida rugosa*; Stationary phases, LC; Polyvinyl alcohol–fatty acids; Enzymes; Lipases

1. Introduction

Lipase from *Candida rugosa* (formerly *Candida cylindracea*) is one of the most used enzymes in biocatalysis because of its catalytic effects in the non specific cleavage of ester bonds of carboxylic esters. It is also used for esterification and transesterification reactions in addition to being a valuable chiral catalyst for the resolution of racemic alcohols and carboxylic acids.

Despite its widespread use in a number of biotechnological preparations, to date lipase produced by *Candida rugosa* has attracted relatively little attention and a complete characterization has yet to be carried out. In 1990 Brahimi-Horn et al. [1] examined the esterase profile of a commercial preparation of *Candida rugosa* lipase and identified six major bands of hydrolytic activity. These correspond to several isoforms of slightly different ionic state and hydrophobicity.

Schrag et al. [2] demonstrated that *Geotricum candidum* and *Candida rugosa* lipases are homologous enzymes and are connected to the catalytic triad of the cholinesterase and serine protease family.

In this work, stationary phases based on cross-linked polyvinyl alcohol with side groups esterified with different fatty acids [3] were developed to carry out a preliminary purification of a commercial

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preparation of *Candida rugosa* lipase [4]. In order to investigate the lipase adsorption mechanism on these hydrophobic interaction stationary phases, a better characterization of these polymers was attempted.

2. Experimental

Candida rugosa lipase (L 1754, listed as *C. cylindracea*), tributyrin 99%, (3-[(3-Cholamidopropyl) - dimethylammonio] - 1 - propane - sulfonate) (CHAPS), ethylenediaminetetraacetic acid (EDTA), N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic) acid (HEPES), 2-(N-Morpholino)ethanesulfonic acid (MES), 2-methyl-4-[(2-methylphenyl)azo]benzenediazonium salt (FAST GARNET GBC), α-naphthyl acetate, *p*-nitrophenyl esters, Triton X-100 and phenolphthalein were purchased from Sigma (St. Louis, MO, USA).

Polyvinyl alcohol (PVA) 100% hydrolyzed M_r 72 000 was purchased from Aldrich (Milwaukee, WI, USA).

Molecular mass marker: HMW calibration kit native, LMW calibration kit; native electrophoresis gel: PhastGel gradient 8–25; electrophoresis gel: PhastGel gradient 10–15; PhastGel native buffer strips and PhastGel silver kit were purchased from Pharmacia (Uppsala, Sweden).

Epichlorohydrin and acyl chlorides were purchased from Fluka (Buchs, Switzerland)

All solvents were purchased from Farmitalia Carlo Erba (Milan, Italy).

2.1. Preparation/characterization of new stationary phases for the purification of enzymes by hydrophobic interaction chromatography (HIC)

The preparation of the esters of cross-linked polyvinyl alcohol (CL-PVA) with fatty acids was accomplished by the reactions reported in Fig. 1. For the cross-linking reaction (step 1) 100 g of PVA was 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 h at 60°C, then was neutralized with 3 *M* HCl. The powder obtained was washed in a Soxhlet extractor with acetone and then with water. Cross-linked polymer (1 mole) was esterified (step 2) by suspending it in pyridine, adding an excess of the appropriate acyl chloride and refluxing for 4 h at 60°C, under mechanical stirring. The esterified CL-PVA was washed in Soxhlet with water and then with acetone. The use of pyridine offered the advantage of increasing the reaction temperature, of neutralizing hydrogen chloride and of assuring a good dispersion of the beads. The fatty acid chlorides used were chosen

