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Chemical modification and immobilisation of lipase B from *Candida antarctica* onto mesoporous silicates

Jessica Forde[a], Alex Vakurov[b], Tim D. Gibson[b], Paul Millner[b], Mícheál Whelehan[a], Ian W. Marison[a], Ciarán Ó'Fágáin[a,*]

[a] School of Biotechnology & National Centre for Sensors Research, Dublin City University, Dublin 9, Ireland
[b] Research Institute of Membrane and Systems Biology, University of Leeds, Leeds LS2 9JT, United Kingdom

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ABSTRACT

The chemical modification and immobilisation of lipase B from *Candida antarctica* (CalB) onto three different types of mesoporous silicate (MPS) were undertaken. Soluble CalB was modified by two bifunctional reagents, ethylene glycol bis(succinimidyl succinate) (EGNHS) and glutaraldehyde, and by the monofunctional citraconic anhydride. Both chemically modified and untreated enzyme were then immobilised onto SBA-15-, CNS- and MCM-type MPS by adsorption. Thermal stabilities of chemically modified CalB in solution and of the immobilised preparations were evaluated and compared. Citraconic anhydride dramatically reduced the stability of CalB whereas both bifunctionals yielded an eightfold increase in stability over the native free CalB at 70 °C. Following immobilisation of the EGNHS-treated preparation onto CNS-MPS, the stability gain increased to over 60-fold and this combination proved to be the most effective stabilisation strategy. CalB also showed a preference for MPS with larger pores, namely SBA-15. Immobilisation of CalB in alginate beads was also stabilising.

1. Introduction

Lipases (EC 3.1.1.3) belong to the hydrolase class of enzymes and catalyse the hydrolysis of esters formed from glycerol and long-chain fatty acids [1,2]. They can accommodate a wide variety of other substrates, however, and their abilities to perform esterifications, transesterification and enatioselective hydrolyses gives them numerous applications throughout the biotechnology industry, including the synthesis of enantiomerically pure pharmaceuticals and fine chemicals, in the dairying industry, in oil processing and in the flavouring of compounds and foodstuffs [3–6]. Lipases are ubiquitous [7], being found in animals [8], plants [9], fungi [10] and bacteria [11,12].

CalB, the B-component lipase from the yeast *Candida antarctica*, is a particularly interesting enzyme and probably the most industrially important member of the family. This is due to its ability to catalyse a diversity of reactions, resistance to organic solvents, reasonable thermal stability, stereospecificity and high enantioselectivity [13,14]. The X-ray structure of CalB lipase was elucidated by Uppenberg et al. [15]; the enzyme comprises 317 amino acids with a molecular weight of approximately 33 kDa and an isoelectric point of 6.0. In comparison with some other lipases, CalB has only a small active site lid [15,16], so its active site is quite exposed to the solvent [15]. With certain substrates, it does not show the typical "interfacial activation" of a lipase [17]. These features enable it to perform a very wide variety of reactions.

Although CalB is reasonably stable, its usefulness in industrial processes would be enhanced by the availability of a re-usable and more stable form to afford more favourable process economics. Immobilisation onto solid particles permits the recovery of enzyme for re-use. In addition, both immobilisation and chemical modification are two popular strategies used to increase enzyme stability. Lipase-relevant examples include the chemical modification of surface residues using polyethylene glycol monomethyl ether (MPEG) [18], immobilisation onto agarose [19], chitosan [20] and green coconut fibres [19,20] and site-specific immobilisation of genetically engineered variants [21]. It is also possible to improve the enantioselectivity of a given lipase by using different immobilisation protocols [22].

Abbreviations: BCA, Bicinchoninic acid; BET, Brunauer–Emmet–Teller; BSA, Bovine serum albumin; CA, Citraconic anhydride; CalB, Lipase B from *Candida antarctica*; CEOS, (2-Cyanoethyl)triethoxysilane; CLEA, Cross-linked enzyme aggregate; CNS, Nitrile-modified silica; CNS-Sox, Soxhlet-refluxed CNS (with retention of the nitrile group); CTAB, Cetyltrimethylammonium bromide; DMSO, Dimethyl sulfoxide; EGNHS, Ethylene glycol bis(succinimidyl succinate); GA, Glutaraldehyde; MCM, Mobil composite matter; MPEG, Polyethylene glycol monomethyl ether; MPS, Mesoporous silicates; 4-NB, 4-Nitrophenyl butyrate; SBA, Santa Barbara amorphous; SEM, Scanning electron microscopy; T_{50}, Temperature of half-inactivation (10 min incubations); TEOS, Tetraethoxysilane; TNBS, 2,4,6-Trinitrobenzenesulfonic acid.

