Carrier-free immobilization of lipase from *Candida rugosa* with polyethyleneimines by carboxyl-activated cross-linking

AUTHOR NAMES

Susana Velasco-Lozano[1], Fernando López-Gallego[2,3], Rafael Vázquez-Duhalt[4], Juan C. Mateos-Días[5], José M. Guisán[6] and Ernesto Favela-Torres[1]*

AUTHOR ADDRESS

[1]Departamento de Biotecnología, Universidad Autónoma Metropolitana Iztapalapa. Av. San Rafael Atlixco #186, Col. Vicentina 09340, D.F. México.

[2]Biofunctional Nanomaterials unit, CIC Biomagune, Parque tecnológico de San Sebastián, Edificio Empresarial "C", Paseo Miramón 182, 20009, Donostia-San Sebastián Guipúzcoa, Spain.

[3]Ikerbasque, Basque foundation for Science, 48011, Bilbao, Spain.

[4]Centro de Nanociencias y Nanotecnología, UNAM, Ensenada, Baja California. 22780, México.

[5]Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco, A.C., Unidad de Biotecnología Industrial, Guadalajara, México.

[6]Departamento de Biocatálisis, Instituto de Catálisis (CSIC), Campus UAM Cantoblanco, 28049, Madrid, Spain.

*Corresponding author info: Universidad Autónoma Metropolitana Iztapalapa,

Tel. +52 55 58 04 65 55 Fax: +52 55 58 04 65 54

E-mail address: favela@xanum.uam.mx (E. Favela-Torres).

KEYWORDS immobilization, lipases, cross-linked enzyme aggregates, carbodiimide, polyethyleneimine, enantioselectivity.

ABSTRACT

Carrier-free immobilization of *Candida rugosa* lipase (CRL) and polymers containing primary amino groups were cross-linked using carbodiimide. To accomplish this, the free carboxyl groups of the enzyme were activated with carbodiimide-succinimide in organic medium, and then the activated proteins were cross-linked with different polyethyleneimines (PEIs). The effect of the cross-linker chain length, the amount of added bovine serum albumin (BSA), and carbodiimide concentration on the catalytic properties of resulting cross-linked enzyme aggregates (CLEAs) was investigated. The CLEAs' size, shape, specific activity, activity recovery, thermostability and enantioselectivity significantly varied according to the preparation procedure. The highest thermostable CRL-CLEA preparation was obtained with 1.3 kDa polyethyleneimine as cross-linker, 10 mg of BSA and 28 mM of carbodiimide. This preparation is 1.3-fold more active and thermostable than CLEAs prepared by the traditional method of amino cross-linking with glutaraldehyde, and retains 60% of residual activity after 22 h at 50°C. Additionally, the CRL-CLEA preparation showed an enantioselectivity of 91% enantiomeric excess (*ee*). This immobilization procedure provides an alternative strategy for CLEA production, particularly for enzymes where the traditional method of cross-linking via lysine residues leads to enzyme inactivation.

INTRODUCTION

Lipases are a group of enzymes with important applications in the energy, food and chemical industries.[1-3] However, these biocatalysts are usually expensive and often not sufficiently stable under industrial process conditions. To address this, immobilization techniques have been developed as tools for stabilization, separation and reutilization of these enzymes, increasing their potential for use as industrial catalysts.[4-6]

Carrier-free immobilization refers to the irreversible insolubilization of enzymes, mediated by cross-linking with bifunctional reagents, to achieve enzyme-enzyme covalent bonds. This kind of immobilization avoids the use of pre-existing supports and offers advantages such as lower production costs and higher specific activity per gram of biocatalyst.[7] One of these immobilization techniques, cross-linked enzyme aggregates (CLEAs) technology, has been used for lipases, proteases, amidases and oxynitrilases[8, 9] and it appears promising for use in the stabilization of proteins and for providing robustness for immobilized biocatalysts.[10, 11]

Nevertheless, the preparation of CLEAs is challenging for enzymes with few lysine residues. This problem can be solved by cross-linking these proteins in the presence of amino-containing polymers, such as polylysine[12] and polyethyleneimine.[13-15] These compounds provide a source of free primary amino groups and facilitate intermolecular cross-linking between proteins and polymers.[16] Additionally, the absolute number of amino groups can be increased by adding bovine serum albumin (BSA) to the CLEA preparation. BSA can facilitate inter-molecular cross-linking, which increases the robustness of the final CLEAs.[17, 18]

Several reports have highlighted the parameters that control the catalytic features of CLEAs.[10, 19, 20] For example, it has been shown that the cross-linker plays a key role during the preparation and the final properties of CLEAs.[21-24] Glutaraldehyde (GA) is the most commonly employed cross-linker for CLEA preparation, since it results in a high degree of cross-linking, is

cheap to produce, and is readily available.[19] However, GA cross-linking cannot be universally employed for proteins with few lysine residues, or for those containing catalytic lysines. This is the case for nitrilases, whose activity is dramatically reduced after GA cross-linking.[25] To avoid enzyme inactivation, several groups have studied alternative cross-linkers, such as dextran polyaldehyde,[7] *p*-benzoquinones[26] and epoxides.[27] In all the latter reported methods, the chemical mechanism is based on the covalent bond between the cross-linker's aldehyde groups and lysine amino groups of enzymes.[28]

As well as lysine amino groups, free carboxyl groups from aspartic and glutamic acids are also potentially reactive and could be involved in cross-linking. In general, these acidic amino acids are more abundant than lysine residues in proteins,[29] thus could be used to increase the number of possible cross-linking sites. Carboxyl groups have also been tested for CLEA preparation. In order to increase the number of possible covalent bonds of lipase from *Candida antarctica* B (CALB), Galvis and coworkers achieved the amination of carboxyl groups of CALB prior to GA cross-linking, thus achieving CALB-CLEAs with improved catalytic rates and thermal stability.[30] Chitosan was also used as cross-linker for the production of a stable laccase-CLEA by cross-linking carboxyl groups after activation with carbodiimide.[31]

This work, to our knowledge, is the first report of CLEA preparation by cross-linking the enzyme surface carboxyl groups with polyethyleneimines, mediated by EDC-NHS. We studied different parameters in order to optimize CLEA preparation. Morphological and functional studies were carried out in order to further characterize these insoluble biocatalysts.

MATERIALS AND METHODS

Reagents. *Candida rugosa* lipase (CRL) TVII was purchased from Sigma-Aldrich (St. Louis, MO, USA). 1-Ethyl-3-[3-dimethylaminopropil]carbodiimide (EDC) and N-hydroxysuccinimide (NHS) were purchased from Pierce Scientific (Mexico City, Mexico). Polyethyleneimine (PEI) with different molecular weights (MW), i.e. 1300 ($PEI_{1.3}$), 25,000 (PEI_{25}), 70,000 (PEI_{70}) and 750,000 (PEI_{750}) Da, hexamethylenediamine (DH), bovine serum albumin (BSA), trioctanoin, *p*-nitrophenyl butyrate (pNPB), glutaraldehyde solution (25% in water) and 2-morpholinoethanesulfonic acid monohydrate (MES) were obtained from Sigma-Aldrich (St. Louis, MO, USA). 2-*O*-butyryl-2-phenylacetic acid (BPA) was prepared as described previously.[32] Cyanogen bromide activated agarose (CNBr-agarose) was purchased from GE Healthcare (Uppsala, Sweden).