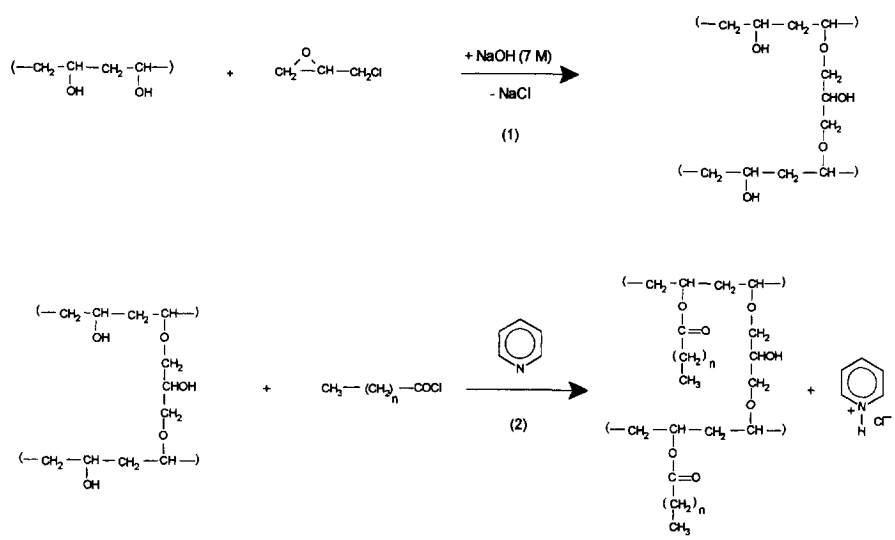

Fig. 1. The two steps for the preparation of esters of cross-linked PVA with fatty acids.

with linear chains of different length that followed the formula $CH_3(CH_2)_nCOCl$ (n=2, 4, 6, 8, 10, 12, 14).

The esters of CL-PVA were identified and characterized by the following methods;

(a) Infrared spectroscopy.
(b) Hydrolysis of ester groups in order to allow the calculation of the esterification ratio. The procedure consisted of the controlled base hydrolysis of a weighed amount of polymer with a known amount of sodium hydroxide under reflux and the titration of unreacted base.
(c) Scanning electron microscopy.
(d) Analysis of swelling properties.

2.2. Characterization of the basic separative properties of the stationary phases versus lipolytic enzymes

The batch experiments were performed by suspending the appropriate amount (25 g) of CL-PVA esterified with dodecanoic acid (CL-PVA–C_{12}) in HEPES–EDTA pH 7.6 buffer solution (300 ml) (20 m*M* HEPES, 2 m*M* EDTA, brought to pH 7.6 with NaOH) and adding a solution of lipase from *Candida rugosa* (200 mg) in the same buffer (5 ml). After 2 h of vigorous stirring the suspension was filtered and washed with the buffer solution. The solid, the filtrate and the washings were tested for lipolytic activity by titration of the acid formed by the hydrolysis of tributyrin.

2.3. Determination of lipase activity

Lipase activity was assayed by the following methods:

2.3.1. Lipolytic activity

The assay mixture, containing 2.5 ml of HEPES–EDTA pH 7.6 buffer solution, 0.5 ml of tributyrin and 100 μl of the enzymatic sample, was shaken for 30 s and incubated at 37°C under magnetic stirring. After 30 min the reaction was stopped with 2.5 ml of ethanol–acetone (1:1). The reaction mixture was titrated with 0.05 *M* NaOH in the presence of phenolphthalein using an automatic burette (Metrohm AG 645 multidosimat). The same assay was performed without enzyme and considered as a blank.

2.3.2. Esterasic activity

The lipase solution was added to the HEPES–EDTA pH 7.6 buffer solution previously mixed in a 9:1 (v/v) ratio with *p*-nitrophenyl ester solution at different concentrations (0.16–1.6 m*M*) in acetonitrile. The *p*-nitrophenoxide released by hydrolysis was measured at 410 nm and at 25°C on a Lambda 5 spectrophotometer (Perkin-Elmer, Norwalk, CT, USA).

2.4. Lipase purification

The purification of crude lipase was performed at 4°C. Lipase was suspended in HEPES–EDTA pH 7.6 buffer at a 100 mg/ml concentration. After centrifugation at 3000 rpm for 30 min at 4°C the pellet was discarded and the supernatant was further purified.