* Corresponding author. Tel.: +353 1 7005288; fax: +353 1 7005288.
E-mail address: ciaran.fagan@dcu.ie (C. Ó'Fágáin).

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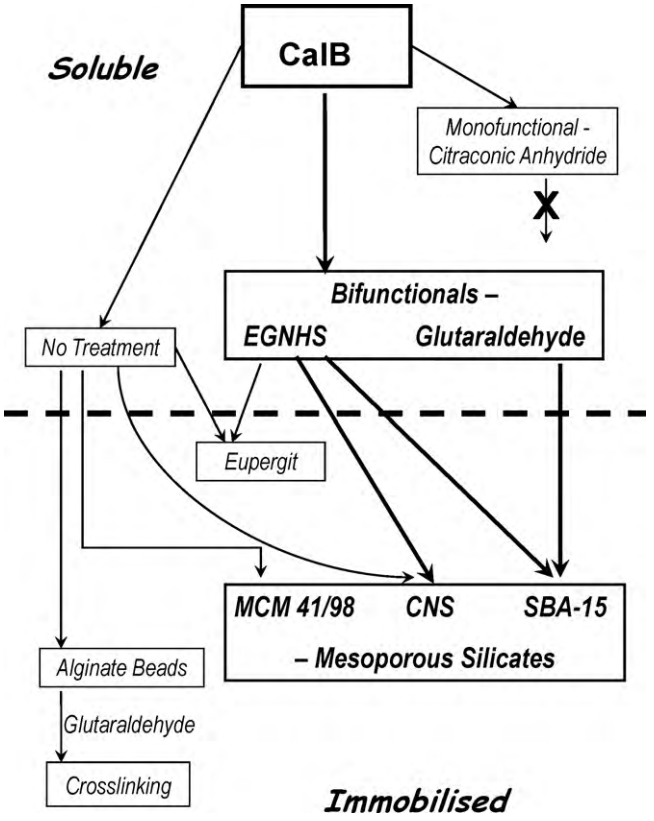

Scheme 1. Schematic of the chemical modification and immobilisation procedures performed on CalB enzyme.

The aim of the present study was to develop an immobilisation strategy for the thermal stabilisation of CalB which could be applied to industrial situations. This involved chemical modification of free, soluble CalB, using monofunctional and bifunctional reagents, followed by its adsorptive immobilisation onto different types of mesoporous silicates (MPS) [23], or within alginate beads (Scheme 1). We were curious whether additional stabilising effects would result from combining the two operations. Our dual approach [23] is quite different from that of Montes et al. [24], who aminated penicillin G acylase before immobilisation on negatively charged supports. That amination was not intended to be stabilising by itself; rather, it was to increase the number of attachment sites between protein and solid phase: the gain in stability correlates with the number of protein-solid links. The converse approach of Hidalgo et al. involved an initial, somewhat-stabilising immobilisation of an oligomeric catalase followed by a further-stabilising cross-linking reaction with dextran aldehyde performed on the immobilised protein [25]. In the present work, each step (modification in solution and immobilisation onto solid phase) was intended to be stabilising on its own, with the hope that the combination would give additional stability gains.

The chosen chemical modification reactions target free amino groups (including lysine side chains) on the enzyme surface. CalB's lysine content comprises approximately 2–3% of the total enzyme surface [26]. Anhydrides have been previously shown to stabilise a number of enzymes including laccase [27] and papain [28]. The present study used acylating citraconic anhydride (2-methylmaleic anhydride; CA) due to its specificity for amino groups, simplicity of use and the ease of deacylation under altered conditions [29,30]. The other two reagents were bifunctional cross-linkers, namely glutaraldehyde and ethylene glycol bis(succinimidyl succinate) (EGNHS). Glutaraldehyde is frequently used to cross-link both *soluble* enzymes (either intra- or inter-molecularly [31]) and to enhance the stability of proteins previously *immobilised* onto supports by simple adsorption [32–35]. Glutaraldehyde is also used in the making of CLEAs, a technique applicable to numerous enzymes [36–39]. EGNHS, a bifunctional reagent, has previously been used successfully to stabilise horseradish peroxidase [40] and trypsin [41].