Production of CLEAs by carboxyl cross-linking. Different amounts (as indicated) of both CRL and BSA powders were suspended in 2 mL of trioctanoin/acetonitrile (0.05 g/25 mL) solution in 15 mL conical tubes placed in an ice bath and allowed to stand for 60 min at 4°C. Then, EDC dissolved in acetonitrile was added drop-by-drop under vortex agitation and allowed to react for 5 min at 25°C and 750 rpm (to dissolve the EDC in acetonitrile, hydrochloric acid was used in a molar ratio of 1:1; this solution was prepared 1 h before it was used and maintained at room temperature). After 5 min of reaction time, NHS dissolved in acetonitrile was added drop-by-drop and left for 5 min under vortex agitation (equimolar concentrations of both EDC and NHS during the cross-linking process were used,[33, 34] i.e. when 28 mM of EDC was employed, the concentration of NHS was also 28 mM). The cross-linker dissolved in distilled water was then added drop-by-drop to reach a final concentration of 3 g/L and allowed to react for 30 min at 25°C and 750 rpm. Afterwards, the mixture was centrifuged at 10,000 rpm at 4°C

for 5 min and the organic supernatant was discarded. The pellet was suspended in hydroxylamine buffered solution five times more concentrated that the EDC concentration employed (0.1 M MES, 0.5 M NaCl buffer, pH 5) and allowed to react for 1 h at 25°C and 750 rpm. CLEAs were centrifuged at 10,000 rpm for 5 min at 4°C and washed three times with the same volume of MES-NaCl buffer. Finally, CLEAs were suspended in 25 mM phosphate buffer (pH 7) and stored at -20°C.

Production of CLEAs by amino cross-linking. CRL and BSA powders (100 and 10 mg, respectively) were suspended in 2 mL of trioctanoin/acetonitrile (0.05 g/25 mL) solution in 15 mL conical tubes placed in an ice bath and allowed to stand for 60 min at 4°C. Then, $PEI_{1.3}$ water solution and GA solution was added drop-by-drop to reach a final concentration of 3 g/L and 14 mM, respectively. The reaction mixture was shaken (750 rpm) for 30 min at 25°C. The mixture was then centrifuged at 10,000 rpm at 4°C for 5 min and the organic supernatant was discarded. The pellet was suspended in 10 mL of a buffered solution (0.1 M MES, 0.5 M NaCl, pH 5). CLEAs were then centrifuged at 10,000 rpm for 5 min at 4°C and washed three times with the same volume of MES-NaCl buffer. Finally, CLEAs were suspended in 25 mM phosphate buffer (pH 7) and stored at -20°C.

Immobilization of CRL on cyanogen bromide activated support. Ten milliliters of CRL solution (10 mg of powder in 10 mL of 25 mM phosphate buffer, pH 7 with 0.05% w/v Triton X-100) were added to 1 g of CNBr-agarose (the use of the detergent was to avoid the attachment of lipase dimers). After 7 min at 4°C, immobilization was stopped by filtration through a POBEL Buchner funnel with perforate plate No. 2. The filtered derivative was suspended in 10 mL of 1 M ethanolamine at pH 7 and incubated for 2 h at 4°C. Then, the immobilized preparation was

filtered and washed with 10 volumes of distilled water and finally rinsed with 25 mM phosphate buffer solution at pH 7.

Protein quantification. Protein was quantified by Bradford's method.[35] Twenty microliters of enzyme solution were mixed with 1 mL of Bradford reagent, incubated at room temperature for 15 min, and then the absorbance was measured at 595 nm. The protein content was estimated using a calibration curve using BSA as a standard.

Activity assay. Lipase activity was determined by the hydrolysis of *p*NPB as follows: 20 µL of 50 mM *p*NPB in acetonitrile was mixed with 2 mL of 25 mM phosphate buffer at pH 7. To start the reaction, 50 µL of an adequately diluted enzyme solution or CLEA suspension was added. The *p*-nitrophenol concentration was estimated as the increase in absorbance at 348 nm. A *p*-nitrophenol standard curve was carried out under the same conditions. One unit of activity (U) was defined as the amount of enzyme required to release one µmol of *p*-nitrophenol per minute. The activity recovery as CLEA was calculated with equation 1.

$$Activity\ recovery\ (\%) = \left(\frac{Total\ activity\ of\ CLEA}{Total\ initial\ activity\ of\ the\ free\ enzyme}\right) x100 \qquad \text{Eq. 1}$$

Thermal stability. Thermal stability was determined by incubating enzyme solutions or CLEA suspensions in 25 mM phosphate buffer at pH 7 at 50°C for 22 h in a water bath. Samples were taken at 0.5, 1, 2, 4, 6, and 22 h of incubation, and their residual activity was measured as above described.

CLEA scanning electron microscopy. Scanning electron micrographs of CLEA suspension in distilled water were obtained with a Hitachi TM-1000 scanning electron microscope (SEM). The size and shape of CLEA were determined.

Enzymatic hydrolysis of 2-O-butyryl-2-phenylacetic acid. Pellets from 10 mL CLEA preparations (30-40 µg of protein per mL) were added to 3 mL of 5 mM racemic 2-*O*-butyryl-2-phenylacetic acid (BPA) in 10 mM sodium acetate buffer at pH 5. Solutions were maintained under mild agitation at 25°C for different time intervals. Substrate and products of the enzymatic reaction were analyzed by HPLC (Spectra System P4000 coupled with an UV-diode array detector, Spectra System SN4000) using a reverse-phase Kromasil C18 (25 cm×0.4 cm) column. Elution was performed with 10 mM ammonium acetate in acetonitrile/water (35:65, v/v) buffer at pH 3.2 at a flow rate of 1 mL/min. Absorbance was measured at 210 nm. One unit of activity (U) was defined as the amount of enzyme releasing one µmol of mandelic acid per minute.