2.4.1. Hydrophobic interaction chromatography (HIC)

For the HIC, cross-linked poly(vinyl alcohol) esterified with dodecanoic acid (CL-PVA–C_{12}) was used as stationary phase. The polymer (about 50 g) was initially equilibrated with HEPES–EDTA pH 7.6 buffer and used to fill a 30 cm×50 mm column (XK50 Pharmacia Biotech, Uppsala, Sweden). The lipase solution was applied to the column; the inlet solution was fed at a 1 ml/min flow-rate and then was recycled through the column for 36 h to ensure equilibration. The unbound material was further washed from the column at a flow-rate of 2.5 ml/min by using the same buffer solution until the absorbance at 280 nm was lower than 0.005 U. The absorbance of the eluate was checked by an UV monitor (Uvicord SII, LKB, Uppsala, Sweden) at a wavelength of 280 nm.

The retained proteins were eluted from the column by stepwise increasing CHAPS concentration in the HEPES–EDTA pH 7.6 elution buffer (1.5, 6.0 and 20.0 mmol/l, corresponding to fractions 1, 2 and 3 respectively).

2.5. Fraction analysis

The fractions obtained were analysed by several methods.

2.5.1. Native electrophoresis

Native electrophoresis was performed using a combination of PhastSystem with ready Phast gel media with a 8–25 gradient in polyacrylamide gel according to the manufacturer's instructions [5]. A HMW marker calibration kit (Pharmacia) was used. Two identical gels were stained in two different ways in order to identify esterase activity and protein composition. α-naphthyl acetate in N,N-dimethylformamide and Fast Garnet GBC were used for the specific esterase activity staining [1]. Protein staining was carried out by the silver stain method [5].

2.5.2. SDS–PAGE

Sodium dodecyl sulfate polyacrylamide electrophoresis (SDS–PAGE) was performed by the use of Phast gel media with 10–15 polyacrylamide gel under reducing conditions following the Laemmli procedure [6]. A LMW electrophoresis calibration kit (Pharmacia) was used as a marker. Electrophoresis and silver gel staining were performed as suggested by the manufacturer [5].

2.5.3. Detection of portein concentration

The determination of the protein concentration was performed using the Bradford method [7].

3. Results and discussion

Crude protein mixtures are put in contact with the adsorbent by using the appropriate conditions to selectively bind the enzyme, while nearly all other proteins are not adsorbed and are removed by washing with buffer solution (unbound fractions).

A subsequent additional resolution step is obtained by gradually reducing the strength by which proteins are adsorbed on the support, so obtaining the sequential elution of the fractions (different lipases and/or contaminating proteins). With this system it was also possible to hypothesize a partial resolution of different isoforms. All the fractions obtained were characterized by different electrophoretic methods.

3.1. Characterization of the new stationary phases

All the esters of CL-PVA appeared as white powders, insoluble in hot or cold water and in organic solvents such as acetone, ether, chloroform, hexane, ethanol and DMSO. The esters differed in many respects from linear PVA. They swelled in water without forming a solution and were more stable towards thermal decomposition. The esters of CL-PVA were characterized by infrared spectroscopy. In Fig. 2 the infrared spectrum of the CL-PVA–C_{12} is reported and compared with that of the CL-PVA. The spectrum shows the sharp absorption of the ester function at 1750 cm^{-1}, which is absent in the CL-PVA spectrum. The spectrum of the ester shows the stretching C–H bonds at 2900–3000 cm^{-1} and those of hydrogen-bonded OH groups at 3000–3500 cm^{-1}. This finding is important, since it demonstrates that not all the hydroxyl groups of CL-PVA were esterified; some of them formed hydrogen bonds. Assessment of the degree of esterification was then necessary in order to further characterize the structure of the polymer. The characterization by infrared spectroscopy of the recovered polymers confirmed the complete disappearance of the ester groups, i.e., complete hydrolysis. The esterification ratios are shown in Table 1. The results demonstrate that the percentages of esterification are quite high and decrease only with

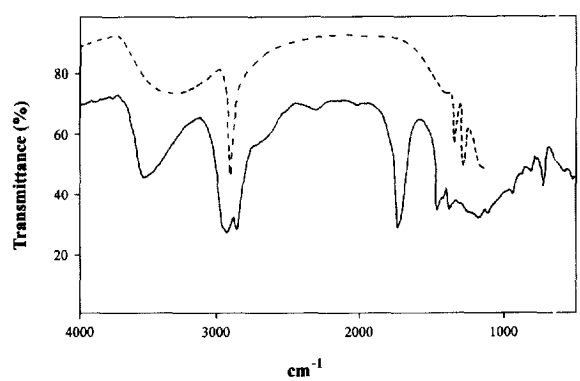

Fig. 2. Infrared spectrum of CL-PVA esterified with dodecanoic acid showing the ester absorption at 1750 cm^{-1} (KBr disk) (continuous line). The infrared spectrum of CL-PVA[1] (KBr disk) (dotted line).