Many reports describe the immobilisation of enzymes (e.g. trypsin [42], chloroperoxidase [43], horseradish peroxidase [44] and glucose oxidase [45]) by adsorption onto MPS. Attractive features of MPS include large surface areas of up to 1000 $m^2\ g^{-1}$ [46], chemical and mechanical stability and resistance to microbial attack. Deere et al. [47] have outlined the factors that influence the adsorption of proteins to MPS. The present study used three types of MPS, namely MCM 41/98, SBA-15 and CNS for adsorptive immobilisation of CalB. The pores of these MPS types, with diameters ranging 4–9 nm, can accommodate a number of different enzymes within their long channels [48]. The pore openings of MPS may also be modified with organosilane groups to further increase the stability of a number of proteins [49].

All our CalB preparations were analysed in terms of their hydrolytic activity, extent of modification and thermal stability at elevated temperatures. The MPS-immobilised preparations were also characterised using nitrogen adsorption and surface area measurement.

2. Materials and methods

2.1. Enzymes and chemicals

Lipase B from *C. antarctica* (CalB), a gift from CLEA Technologies (Delft, the Netherlands), was used as supplied. 4-Nitrophenyl butyrate (4-NB), Tris, HCl, Triton X-100, sodium tetraborate, potassium phosphates (mono- and di-basic), 2,4,6-trinitrobenzenesulfonic acid (TNBS), dimethyl sulfoxide (DMSO), citraconic anhydride (CA) and ethylene glycol bis(succinimidyl succinate) (EGNHS) were all purchased from Sigma–Aldrich. Glutaraldehyde (25%, v/v), also from Sigma–Aldrich, was subjected before use to a simple distillation (100 °C using a Quickfit glass distillation apparatus) to obtain the monomer. Analytical grade $CaCl_2$, 3-(*N*-Morpholino)propanesulfonic acid (MOPS), Tween 80 and NaCl came from Fluka (Buchs, Switzerland). SBA-15 mesoporous silicates (MPS; average particle size 9.8 μm) were purchased from Glantreo Ltd., Cork, Ireland, and all other MPS were synthesised according to Ref. [42]. Sodium alginate (Keltone LV) was from Inotech Biotechnologies (Basel, Switzerland). Pierce supplied BCA protein estimation kits. Econo-pac 10DG desalting chromatography columns were from BioRad. Eupergit CM was from Degussa (Darmstadt, Germany).

2.2. CalB activity assay

To 980 μL of 100 mM Tris–HCl buffer pH 7.0, 10 μL of 4-nitrophenyl butyrate (4-NB; 10 μL, 200 mM) was added and the reaction was allowed to proceed for 2 min at room temperature. The increase in absorbance at 410 nm permits estimation of CalB activity.

2.3. Protein determination

Protein determination was via the BCA standard microtitre plate protocol according to the Pierce kit insert, using bovine serum albumin (BSA) as standard [50].

2.4. Temperature profile and effect of temperature on activity and stability

Prior to chemical modification or immobilisation, the T_{50} of CalB was estimated by performing a thermal profile on the unmodified enzymes. An enzyme sample was incubated (10 min) at temperatures ranging 20–80 °C, in 5 °C increments. After each incubation period, a 20-μL sample of enzyme was removed, transferred to a microtitre plate on ice and, immediately after re-warming to room temperature, assayed using the standard protocol. A plot of % activity remaining (room temperature activity = 100%) permitted estimation of T_{50} (70 °C) by visual inspection. To assess whether modification and/or immobilisation improved CalB stability, thermoinactivation of untreated and of modified samples was performed at 70 °C. Samples were removed at 10 min intervals, transferred to a microtitre plate, placed on ice and, following re-warming to room temperature, assayed using the standard method. For the immobilised samples, 5–10 mg of the immobilised MPS was pre-weighed into an Eppendorf tube for each specified time interval, incubated for that time, removed, placed on ice and assayed (following re-warming) according to the standard protocol. Where possible, thermoinactivation data (% activity remaining versus time) were fitted to a first-order decay equation, to estimate the rate constant k and the half-life at 70 °C.