Enantiomeric excess (ee) and enantiomeric ratio (E) determinations. The enantiomeric excess of the released acid was analyzed using Chiral Reverse Phase HPLC. Enzyme concentrations yielding conversion degrees from 10 to 15% were used to assure that the enzyme kinetics were in the first-order region. The column used was a Chiralcel OD-R, the mobile phase was an isocratic mixture of 10 mM ammonium phosphate in acetonitrile/water (5%/95% v/v) buffer, at pH 2.3 and a flow rate of 0.5 mL/min. Absorbance was measured at 210 nm. Retention times for the *S* and *R* mandelic acids were 28.5 and 32.5 min respectively. The enantiomeric ratio (*E*) was directly calculated from the ratio between the reaction rates of both isomers. Calibration curves for both isomers of mandelic acid were determined for concentrations of 1, 10, 100, and 1000 ppm.[36] At concentrations of 100 ppm a correction factor of 1.037 was applied for the *S*-isomer, which displayed slightly lower response than the *R*-isomer. The *ee* was calculated using the equations by Chen et al.[37]

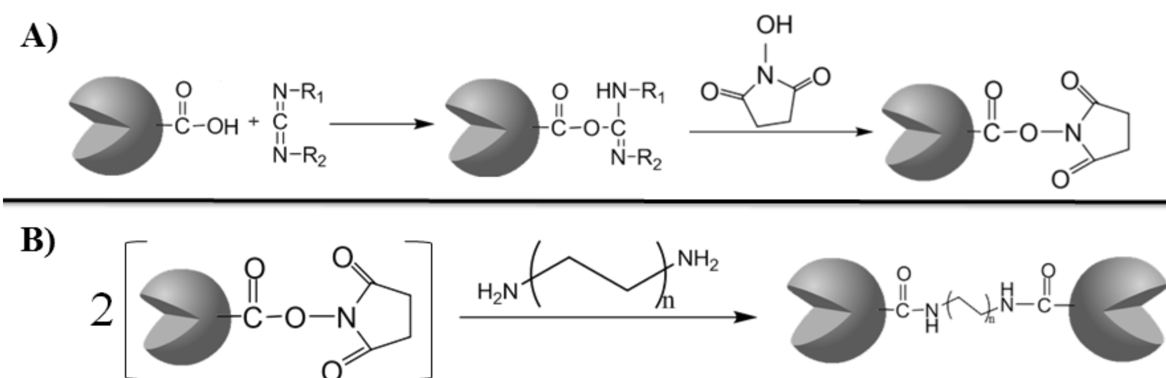
RESULTS AND DISCUSSION

Candida rugosa lipase (CRL) was selected as a model system due to its high content (2.6-fold more than the amino groups) of carboxylic acid groups (Data obtained from the reported 3D structure from CRL at Protein Data Bank (PDB) with PDB code 1LPS). Additionally, these carboxyl groups are well spread in the enzyme molecule (Supporting information Fig. 1S). Furthermore, successful production of CLEAs of lipase from *Candida rugosa* (CLR) cross-linked with GA has been well documented.[8, 9, 21, 38] Aminated polymers were used to evaluate the effect of the cross-linker length on the catalytic properties of carboxyl cross-linked CLEAs (carboxyl-CLEA).

CLEA production was carried out without a previous precipitation step. Instead, powdered enzyme was directly suspended in acetonitrile and then cross-linked. In order to maintain the active conformational state of lipase during its immobilization, CLEA production was performed in the presence of trioctanoin, a well-documented substrate for lipases; this approach has demonstrated to be useful to preserve the active conformational state of enzymes during their immobilization.[39] Different concentrations of trioctanoin were evaluated and the addition of 50 mM trioctanoin provided two-fold more active CLEAs (Supporting information Fig. 2S).

The preparation of carboxyl-CLEAs was performed in two steps based on the well-known chemistry of carbodiimide. First, the carboxyl groups of the enzyme were activated with 1-ethyl-3-[3-dimethylaminopropil] carbodiimide (EDC), followed by N-hydroxysuccinimide (NHS) to enable them to react with primary amines.[40] The activated carboxyl groups were then covalently bonded to the primary amino groups of the polyethyleneimine (PEI) (Scheme 1). Polyethyleneimines are multi-amino polymers containing numerous primary, secondary and tertiary amino groups. Because of their capacity to establish electrostatic interactions with

proteins, these charged polymers have been employed in purification,[41] immobilization,[42] and as coating-agents[43] of enzymes.



Scheme 1. Cross-linking of enzymes through their carboxyl groups. A) Activation of enzyme carboxyl groups by the addition of EDC-NHS. B) Amide bond formation between the amino-containing polymer and enzymes.

Different conditions for the production of carboxyl-CLEAs were assessed in order to yield CLEA with both high specific activity and thermostability. Enantioselectivity was also evaluated for the different lipase-CLEA preparations, since lipases are well-known as enantioselective biocatalysts. 2-*O*-butyryl-2-phenyl acetic acid (BPA) was chosen as a model substrate to study enzyme enatioselectivity because the resulting product–mandelic acid–is an important precursor in the production of a great variety of antibiotics,[44, 45] anti-tumoral agents,[46] and chiral alcohols.[47] We also compared the catalytic properties of these novel protein aggregates to CRL immobilized on pre-existing carriers such as cyanogen bromide agarose. This latter immobilization procedure allows for one point attachment of the enzyme,[48] resulting in fewer structural modifications and showing an E (S) = 9.5.

Effect of different preparation conditions on CLEAs' properties

Extent of cross-linking. The effect of the concentration of the EDC used to activate the carboxyl groups of both BSA and CRL was evaluated (Table 1). The variation of the EDC concentration modulates the extent of cross-linking by controlling the degree of activation of the carboxyl groups of the protein. Both specific activity and activity recovery decreased when the carbodiimide concentrations increased. The higher EDC concentration lowered the activity recovery; this is the result of a higher rigidification of enzymes with the consecutive loss of enzyme flexibility. However, under low EDC concentration we did not observe CLEA formation. A similar trend was observed for GA lipase CLEA preparations[22, 38] and for other cross-linking agents like *p*-benzoquinones.[26] These results show that CLEA optimization requires a commitment between the extent of cross-linking (EDC concentration) and activity recovery. The highest activity recovered after CLEA production was 15% of the initial activity present in the free enzyme (Table 1) by using 13-20 mM of EDC. This value was comparable to that found by Kartal *et al*. who recovered 26% of the initial activity after optimization of CRL-CLEA cross-linked with glutaraldehyde.[38] CRL-CLEA preparation at 50 and 100 mM of EDC did not improve the specific activity and activity recovery presented in table 1 (data not shown). Additionally, EDC concentrations higher than 80 mM resulted in nearly 50% loss of free CRL activity (data not shown).

Table 1. Effect of EDC concentration on CLEAs' properties.

EDC (mM)	CLEA diameter[1] (μm)	pNPB Specific activity[2] (U/mg)	Activity recovery[3] (%)	BPA Specific activity[4] (U/g)	*ee* *(%)*	*E*[5] (*S*)
13	20-80	75[a]	15[a]	5.8[a]	82[a]	10[a]
20	20-100	71[a]	15[a]	4.5[b]	86[b]	13[a,b]
28	50-100	33[b]	12[b]	3.1[c]	91[c]	21[c]
35	50-150	18[c]	11[b,c]	2.0[c,d]	86[b]	13[a,b]
40	50-150	13[c]	9[c]	1.1[d]	88[b,c]	15[b]

All CLEAs were prepared at 30 mg/mL [enzyme], 3 g/L [$PEI_{1.3}$] and 10 mg of BSA. [1]CLEA diameters were estimated from SEM images. [2]Reaction conditions were 0.5 mM of pNPB in phosphate buffered solution (25 mM, pH 7.0) at 25°C. [3]Activity recovery was estimated from CLEA activity with pNPB as substrate. [4]Reaction conditions were 5 mM of BPA in acetate buffered solution (10 mM, pH 5.0) at 25°C. [5]*E* was calculated from the reaction rates ratio *S*-mandelic acid/*R*-mandelic acid. [a,b,c,d,e]Numbers with different letters are statistically different ($\alpha > 0.05$). Values are the average of three independent assays.