Table 1
Esterification percentage as a function of acylic chain length of the acylic chlorides used

	Fatty acid carbon atom number				
	C_6	C_8	C_{10}	C_{12}	C_{16}
CL-PVA–C_n (mmol acid/g)	6.05	5.50	4.64	4.18	3.03
Esterification (%)	67	81	72	79	51

palmitic acid, probably because of the steric repulsion between carbon chains.

The morphology of the polymers was investigated by scanning electron microscopy (Fig. 3). The beads had a wide range of pore diameters and were sufficiently compact and uniform to appear ideal as stationary phases.

The degree of swelling was inversely proportional to the length of the acyl chain, as shown in Table 2. Saturated alkyl chains would be compactly arranged because of strong hydrophobic interactions between the hydrocarbon chains, resulting in decreasing water adsorption. However, as the hindrance of the carbon chains decreased, the inter-chain hydrogen bonds between hydroxyl groups decreased, leading to a greater capacity for hydration of the free O–H groups. Thus an equilibrium was reached for the long-chain terms. As expected, the water retained in CL-PVA esters was markedly less than that in the simple CL-PVA–X-40 polymer.

The properties of CL-PVA esters with the corresponding esters of linear PVA were also compared. These products are intractable gums, although extensive esterification occurred.

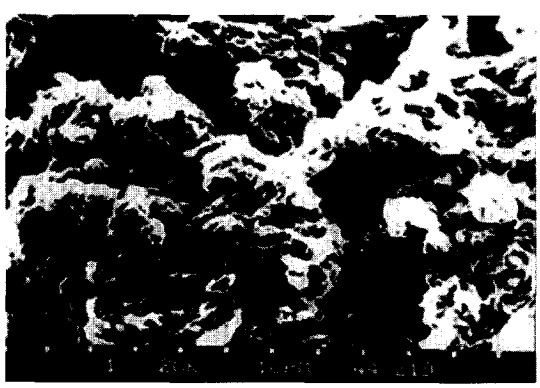

Fig. 3. Morphological state of CL-PVA esterified with dodecanoic acid using the scanning electron microscope (1000×).

3.2. Basic separation properties of the stationary phases with regard to lipolytic enzymes

The batch experiments showed that some 10% of enzyme activity was not adsorbed. The remaining 90% of the activity was recovered in the retained fraction eluted with HEPES–EDTA pH 7.6 buffer solution containing 1% Triton X-100. Adsorption experiments on simple CL-PVA showed that the lipase did not interact with this polymer and that more than 90% activity was recovered in the unretained fraction. These results suggest that a strong hydrophobic interaction occurred between the hydrophobic chains of the polymer (CL-PVA–C_{12}) and the enzyme. The above interaction was also confirmed by the infrared spectrum of the solid which showed the typical signals of the polymer and of the adsorbed enzyme (Fig. 4). The polymer showed its absorptions at 3200–3500, 2750–3000 and 1740 cm^{-1}. The enzyme showed the signals typical of proteins: NH band (3400 cm^{-1}) and peptide band

Table 2
Swelling properties of CL-PVA polymers esterified with linear fatty acids

Fatty acid carbon atom number	Water regain (g H_2O/100g dry polymer)	Water of hydration (g H_2O/100g dry polymer)
-	1.88	3.90
6	1.33	2.70
8	1.26	2.65
10	1.19	2.60
12	1.14	2.60