2.5. Chemical modification

Before modification, a portion of the commercial CalB preparation was transferred into the appropriate buffer for the reaction in question by means of a PD10 desalting column (BioRad). To CalB diluted in 100 mM potassium phosphate buffer pH 7.4 (final CalB concentration 0.4–0.8 mg/mL) was added freshly distilled glutaraldehyde (1 μL/mL CalB solution). Reaction was allowed to proceed for 30 min and was terminated by passage through a desalting column.

Similarly, 1 mg EGNHS dissolved in 200 μL DMSO was added to 1 mL unmodified CalB (0.6 mg/mL) in 100 mM potassium phosphate buffer pH 7.4, allowed to react at room temperature for 30 min and the reaction then terminated as above.

For citraconic anhydride, a concentration of 2 μL citraconic anhydride per mL of CalB solution was added slowly dropwise to CalB (0.5–1 mg/mL) in 100 mM borate buffer pH 9.0 while stirring continuously and monitoring the pH. Reaction was terminated as above after 30 min.

All modified enzymes were then characterised by standard activity assay, TNBS and BCA methods and subjected to thermoinactivation to assess stability.

2.6. Determination of free amino groups

The number of free amino groups of native CalB and of each modified preparation was determined using 2,4,6-trinitrobenzenesulfonic acid (TNBS) [51]. To 500 μL of 100 mM borate buffer pH 9.5 (prepared in 0.1 M NaOH), 400 μL of ultra pure distilled water and 100 μL of enzyme (0.5 mg/mL) were added. The reaction was initiated by the addition of 20 μL TNBS and allowed to proceed for 5 min exactly before termination with 2 mL sodium sulphite. Absorbance was then read at 420 nm. The difference in free amino groups and, hence, the extent of modification was calculated from a standard curve prepared with α-*N*-acetyl-L-lysine (10–0.05 μM).

2.7. Immobilisation of CalB on mesoporous silicates

Prior to immobilisation, all MPS were refluxed in a Soxhlet extractor for at least 6 h in 95% methanol/5% HCl (v/v) to remove any residual surfactant and were then sonicated (28 kHz, 240 W, room temperature) for 10 min.

MPS (20–100 mg/mL of CalB solution) was added to the enzyme solution (typically 0.5 mg CalB/mL) for both chemically modified and native forms) and maintained at 4 °C while stirring continuously for 16 h to enable immobilisation by adsorption. The suspension was then centrifuged at 20,000 × *g*, 4 °C, for 10 min. The supernatant was decanted and its CalB activity and protein concentration were assayed in order to estimate the amount of enzyme immobilised. The pellet was resuspended, filtered through sintered glass funnels (pore size 3) and washed with distilled water. All of the immobilised MPS were characterised by N_2 adsorption (see Section 3 and Table 2).

2.8. Preparation of calcium alginate beads

Sodium alginate stock solutions, used to produce calcium alginate beads, were prepared by dissolving 15 g/L of sodium alginate powder in MOPS buffer (10 mM MOPS, 0.85% (w/v) NaCl, pH 7.0) and mixing overnight on a magnetic stirrer at 4 °C. After solubilisation, the alginate solution was filtered using a 0.45- and 0.22-μm cellulose acetate filter membrane (Whatman, Dassel, Germany) under a pressure of 2 bar.

Monodisperse calcium alginate beads were prepared using an Inotech encapsulator IE-50R (Inotech Biotechnologies, Basel, Switzerland). The encapsulator was fitted with a nozzle with a diameter of 400 μm and sodium alginate was supplied to the nozzle using an air pressure regulation system, which enabled flow rates of 10–15 mL/min to be generated using a maximum head pressure of 0.5 bar. The desired flow rate was set using a pressure reduction valve. Spherical beads were obtained by the application of a set vibrational frequency, with defined amplitude, to the extruded alginate jet. This resulted in the jet breaking up into droplets, which fell into a magnetically stirred gelling bath placed 15 cm below the nozzle, where the droplets gelled to produce calcium alginate beads. The gelling bath consisted of 32 g/L $CaCl_2$, 10 mM MOPS pH 7.0 and 0.1–0.2% (v/v) Tween 80, which was added to reduce the surface tension of the gelation solution. Beads were allowed to harden for at least 30 min to ensure complete gelation and were then washed and filtered using a porous steel mesh to remove any unreacted components.