In morphological terms, CLEAs made with low EDC concentration (< 20 mM) were smaller and presented as sparse structures whereas bigger, denser and more grained structures were obtained with higher EDC concentrations (>28 mM) (Supporting Information Fig. 3S).

The highest enantioselectivity value (*ee* 91%) was obtained with 28 mM EDC as cross-linker (Table 1). Nevertheless, both lower enantioselective and active CLEA were found to have a high degree of cross-linking, showing a compromise between specific activity and enantioselectivity. These results strongly agree with the results of CLEAs of *Burkholderia cepacia* lipase prepared with glutaraldehyde as cross-linker.[22]

Finally, we also evaluated the thermal stability of the different preparations (Fig. 1). They showed higher thermostability than the free enzyme in all cases. CLEAs prepared with 28-35 mM EDC showed the highest thermal stability, retaining 63% of their initial activity after 22 h incubation at 50ºC and pH 7 (Fig. 1). These results suggest that a higher degree of protein cross-linking increases the enzyme structural rigidity (over the entire tertiary protein structure),

consequently providing higher thermal stability. At higher cross-linker concentration (40 mM), the chemical modification of the protein might result on structural distortions with negative effect on the thermal stability of the protein. The stability of an enzyme might depend on flexibility and rigidity of its active site. It has been demonstrated that interactions as hydrophobic ones; main chain-main chain hydrogen bonds, side chain-main chain hydrogen bonds, side chain-side chain hydrogen bonds, ionic interactions and aromatic-aromatic interactions play an important role in the thermal stability of some lipases.[49] Improved thermal stability in CLEAs is also related to the degree of cross-linking when glutaraldehyde was employed as the cross-linker.[22]

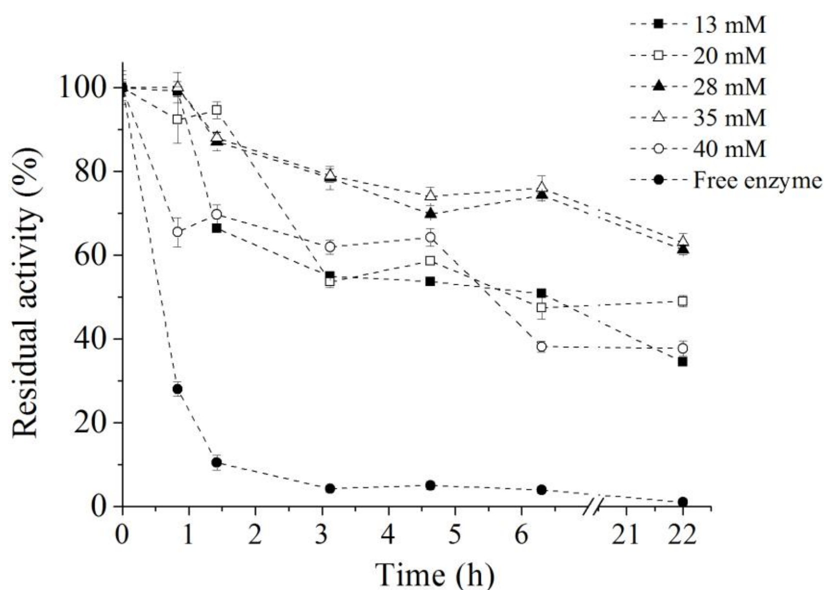


Figure 1. Effect of EDC concentration on thermal inactivation of free CRL and CRL-CLEA at 50°C pH 7. Symbols represent the mean value of two independent assays ± standard deviation.

BSA as an aid to increase the cross-linking efficiency. In order to evaluate the effect of the addition of BSA in CLEA formation, we chose 28 mM EDC as the optimal cross-linking condition based on the results obtained previously. Benefits of using BSA as a protein feeder for production of CLEAs are well documented.[17, 18] Different amounts of BSA were added, and then

the catalytic properties of CLEA preparations were evaluated. Firstly, adding BSA during CLEA preparation notably affected both size and catalytic properties (Table 2). A strong positive effect on specific activity and enantioselectivity was observed when low amounts (< 10 mg) of BSA were present during the production of CLEAs. Although CLEA morphology was the same at different concentrations of BSA (Supporting information Fig. 4S), at lower BSA concentrations, CLEA were smaller and more active.

Table 2. Effect of the amount of added BSA on CLEAs' properties.

BSA (mg)	CLEA diameter[1] (μm)	pNPB specific activity[2] (U/mg)	Activity recovery[3] (%)	BPA specific activity[4] (U/g)	*ee* *(%)*	E^5 (*S*)
0	20-100	36[a]	13[a]	3.1[a]	92[a]	24[a]
5	50-100	32[b]	12[a]	4.5[b]	92[a]	23[a]
10	50-100	33[b]	12[a]	3.0[a]	91[a,b]	21[a]
30	100-200	11[c]	9[b]	1.2[c]	87[b,c]	15[b]
50	150-250	3[d]	9[b]	0.2[d]	84[c]	11[b]

All CLEAs were prepared at 30 mg/mL [enzyme], 3 g/L [$PEI_{1.3}$] and 28 mM [EDC]. [1]CLEA diameters were estimated from SEM images. [2]Reaction conditions were 0.5 mM of pNPB in phosphate buffered solution (25 mM, pH 7.0) at 25°C. [3]Activity recovery was estimated from CLEA activity with pNPB as substrate. [4]Reaction conditions were 5 mM of BPA in acetate buffered solution (10 mM, pH 5.0) at 25°C. [5]*E* was calculated from the reaction rates ratio *S*-mandelic acid/*R*-mandelic acid. [a,b,c,d]Numbers with different letters are statistically different ($\alpha > 0.05$). Values are the average of three independent assays.

It has been reported that CLEAs prepared with GA in the presence of BSA retained higher catalytic activity than those prepared without BSA.[50] For example, CLEAs of lipases from *Pseudomonas cepacia* prepared with GA in the presence of BSA retained 100% of their native activity, while the same immobilized enzymes with GA in the absence of BSA retained only 4% of the initial activity.[17] In contrast, our results showed that specific activity and activity recovery are nearly independent of BSA addition at low BSA concentrations (<10 mg). BSA did not seem to induce any positive effect on enzyme specific activity of the final CLEA preparations, as previously reported for CLEAs prepared with GA for different enzymes, including CRL.[38, 51, 52]

The CLEAs' enantioselectivity at different concentrations of BSA was also investigated. A substantial effect on enantioselectivity was observed at high BSA load (above 10 mg) during CLEA preparation. Similar enantioselectivities were obtained with 0, 5, and 10 mg of added BSA, whilst 30 and 50 mg of BSA resulted in lower enantioselectivities (Table 2). Conversely, low specific activity values, during the hydrolysis of BPA, were observed at high BSA concentrations, with 22-fold more active CLEAs when 5 mg of BSA were added as compared with the specific activity obtained when 50 mg of BSA were loaded (Table 2).