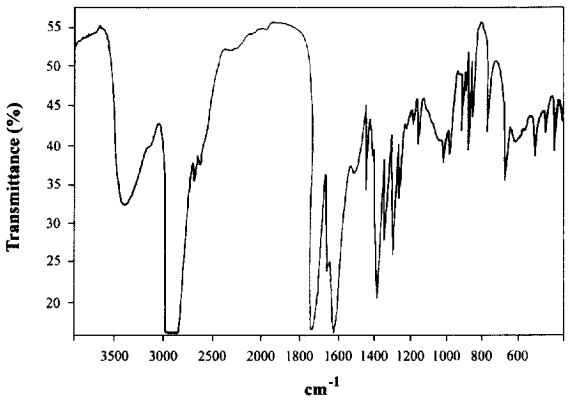

Fig. 4. Infrared spectrum of the lipase from *Candida rugosa* adsorbed on CL-PVA esterified with dodecanoic acid (KBr disk).

(1625 and 1074 cm^{-1}). Furthermore, the spectrum of Fig. 4, below 1625 cm^{-1}, is quite similar to commercial lipase.

The polymer-enzyme interaction was also studied by measuring the adsorption of the lipase on 1 g of polymer versus the interaction time and versus lipase concentration. Adsorption of a fixed concentration of lipase was also studied versus different amounts of polymer after 1 h of stirring (Table 3, Table 4 and Table 5). The results showed that the major part of lipase activity was adsorbed within the first 30 min and that 4 g of polymer is needed to obtain saturation, starting from 10 ml of 10 mg/ml lipase solution. The highest adsorptions of lipase were observed for the C_8–C_{12} esters of the polymer. These results are consistent with the observation showing a higher lipase activity for esters with C_8–C_{12} acyl chains as substrates.

Table 3
Adsorption[a] of lipase[b] from *Candida rugosa* on CL-PVA esterified with fatty acids of different acylic chain length versus contact time

Polymer (1 g)	0.5 h	1 h	6 h	16 h	24 h
CL-PVA–C_6	31	32	30	33	36
CL-PVA–C_8	29	33	37	45	45
CL-PVA–C_{10}	21	23	28	33	35
CL-PVA–C_{12}	20	26	28	32	36
CL-PVA–C_{16}	9	10	13	13	13

[a] Values expressed as a percentage.
[b] 10 ml of lipase solution, 10 mg/ml.

Table 4
Adsorption[a] of lipase[b] from *Candida rugosa* on CL-PVA esterified with fatty acids of different acylic chain length versus the amount of polymer

Polymer	1 g	2 g	3 g	4 g	5 g
CL-PVA–C_6	32	54	68	75	74
CL-PVA–C_8	33	64	72	81	81
CL-PVA–C_{10}	23	47	68	76	79
CL-PVA–C_{12}	26	51	71	82	83
CL-PVA–C_{16}	10	21	45	45	45

Contact time 1 h.
[a] Values expressed as a percentage.
[b] 10 ml of lipase solution, 10 mg/ml.

3.3. Choice of the stationary phase

In order to prepare a suitable stationary phase for purification of lipase, preliminary experiments were required to collect information on the affinity of *Candida rugosa* lipase for different substrates. A colorimetric method was used based on the hydrolysis of *p*-nitrophenyl esters of various acyl chain length (from C_4 to C_{18}) in homogeneous solutions (acetonitrile–water). Using crude lipase preparations, we obtained Michaelis–Menten kinetics which provided an approximate measure of the affinity of lipase for fatty acids of different acyl chain lengths. In Table 6 the K_m and V_{max} obtained by using *p*-nitrophenyl esters of different chain lengths are shown. The affinity of *Candida rugosa* lipase (esterase) is higher for esters with a chain length ranging from 8 to 14 carbon atoms. Since a good stationary phase for HIC should provide both specificity and

Table 5
Adsorption[a] of lipase from *Candida rugosa* on 1 g of CL-PVA esterified with fatty acids of different acylic chain length (C_n) as a function of initial solution enzyme concentration

Enzyme (mg)	C_6	C_8	C_{10}	C_{12}	C_{14}
100	96	95	97	96	96
200	192	188	193	192	146
300	232	283	291	261	218
400	308	331	384	324	264
500	338	387	474	369	306
600	363	432	499	373	360
1000	450	671	768	404	452

Contact time 1 h.
[a] Values expressed in mg.