2.9. Immobilisation of CalB onto calcium alginate beads by adsorption

Wet calcium alginate beads (3 g) were incubated overnight at room temperature in 30 mL 100 mM Tris–HCl buffer pH 7.0 containing 0.36 mg/mL CalB. The size of the beads was 852 μm ± 1.5%. The size and size distribution of the beads were determined using a camera (model DP3OBW, Olympus, Japan) attached to a light microscope (model B-X-51, Olympus, Japan) interfaced to a PC operating with Cell^F image analysis software (Olympus, Japan). Up to 100 beads were analysed for each batch manufactured (using a magnification of 40×) and the mean size and standard deviation were determined. All size measurements were made in liquids to avoid swelling/shrinkage effects. The beads were then filtered through a sintered glass mesh to remove any water or liquid material.

2.10. Immobilisation of CalB onto calcium alginate beads by adsorption and cross-linking with glutaraldehyde

The procedure was as above for adsorption, except that the final washed preparation was filtered and resuspended in a solution of 5.6 mM glutaraldehyde at room temperature for 10 min.

Table 1
Chemical modification of lipase B from *Candida antarctica* (CalB) in free solution.

Type of modification	Specific activity before modification (U/mg)	% recovery	Percent (%) modification of reactive amino groups	Half-life $t_{1/2}$ (min) 70 °C	*k*-Value
Untreated CalB (control)	137	100	0	*2*	Not first order
EGNHS	140	Approximately 100	100%	14	0.048 ± 0.001
Glutaraldehyde	145	Approximately 100	100%	16	0.044 ± 0.001

Note: The figure in italics is not a true half-life; rather, it is a half-inactivation time (i.e. time for a 50% decrease in initial activity) estimated from inspection of Fig. 1. Amino groups were determined by the TNBS method [51], where errors did not exceed 10%.

2.11. Nitrogen adsorption

All MPS used were characterised by nitrogen gas adsorption/desorption isotherms. Samples were preheated at 70–150 °C under vacuum to remove any H_2O. The pore size data were analysed before and after enzyme loading by the thermodynamically based Barrett–Joyner–Halenda (BJH) method [52]. Surface areas of the MPS were also determined using the Brunauer–Emmett–Teller (BET) method, a surface area measurement technique based on Langmuir theory [53].

3. Results

3.1. Chemical modification of CalB

The TNBS assay indicated a free amino group content of approximately 4 per CalB molecule. This value was unexpectedly low: the crystal structure [15] showed a total lysine content of 9 plus the N-terminus, although it is not clear whether all of these are free and available for cross-linking. Nevertheless, all three reagents successfully modified all of the available CalB aminos (Table 1). Stability was enhanced only by the bifunctionals EGNHS and glutaraldehyde, with scarcely any loss of enzyme activity (i.e. approximately 100% recovery of pre-modification activity; Table 1). Citraconic anhydride afforded no significant stability gain (Fig. 1) and gave poor recovery of activity (only 20–30%). Half-lives of EGNHS- and glutaraldehyde-treated CalB were 15 and 16 min respectively (Table 1). Thermal decay of untreated CalB was not first order but visual inspection of the % activity versus time plot indicated a time of just 2 min for a 50% activity loss (Fig. 1), so the treatment with bifunctionals affords a significant stability gain.

3.2. Immobilisation of CalB onto mesoporous silicates

CalB immobilised successfully onto all of the MPS with one exception: CNS (nitrile group functionalised silica) MPS that had been calcined at 650 °C for 6–8 h to remove the nitrile group. Calcination may lead to a slight reduction of pore sizes [54,55]. The most successful procedure was immobilisation of native CalB onto CNS MPS that had been refluxed in a Soxhlet extractor to remove any remaining surfactant (this milder procedure preserves the nitrile group; see Fig. 2). From this distinction, it is clear that CalB prefers the larger pore sizes of the CNS MPS with the nitrile group in place (Table 2). Thermoinactivation data of native CalB immobilised on CNS MPS did not fit a first order decay. The EGNHS-treated enzyme on CNS had a "half-life" of 119 min, nearly a 60-fold increase over soluble CalB. Eupergit CM, a commercial enzyme carrier, was used for comparison purposes according to the manufacturer's standard recommended protocol. It immobilised the greatest amount of native enzyme (load 540 μmol untreated CalB/g solid) but gave only a 10-fold stability increase over soluble CalB (Table 2). We did not attempt to achieve maximal immobilisation on Eupergit via the optimised protocol of Mateo et al. [56]. The standard method may have yielded only a limited number of enzyme-support links. Only a limited amount of EGNHS-modified CalB immobilised on Eupergit (Table 2).