We have observed that optimal addition of BSA (10 mg) yielded CLEAs with significantly higher thermal stability (Fig. 2). The same BSA effect was found in laccase CLEAs prepared at different BSA/enzyme ratios,[51] as well as for penicillin acylase CLEAs.[17] However, higher BSA concentrations (> 10 mg) resulted in a decrease of thermal stability, as shown for CLEA prepared with 30 and 50 mg of BSA, probably due to a lower degree of enzyme cross-linking. This fact is supported by the high concentration of released protein after the leakage stability test (heating at 95 ºC, for 5 min) of CLEAs produced with high amounts of BSA (>10 mg) (Supporting information Fig. 5S).

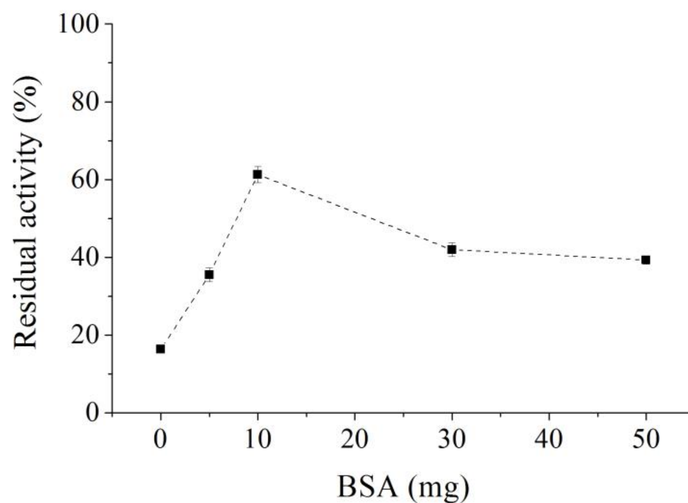


Figure 2. Effect of the amount of BSA on thermal inactivation of CRL-CLEA at 50°C pH 7 for 22 h. Symbols represent the mean value of two independent assays ± standard deviation.

Effect of cross-linker length. We studied the effect of polyethyleneimines (PEIs) chain length on CRL-CLEA properties by preparing them with 10 mg of BSA and 28 mM EDC as optimal conditions. Medium polymeric cross-linker ($PEI_{1.3}$) yielded CLEAs with higher specific activity than with the shortest cross-linker (DH) (Table 3). This effect could be attributed to the formation of less compact CLEAs due to the longer cross-linker, which enables larger intra-molecular spacing compared to the shortest cross-linker.[53] However, the larger cross-linkers (PEI_{70} and PEI_{750}) tested, resulted in bigger and less active CLEAs than those obtained with the $PEI_{1.3}$. This effect was also reported with dextran-aldehyde (100-200 kDa), which produced less adequate CLEAs than glutaraldehyde (0.1 kDa).[23]

Table 3. Effect of the cross-linker molecular weight on CLEAs' properties.

Cross-linker	Cross-linker Mw (kDa)	CLEA diameter[1] (μm)	pNPB specific activity[2] (U/mg)	Activity recovery[3] (%)	BPA specific activity[4] (U/g)	*ee* *(%)*	E[5] (*S*)
DH	0.12	10-70	30^{a}	9^{a}	3.0^{a}	87^{a}	14^{a}
$PEI_{1.3}$	1.3	50-100	33^{a}	12^{b}	3.3^{a}	91^{b}	22^{b}
PEI_{70}	70	100-200	24^{b}	7^{c}	3.7^{a}	93^{b}	26^{c}
PEI_{750}	750	100-300	22^{b}	11^{d}	1.8^{b}	91^{b}	21^{b}

All CLEAs were prepared at 30 mg/mL [enzyme], 28 mM [EDC], 10 mg of BSA and 3 g/L of each cross-linker. [1]CLEA diameters were estimated from SEM images. [2]Reaction conditions were 0.5 mM of pNPB in phosphate buffered solution (25 mM, pH 7.0) at 25°C. [3]Activity recovery was estimated from CLEA activity with pNPB as substrate. [4]Reaction conditions were 5 mM of BPA in acetate buffered solution (10 mM, pH 5.0) at 25°C. [5]*E* was calculated from the reaction rates ratio *S*-mandelic acid/*R*-mandelic acid. [a,b,c,d]Numbers with different letters are statistically different ($\alpha > 0.05$). Values are the average of three independent assays.

Regarding the enantioselectivity, although specific activity of CLEAs towards BPA is nearly independent of the cross-linker length, the *E* value increased with larger cross-linker lengths (Table 3). The polyethyleneimines employed in this work are ionic polymers providing a

positively charged microenvironment around the enzyme and are responsible for hydrophilic and electrostatic conditions in the surroundings of the enzymes. Therefore, different degree of polymerization of PEI will modify the open-closed equilibrium of the lipase´s lid and the conformation of the binding pocket, resulting in different enzyme selectivity.[13-15]

The open-close equilibrium of lipases is also mediated by electrostatic interactions between the enzyme surface and the external face of the lid when it is open. These interactions might be modified in presence of hydrophilic polymers around the enzyme. Therefore, in this particular case, longer cross-linkers provided a major positively charged microenvironment resulting in conformational changes in the enzyme structure. As a result of this, an improved enantioselectivity was observed in the tested hydrolytic resolution (Table 3).

For example, CLEAs preparations that contain DH (DH-CLEAs), showed similar enantioselectivity to the CRL immobilized on agarose activated with cyanogen bromide groups ($E_{(S)}$ = 9.5) that provide hydrophilic but neutral environments for the protein. Instead, $PEI_{1.3}$- and PEI_{70}-CLEAs provide higher hydrophilic and positively charged environments, showing improved enantioselectivity than the DH-CLEAs and the CRL-CNBr. Nevertheless, an overly increase in the positive charge that surrounds the enzyme (i.e. when PEI_{750} was used) decreased the enzyme enantioselectivity. We found that CLEAs produced with PEI_{750} were less enantioselective than CLEAs produced with PEI_{70}. In order to improve the enantioselectivity, we used shorter PEIs (MW 25 kDa), that did not improve the enantioselectivity values found for the CLEAs containing $PEI_{1.3}$ (data not shown).

Length of cross-linkers also showed marked effects on thermal deactivation of CLEAs (Fig. 3). Small and compact CLEAs are more thermostable than bigger and sparse CLEAs. The highest thermal stability was obtained with $PEI_{1.3}$-CLEA, prepared with 10 mg of BSA at 28 mM EDC.

Under these production conditions, $PEI_{1.3}$-CLEA maintained 61% of its initial activity after 22 h under the assayed conditions, while the free enzyme lost 50% of its initial activity in less than 2 h (Fig. 3).