Table 6
Kinetic parameters of the esterasic activity of *Candida rugosa* lipase measured on *p*-nitrophenyl esters with different chain lengths

Chain length (carbon atom number)	K_m (μmol/l)	V_{max} (nmol/min)
4	210	1.7
6	130	2.1
8	453	2.3
10	12	1.4
12	20	1.6
14	50	0.3
16	240	0.2
18	250	0.03

high-affinity for the substances to be separated, together with mild elution conditions, CL-PVA esterified with dodecanoic acid was used. This choice was also based on the batch experiments, which indicated a stronger interaction between the lipase and the CL-PVA–C_{12} and a high yield in the preparation of the polymer. Polymers esterified with fatty acids with 8, 10 or 14 carbon atoms are equally good stationary phases for the purification of *Candida rugosa* lipase. Preliminary experiments indicated a loss of enzymatic activity due to the presence of Triton X-100. This surfactant was substituted with CHAPS, which shows similar desorption efficiency but few, if any, denaturing characteristics.

Between two consecutive chromatographic separations, it is necessary to wash the polymers with acetone followed by re-equilibration with the elution buffer. If this is done, then the CL-PVA–C_{12} can be used several times (more than fifteen) without apparent loss of performance. This fact indicates that during chromatography the hydrolysis of ester bonds does not occur. In this respect the use of the polymer esterified with octanoic acid is not advisable, since the high V_{max} measured for esterase activity for the C_8 ester could cause considerable hydrolysis of the stationary phase during chromatography.

3.4. Lipase purification

Lipase purification was performed using HIC on CL-PVA–C_{12}. The separation obtained by hydrophobic chromatography gave three fractions with lipolytic activity as shown in Fig. 5. The recovery of applied activity was about 100%. Most of the activity, however, was lost during the various desalting procedures (dialysis, Sephadex G-25 gel permeation). The native electrophoresis profile of the unbound material and of the eluted fractions from HIC (Fig. 6) displayed almost 6 bands with esterase activity and the almost complete absence of proteic and nucleic impurities present in the crude preparation. It is possible to observe a band with esterase activity in the unbound material lane, not seen in the other lanes, indicating the selectivity of the polymeric stationary phase used for the different isoforms in the crude preparation. It is interesting to note that

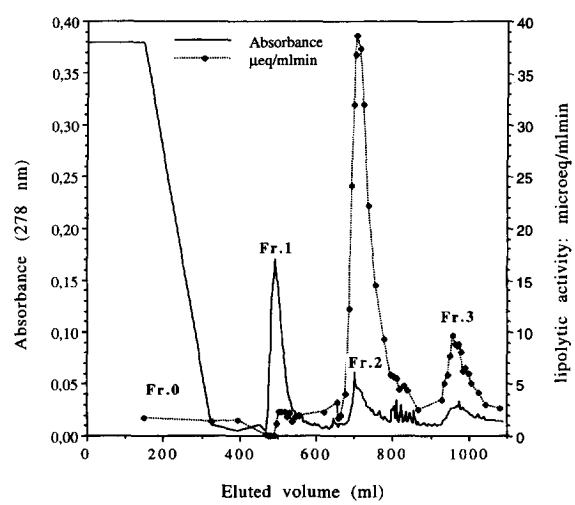

Fig. 5. Hydrophobic interaction chromatography on CL-PVA–C_{12} of a commercial preparation of *Candida rugosa* lipase. Elution by steps: fraction 0, 20 mmol/l HEPES–2 mmol/l EDTA pH 7.6; fraction 1, 1.5 mmol/l CHAPS in 20 mmol/l HEPES–2 mmol/l EDTA pH 7.6; fraction 2, 6.0 mmol/l CHAPS in 20 mmol/l HEPES–2 mmol/l EDTA pH 7.6; fraction 3, 20 mmol/l CHAPS in 20 mmol/l HEPES–2 mmol/l EDTA pH 7.6.