Nitrogen adsorption studies showed decreased pore volumes following enzyme loading (Table 3) indicating that CalB had immobilised inside the pores and not merely on the MPS surface. Fig. 3 shows a typical profile obtained with the SBA-15, indicating a clear difference in the pore volumes before and after CalB immobilisation. Entry of the enzyme into the pores of the SBA-15 creates a more stable environment for the protein and prevents damage by external shear forces.

Immobilisation of CalB onto calcium alginate beads was also performed. These beads (diameter 852 μm ± 1.5%) positively affected

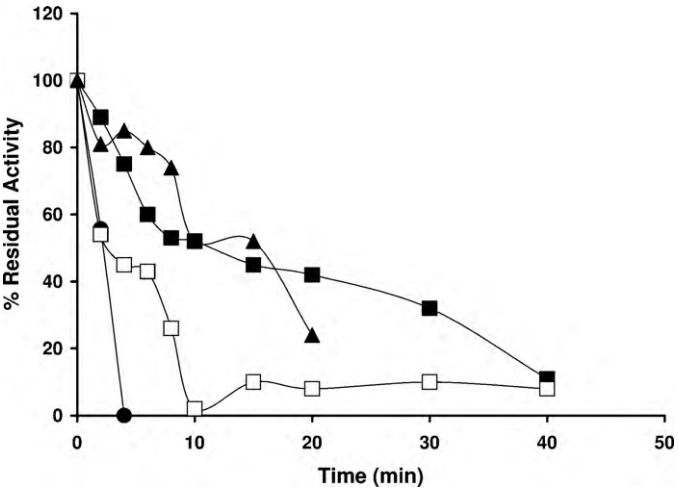

Fig. 1. Thermoinactivation of native and chemically modified soluble CalB at 70 °C in 100 mM Tris–HCl pH 8.0; (●), untreated free enzyme; modified with (■), bifunctional EG-NHS; (□), citraconic anhydride and (▲), glutaraldehyde. Experiments were performed in triplicate with a standard error not exceeding 10%.

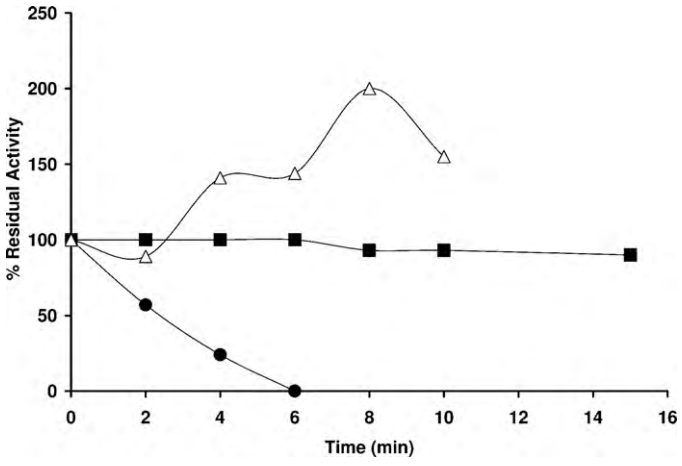

Fig. 2. Thermoinactivation at 70 °C, pH 8.0, of CalB immobilised on methanol/HCl-extracted CNS-type MPS. Native CalB (△); EGNHS-modified CalB (■); untreated, non-immobilised (soluble) CalB (●). Experiments were performed in triplicate. Standard error was 5–10%.

Table 2
Immobilisation of CalB onto MPS and onto Eupergit.

Type of MPS	CalB Pre-treatment	Amount immobilised (μmol/g)	Half-life $t_{1/2}$ (min) 70 °C	*k*-Value
MCM 41/98	Untreated CalB*	114	8	0.086 ± 0.001
CNS	Untreated CalB	120	*20+*	ND
CNS	EGNHS*	33	119	0.0058 ± 0.001
SBA-15	Glutaraldehyde	452	*60+*	ND
SBA-15	EGNHS*	264	10	0.07 ± 0.001
Eupergit CM	Untreated CalB	540	*25*	ND
Eupergit CM	EGNHS	30	*10*	ND

(Eupergit is not a MPS but is included in this Table for comparison). Amounts immobilised are calculated from BCA [50] protein determinations, where errors did not exceed 5%. ND, not determined: thermoinactivation data at 70 °C were not first-order decay. *Note*: The figures in italics are not true half-lives; rather, they are half-inactivation times (i.e. time for a 50% decrease in initial activity) estimated from inspection of plots of % activity versus time (not shown).