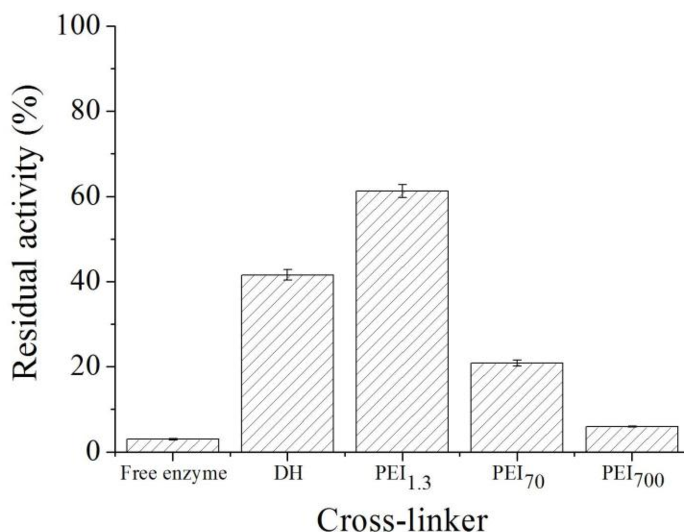


Figure 3. Effect of the cross-linker on thermal inactivation of CRL and CRL-CLEA at 50°C after 22 h at pH 7. Bars represent the mean value of two independent assays ± standard deviation.

Besides the improvement in thermal stability, PEIs have been used for coating immobilized enzymes on pre-existing carriers and have been successfully employed in order to enhance the stability of the biocatalyst during the synthesis of hydrophobic compounds in organic solvents.[54] The stabilization effect may be attributed to the micro-hydrophilic environment created around the enzyme. This environment may keep the water layer surrounding the enzyme. This layer is crucial for enzyme flexibility and would decrease the organic concentration in a potential two-phase system.[43]

An important property of CLEAs for their application in a large scale is their size, which is directly implicated by their mass transfer and filterability.[19] Preferably, CLEAs with smaller

particle size and more porosity are favorable because they enable higher enzyme-substrate contact, whilst bigger particle sizes diminish the formation of enzyme-substrate complexes of the inner enzymes thus wasting their activity.[20] Typically, the particle size of CLEAs varies from 0.1-200 µm. However, during recycling, the use of filtration and centrifugation operations leads to the formation of clusters (aggregated CLEAs), resulting in mass transfer limitations with a consequent reduction in overall specific activity.[55] Among the factors governing CLEA size, enzyme and cross-linking agent concentrations are important variables. Both parameters can also modify the recovery of enzymatic activity.[23]

The effect on the CLEA particle size was estimated by SEM. The images revealed that the size of the obtained CLEAs was strongly dependent on the type and size of the cross-linker agent used (Fig. 4).

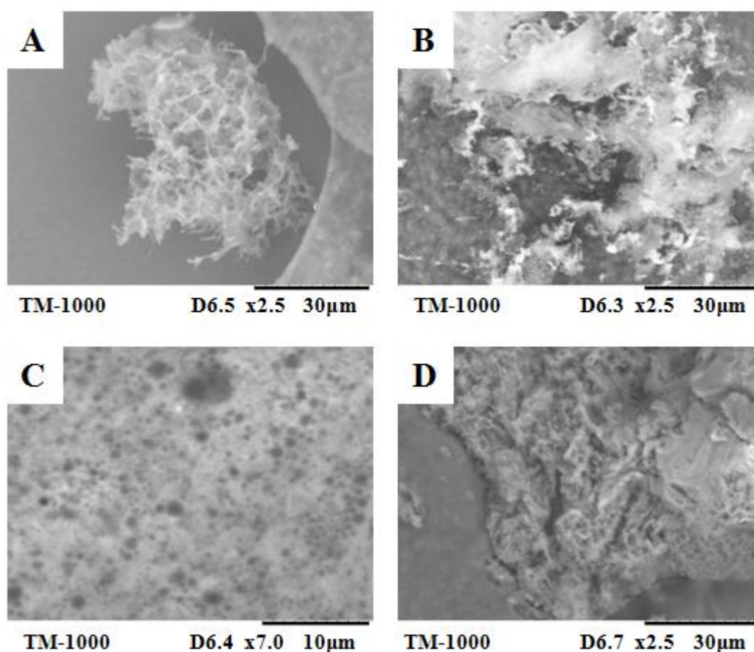


Figure 4. Differences in CLEA morphology and size due to the type and size of the cross-linker. A) DH-CLEA, B) $PEI_{1.3}$-CLEA, C) PEI_{70}-CLEA, and D) PEI_{750}-CLEA.

As expected, smaller CLEAs were obtained with the shorter cross-linkers and bigger CLEAs were achieved with the larger cross-linkers (Table 3). Moreover, cross-linker length also affected the form of CLEA. Small (DH) and medium size ($PEI_{1.3}$) cross-linkers yielded aggregates with a curly-network branched form, whilst CLEAs obtained with long (PEI_{70} and PEI_{750}) cross-linkers were shaped as a very large and homogeneous porous mesh (Fig. 4).

In view of the specific activity and the activity recovery, thermal deactivation and enantioselectivity, the best conditions for CLEA production were: $PEI_{1.3}$ as the cross-linker, 10 mg of added BSA, and a 28 mM concentration of EDC. Although PEI_{70}-CLEA showed higher enantioselectivity than $PEI_{1.3}$-CLEA, the latter was three-fold more thermostable (Fig. 4), whereas the improvement in selectivity of PEI_{70}-CLEA was not so marked (1.2 times higher) as the improvement in thermal stability. The CLEAs prepared under the above-mentioned conditions were compared with glutaraldehyde cross-linked CLEAs in terms of specific activity, activity recovery, thermal deactivation and enantioselectivity.

Comparison of amino and carboxyl cross-linked CLEA. Traditionally, glutaraldehyde is the cross-linker choice during CLEA preparation. Hence, amino-CLEA (obtained by GA cross-linking) and carboxyl-CLEA were compared. Both CLEAs were prepared in the presence of $PEI_{1.3}$ (at the same concentration), 10 mg of added BSA and with equal cross-linking time.

Hydrolysis of *p*NPB and 2-*O*-butyryl-2-phenylacetic acid was 1.3 and 2.3 times more active for carboxyl-CLEA than for amino-CLEA, respectively (Table 4). Carboxyl-CLEA was more thermostable than amino-CLEA (Table 4). This increase in stability can be explained in terms of molecular rigidity, due the higher degree of cross-linking achieved by the cross-linking of carboxyl groups compared to the less abundant amino groups. This effect was also found in

CLEA of CALB cross-linked with glutaraldehyde before the amination of its carboxyl groups with ethylenediamine when compared with CALB-CLEA cross-linked with glutaraldehyde without prior amination.[30] Regarding selectivity, both CLEAs exhibited similar enantioselectivity towards the resolution of *S*-mandelic acid, and the same enantiomeric excesses were reached (Table 4).