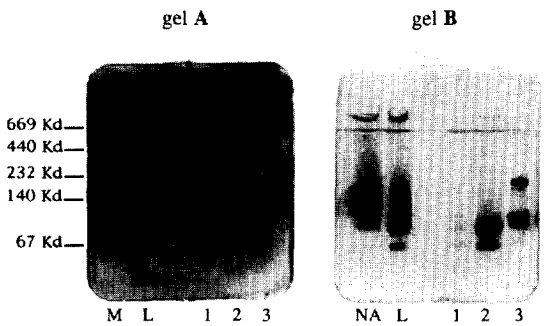

Fig. 6. Native electrophoresis. Gel A: protein staining; Gel B: esterasic activity staining; M: native marker HMV; L: raw lipase preparation; NA: not adsorbed fraction on CL-PVA-C12; 1: fraction 1 eluted with 1.5 mmol/l CHAPS; 2: fraction 2 eluted with 6.0 mmol/l CHAPS; 3: fraction 3 eluted with 20 mmol/l CHAPS; Kd: kilodaltons.

the major part of the lipolytic activity and the highest specific activity are in fraction 2. This fraction showed only two spots (one is stronger) which differ both for charge and molecular mass, while fraction 1 presented three bands and fraction 3 several bands (almost five).

The corresponding silver stained gel showed the loss of material at high and low electrophoretic mobility. For fraction 2, it is possible to observe the presence of a strong band with the same mobility as that obtained in the gel stained for esterase activity. Concerning fraction 3, in the protein stained gel there is a band in the same position as the spot at low electrophoretic mobility (in the esterase activity stained gel). In any case, no bands corresponding to the strongest stained band at higher electrophoretic mobility were detected. This emphasizes the very high specific activity of this isoform.

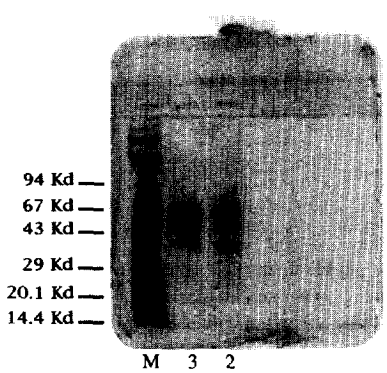

Fig. 7. SDS–PAGE. M: Marker; 3: fraction 3 from hydrophobic chromatography; 2: fraction 2 from hydrophobic chromatography.

The SDS-PAGE analysis of fractions 2 and 3 is presented in Fig. 7 and shows that all the fractions displayed a major band of M_r~60 000 and a lighter one at M_r~40 000.

4. Conclusion

The major finding presented in this study is the use of PVA polymer derivatives which proved to be good stationary phases for the HIC of *Candida rugosa* lipase. In particular CL-PVA–C_{12} gave a good performance in the separation of different isoforms of *Candida rugosa* lipase. The use of this polymer with crude commercial lipase provided three fractions with lipolytic activity with almost total recovery of enzyme activity. This indicates that there are lipases in crude commercial material that could have different affinities for the C_{12} hydrophobic arm. This finding could correspond with the different kinetic properties of these fractions.

In particular, the mechanism of interaction and the "affinity" characteristics of the support for the lipases suggest the hypothesis of a separation mainly in the adsorption step rather than in the more conventional chromatographic resolution by elution in the desorption stage. Similar polymers, differently functionalized, might be useful in the purification of lipases from different sources. In these cases, a preliminary kinetic study could indicate the best fatty acids to be utilized.

Our general conclusions are in agreement with those of Rua et al. [8] who report that the different isoforms in the commercial preparations of *Candida rugosa* lipase differ in their properties. The presence of multiple isoforms has also been observed in other microbial lipases [9–11]. These isoforms either have similar sequences or differ in post-transcriptional processing. Their significance is far from being completely understood, but could reflect the responce of the microorganism to different substrates and/or different environmental situations. This could correspond with different lipase genes detected on the *Candida rugosa* genome [12].

This multiple profile of lipase activity should encourage great care in the use of crude commercial

materials in order to ensure the reproducibility of the assay and for the interpretation of chemical studies of lipase stereospecificity towards different substrates. The use of purified and characterized enzymes is therefore mandatory. HIC similar to that described in this paper could be employed as a fast and easy procedure in isoform separation.

Acknowledgments

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