Table 3
Physical Characteristics of Mesoporous Silicates and Nitrogen Adsorption Data from the Immobilisation of CalB onto Different Mesoporous Silicates and onto Eupergit.

Type of MPS	Lattice structure	Pore size diameter (nm)	BET surface area ($m^2\ g^{-1}$)	Pore volume ($cm^3\ g^{-1}$)
SBA-15	Hexagonal	9	653	0.77
SBA-15/native CalB			38	0.08
MCM-41	Hexagonal	6	400	2.25
MCM-41/native CalB			217	0.125
MCM-41/GA CalB			243	0.17
MCM 48	Cubic	5–6	ND	ND
CNS	Disordered	–	ND	ND
Eupergit CM	Not disclosed	Not disclosed	*123*	*0.49*

GA CalB, glutaraldehyde-treated Cal B. Errors in pore size/volume and surface area did not exceed 5%. The average size of SBA-15 particles was 9.8 μm (data from supplier Glantreo Ltd.); particle sizes of the other MPS were not determined.

CalB thermostability at 70 °C (Fig. 4). We observed no significant difference between the stability gain resulting from simple adsorption and that from adsorption followed by glutaraldehyde treatment.

4. Discussion

The TNBS assay showed that all of the available CalB lysines were successfully modified by each of the three chemical modifiers used. CA gave no improvement in thermal stability, unlike the bifunctionals EGNHS or glutaraldehyde. CA, a monofunctional reagent that cannot form a cross-link, also reduced the catalytic activity of the enzyme to 20–30% of the pre-modification value, whereas both the glutaraldehyde and the EGNHS treatments maintained approximately 100% CalB activity. The poor recovery following CA treatment may be due to the higher pH of that reaction (9.0) compared with that used for the bifunctionals (7.4). Similarly, Bianchi et al. [57] showed that the stereoselectivity and activity of lipase from *P. cepacia* was dramatically reduced following modification with acetic anhydride, which is similar in size to citraconic anhydride (molecular weights of 102 and 112 respectively). They concluded that these small modifiers may have the ability to enter the active site, altering it chemically and thus destabilising the enzyme. It is likely that the bifunctional reagents have formed one or more internal (intramolecular) cross-links within the CalB protein (although this awaits confirmation), leading to the observed increases in thermal stability. The reaction conditions used here (low protein concentrations, excess of modifier, chain length of bifunctional) tend to favour intramolecular links over intermolecular ones [58]. Use of EGNHS on Horseradish peroxidase under similar conditions led to formation of an intramolecular link only [40]. Viewing of CalB (PDB structure 1TCA) in the DeepView modelling programme (not shown) indicates that four Lys pairs within CalB are located within 16 Å of each other, i.e. within the potential span of bifunctional EGNHS, namely Lys 13–124, 32–98, 98–124 and 136–308. Thus, formation of a cross-link, as demonstrated in Ref. [40], appears to be

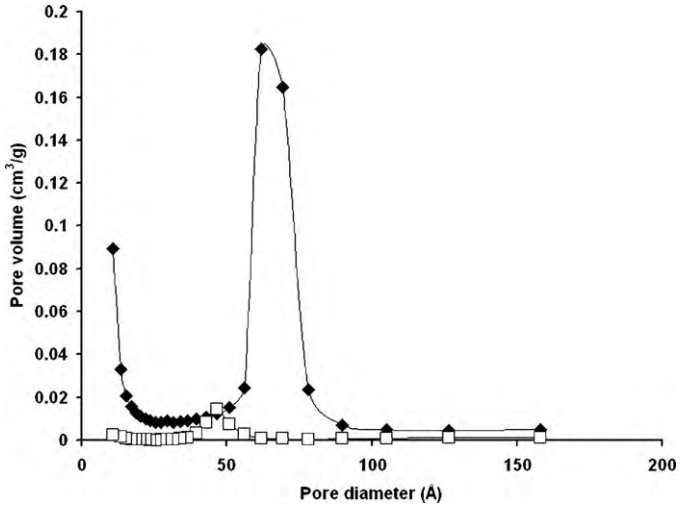

Fig. 3. Nitrogen adsorption isotherm showing the pore volume of SBA-15 mesoporous silicate particles before (♦) and after (□) immobilisation of CalB.