Table 4. Effect of different cross-linker binding sites during CRL-CLEA preparation

Cross-linking binding site	pNPB specific activity[1] (U/mg)	Activity recovery[2] (%)	Residual activity[3] (%)	BPA specific activity[4] (U/g)	*ee* (%)	*E*[5] (*S*)
Carboxyl groups	33[a]	12[a]	61	3.2[a]	91[a]	22[a]
Amino groups	24[b]	12[a]	19	1.4[b]	92[a]	26[b]

All CLEAs were prepared at 30 mg/mL [enzyme], [$PEI_{1.3}$] 3 g/L, 10 mg of BSA and [EDC] = 28 mM or [GA] = 14 mM. [1]Reaction conditions were 0.5 mM of pNPB in phosphate buffered solution (25 mM, pH 7.0) at 25°C. [2]Activity recovery was estimated from CLEA activity with pNPB as substrate. [3]Thermal stability conducted by incubation at 50°C and pH 7.0 for 22 h. [4]Reaction conditions were 5 mM of BPA in acetate buffered solution (10 mM, pH 5.0) at 25°C. [5]*E* was calculated from the reaction rates ratio *S*-mandelic acid/*R*-mandelic acid. [a,b]Numbers with different letters are statistically different ($\alpha > 0.05$). Values are the average of three independent assays.

Finally, carboxyl-CLEAs were found to be potentially useful for enzyme cross-linking because they show better specific activity and thermal stability than amino-CLEAs, while both CLEAs exhibited similar selectivity towards *S*-mandelic acid.

CONCLUSIONS

Production of CLEA by immobilization of the *Candida rugosa* lipase was successfully achieved by cross-linking the carboxyl groups of lipase with polyethyleneimines. CLEA preparation was carried out by activating the carboxyl groups of the enzyme after the addition of EDC-NHS. The best CRL-CLEA exhibited some advantages such as higher enantioselectivity and thermal stability compared to the free enzyme. The cross-linker chain length and the addition of BSA

have an important effect on morphology, specific activity, thermostability, and enantioselectivity of the obtained CLEAs. Additionally, carboxyl-CLEAs were more active and thermostable than amino-CLEAs obtained via glutaraldehyde cross-linking. Therefore, the approach used in this study represents a potential option for CLEA preparation of other enzymes, which are inactivated with GA. The results obtained open up a new field of research related to the features that control the final performance of CLEAs for profitable preparation and improved industrial applications.

ACKNOWLEDGMENTS

The Mexican Council for Science and Technology (CONACyT) supported this work (Project No. 154004). S. Velasco is grateful for the scholarship received from CONACyT. We would like to thank the Spanish Government for the Juan de la Cierva fellowship and Basque country government for the Ikerbasque fellowship that funded PhD Fernando López-Gallego.

DECLARATION OF INTEREST

The authors declare no competing financial interest.

SUPPORTING INFORMATION AVAILABLE

The supporting information section provides: 1) The distribution of aspartic, glutamic and lysine residues in the CRL 3D structure, 2) The effect on the addition of trioctanoin during CLEA preparation, 3) Scanning Electron Microscopy images of CLEA preparations according to the variation in the amount of added BSA and the EDC concentration, and 4) SDS-PAGE gel analysis of CRL-CLEA at different concentrations of BSA. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

(1) Adlercreutz, P. *Chem. Soc. Rev.* **2013**, 42, 6406-6436.
(2) Ferreira-Dias, S.; Sandoval, G.; Plou, F.;Valero, F. *Electron. J. Biotechnol.* **2013**, 16, doi: 10.2225/vol16-issue3-fulltext-5.
(3) Andualema, B.;Gessesse, A. *Biotechnology* **2012**, 11, 100-118.
(4) Stepankova, V.; Bidmanova, S.; Koudelakova, T.; Prokop, Z.; Chaloupkova, R.;Damborsky, J. *ACS Catal* **2013**, 3, 2823-2836.
(5) Cantone, S.; Ferrario, V.; Corici, L.; Ebert, C.; Fattor, D.; Spizzo, P.;Gardossi, L. *Chem. Soc. Rev.* **2013**, 42, 6262-6276.
(6) Garcia-Galan, C.; Berenguer-Murcia, A.; Fernandez-Lafuente, R.;Rodrigues, R. C. *Adv. Synth. Catal.* **2011**, 353, 2885-2904.
(7) Mateo, C.; Palomo, J. M.; Van Langen, L. M.; Van Rantwijk, F.;Sheldon, R. A. *Biotechnol. Bioeng.* **2004**, 86, 273-276.
(8) López-Serrano, P.; Cao, L.; Van Rantwijk, F.;Sheldon, R. A. *Biotechnol. Lett.* **2002**, 24, 1379-1383.
(9) Schoevaart, R.; Wolbers, M. W.; Golubovic, M.; Ottens, M.; Kieboom, A. P. G.; Van Rantwijk, F.; Van Der Wielen, L. A. M.;Sheldon, R. A. *Biotechnol. Bioeng.* **2004**, 87, 754-762.
(10) Roessl, U.; Nahálka, J.;Nidetzky, B. *Biotechnol. Lett.* **2010**, 32, 341-350.
(11) Sheldon, R. A. *Org. Process Res. Dev.* **2011**, 15, 213-223.
(12) Yamaguchi, H.; Miyazaki, M.; Asanomi, Y.;Maeda, H. *Catal. Sci. Technol.* **2011**, 1, 1256-1261.
(13) López-Gallego, F.; Betancor, L.; Hidalgo, A.; Alonso, N.; Fernández-Lafuente, R.;Guisán, J. M. *Biomacromolecules* **2005**, 6, 1839-1842.
(14) Wilson, L.; Fernández-Lorente, G.; Fernández-Lafuente, R.; Illanes, A.; Guisán, J. M.;Palomo, J. M. *Enzyme Microb. Technol.* **2006**, 39, 750-755.
(15) Vaidya, B. K.; Kuwar, S. S.; Golegaonkar, S. B.;Nene, S. N. *J. Mol. Catal. B: Enzym.* **2012**, 74, 184-191.
(16) Pan, J.; Kong, X. D.; Li, C. X.; Ye, Q.; Xu, J. H.;Imanaka, T. *J. Mol. Catal. B: Enzym.* **2011**, 68, 256-261.
(17) Shah, S.; Sharma, A.;Gupta, M. N. *Anal. Biochem.* **2006**, 351, 207-213.
(18) Cruz, J.; Barbosa, O.; Rodrigues, R. C.; Fernandez-Lafuente, R.; Torres, R.;Ortiz, C. *J. Mol. Catal. B: Enzym.* **2012**, 80, 7-14.
(19) Sheldon, R. A. *Appl. Microbiol. Biotechnol.* **2011**, 92, 467-477.
(20) Talekar, S.; Joshi, A.; Joshi, G.; Kamat, P.; Haripurkar, R.;Kambale, S. *RSC Advances* **2013**, 3, 12485-12511.
(21) Yu, H. W.; Chen, H.; Wang, X.; Yang, Y. Y.;Ching, C. B. *J. Mol. Catal. B: Enzym.* **2006**, 43, 124-127.
(22) Majumder, A. B.; Mondal, K.; Singh, T. P.;Gupta, M. N. *Biocatal. Biotransform.* **2008**, 26, 235-242.
(23) Valdés, E. C.; Soto, L. W.;Arcaya, G. A. *Electron. J. Biotechnol.* **2011**, 14, http://dx.doi.org/10.2225/vol14-issue3-fulltext-1.
(24) Pchelintsev, N. A.; Youshko, M. I.;Švedas, V. K. *J. Mol. Catal. B: Enzym.* **2009**, 56, 202-207.
(25) Sheldon, R. A. *Adv. Synth. Catal.* **2007**, 349, 1289-1307.

(26) Wang, A.; Zhang, F.; Chen, F.; Wang, M.; Li, H.; Zeng, Z.; Xie, T.;Chen, Z. *Korean J. Chem. Eng.* **2011**, 28, 1090-1095.
(27) Miletić, N.;Loos, K. *Aust. J. Chem.* **2009**, 62, 799-805.
(28) Wine, Y.; Cohen-Hadar, N.; Freeman, A.;Frolow, F. *Biotechnol. Bioeng.* **2007**, 98, 711-718.
(29) Klapper, M. H. *Biochem. Biophys. Res. Commun.* **1977**, 78, 1018-1024.
(30) Galvis, M.; Barbosa, O.; Ruiz, M.; Cruz, J.; Ortiz, C.; Torres, R.;Fernandez-Lafuente, R. *Process Biochem.* **2012**, 47, 2373-2378.
(31) Arsenault, A.; Cabana, H.;Jones, J. P. *Enzyme Research* **2011**, 2011, art. no. 376015.
(32) Palomo, J. M.; Segura, R. L.; Mateo, C.; Fernandez-Lafuente, R.;Guisan, J. M. *Biomacromolecules* **2004**, 5, 249-254.
(33) Usha, R.; Sreeram, K. J.;Rajaram, A. *Colloids Surf. B. Biointerfaces* **2012**, 90, 83-90.
(34) Sam, S.; Touahir, L.; Salvador Andresa, J.; Allongue, P.; Chazalviel, J. N.; Gouget-Laemmel, A. C.; De Villeneuve, C. H.; Moraillon, A.; Ozanam, F.; Gabouze, N.;Djebbar, S. *Langmuir* **2010**, 26, 809-814.
(35) Bradford, M. M. *Anal. Biochem.* **1976**, 72, 248-254.
(36) Wernerova, M.;Hudlicky, T. *Synlett* **2010**, 2701-2707.
(37) Chen, C. S.; Fujimoto, Y.; Girdaukas, G.;Sih, C. J. *J. Am. Chem. Soc.* **1982**, 104, 7294-7299.
(38) Kartal, F.; Janssen, M. H. A.; Hollmann, F.; Sheldon, R. A.;Kilinc, A. *J. Mol. Catal. B: Enzym.* **2011**, 71, 85-89.
(39) Fishman, A.;Cogan, U. *J. Mol. Catal. B: Enzym.* **2003**, 22, 193-202.
(40) Grabarek, Z.;Gergely, J. *Anal. Biochem.* **1990**, 185, 131-135.
(41) Bassani, G.; Farruggia, B.;Picó, G. *Int. J. Biol. Macromol.* **2011**, 49, 351-355.
(42) Torres, R.; Ortiz, C.; Pessela, B. C. C.; Palomo, J. M.; Mateo, C.; Guisán, J. M.;Fernández-Lafuente, R. *Enzyme Microb. Technol.* **2006**, 39, 167-171.
(43) Cabrera, Z.; Gutarra, M. L. E.; Guisan, J. M.;Palomo, J. M. *Catal. Commun.* **2010**, 11, 964-967.
(44) Tang, K.; Yi, J.; Huang, K.;Zhang, G. *Chirality* **2009**, 21, 390-395.
(45) Xue, Y. P.; Xu, S. Z.; Liu, Z. Q.; Zheng, Y. G.;Shen, Y. C. *J. Ind. Microbiol. Biotechnol.* **2011**, 38, 337-345.
(46) Grover, P. T.; Bhongle, N. N.; Wald, S. A.;Senanayake, C. H. *J. Org. Chem.* **2000**, 65, 6283-6287.
(47) Whitesell, J. K.;Reynolds, D. *J. Org. Chem.* **1983**, 48, 3548-3551.
(48) Fernández-Lorente, G.; Palomo, J. M.; Cabrera, Z.; Guisán, J. M.;Fernández-Lafuente, R. *Enzyme Microb. Technol.* **2007**, 41, 565-569.
(49) Xie, Y.; An, J.; Yang, G.; Wu, G.; Zhang, Y.; Cui, L.;Feng, Y. *J. Biol. Chem.* **2014**, 289, 7994-8006.
(50) García-García, M. I.; Sola-Carvajal, A.; Sánchez-Carrón, G.; García-Carmona, F.;Sánchez-Ferrer, Á. *Bioresour. Technol.* **2011**, 102, 6186-6191.
(51) Cabana, H.; Jones, J. P.;Agathos, S. N. *J. Biotechnol.* **2007**, 132, 23-31.
(52) Matijošyte, I.; Arends, I. W. C. E.; de Vries, S.;Sheldon, R. A. *J. Mol. Catal. B: Enzym.* **2010**, 62, 142-148.
(53) Zhen, Q.; Wang, M.; Qi, W.; Su, R.;He, Z. *Catal. Sci. Technol.* **2013**, 3, 1937-1941.

(54) Guisan, J. M.; Sabuquillo, P.; Fernandez-Lafuente, R.; Fernandez-Lorente, G.; Mateo, C.; Halling, P. J.; Kennedy, D.; Miyata, E.;Re, D. *J. Mol. Catal. B: Enzym.* **2001**, 11, 817-824.
(55) Aytar, B. S.;Bakir, U. *Process Biochem.* **2008**, 43, 125-131.

ABBREVIATIONS

PEI, polyethyleneimine; BSA, bovine serum albumin; CLEAs, cross-linked enzyme aggregates; GA, glutaraldehyde; DP, dextran-polyaldehyde; EDC, 1-Ethyl-3-[3-dimethylaminopropil]carbodiimide; NHS, N-hydroxysuccinimide; CRL, *Candida rugosa* lipase; MW, molecular weight; $PEI_{1.3}$, polyethyleneimine with molecular weight of 1.3 kDa; PEI_{25}, polyethyleneimine with molecular weight of 25 kDa; PEI_{70}, polyethyleneimine with molecular weight of 70 kDa; PEI_{750}, polyethyleneimine with molecular weight of 750 kDa; DH, hexamethylenediamine; CNBr-agarose, cyanogen bromide activated agarose; MES, 2-morpholinoethanesulfonic acid monohydrate; NaCl, sodium chloride; pNPB, *p*-nitrophenyl butyrate; BPA, 2-*O*-butyryl-2-phenylacetic acid; HPLC, high-performance liquid chromatography; SEM, scanning electron microscopy; *Candida antarctica* B, CALB; enantiomeric excess, *ee*; enantiomeric ratio, *E;* DH-CLEAs, cross-linked enzyme aggregates with hexamethylenediamine; $PEI_{1.3}$-CLEAs, cross-linked enzyme aggregates with polyethyleneimine with molecular weight of 1.3 kDa; PEI_{70}-CLEAs, cross-linked enzyme aggregates with polyethyleneimine with molecular weight of 70 kDa.