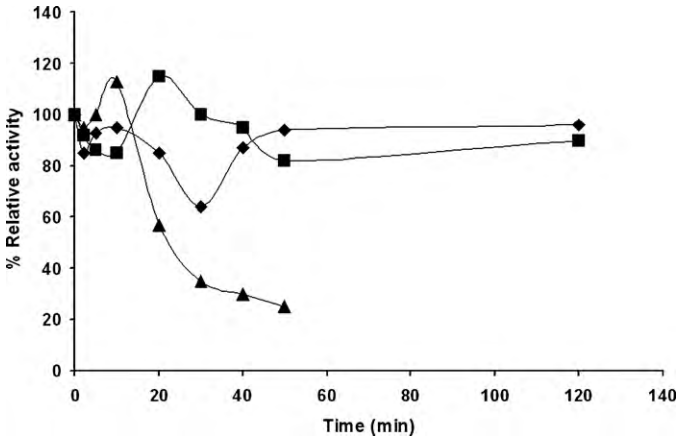

Fig. 4. Thermoinactivation at 70 °C of CalB immobilised on calcium alginate beads by adsorption (♦) and by adsorption followed by cross-linking (■). Free, non-immobilised (soluble) CalB is also shown (▲). Experiments were performed in duplicate in 100 mM potassium phosphate buffer pH 7.0; standard error was 10%.

quite feasible. It is possible however, that our reaction conditions may have led to single-point, non-cross-linking modifications of the protein surface. Some (at least) of the observed stability gains may arise from altered surface polarity of the polypeptide.

Previous studies have highlighted the stabilisation effect on lipase of cross-linking with glutaraldehyde. Palomo et al. [59] found that cross-linking CalB with glutaraldehyde not only improved its stability but also improved its enantioselectivity by up to 400-fold. Their findings also suggest that immobilisation of lipase, and the orientation in which it is immobilised, strongly affect its catalytic activity. Treatment of CalB with glutaraldehyde on aminated supports, *in the presence of detergent*, led to greater activity and enantioselectivities against various substrates, probably via glutaraldehyde-induced stabilisation of the open, active form of the enzyme [60]. Also, cross-linking of lipase in the presence of detergents and its immobilisation onto aminated supports leads to increased catalytic activity and stability by producing a "hyperactivated" form of lipase [22,36]. The present treatment of CalB with monomeric glutaraldehyde (obtained by distillation of the commercially sourced reagent) increased the half-life by approximately eightfold (see Table 1). Subsequent immobilisation of this preparation on SBA-15 MPS further improved the stability approximately 30-fold over untreated soluble CalB. It seems that CalB's orientation within the pore of SBA-15 was such that the active site was exposed for reaction with the nitrophenyl butyrate substrate.

Our most successful procedure involved EGNHS treatment followed by immobilisation onto CNS MPS that had been refluxed in a Soxhlet extractor ("CNS-Sox"), which relatively mild procedure preserves the CNS nitrile group (Fig. 2). Immobilisation of free CalB onto CNS-Sox also produced an apparent activation effect, namely an increase in activity during the first 8 min. This could be due to the hydrophobic surface chemistry of the CNS particle [42]. Previous studies on the immobilisation of CalB onto hydrophobic surfaces have also noted an apparent increase in catalytic activity upon immobilisation [61,62].

CalB is quite a small protein (33 kDa) with a spherical diameter of 3.9 nm. Both the SBA-15 and CNS possessed the largest pore diameters of 9 and 13 nm respectively, while MCM-41 possesses a smaller pore size of 5 nm. Theoretically, MCM-41 has a sufficiently large pore size to accommodate the enzyme, yet only native CalB immobilised successfully on this MPS and none of the chemically treated forms entered the MCM-41 pores. In contrast, all modified CalB forms adsorbed successfully onto the larger-pored CNS and SBA-15 particles (Table 3). Possibly, CalB prefers larger pores: not only would these permit easier access for the enzyme but also they would allow more room for substrate entry and for reaction to occur. Indeed, it has been shown that a pore size only slightly larger than the maximum dimensions of the enzyme is sometimes inadequate [63]. If a single CalB molecule (3.9 nm) immobilises inside an MCM pore of 5 nm diameter, no second CalB molecule can enter that pore and thus the enzyme loading can be expected to be very low in this type of MPS. This could call into question the suitability of MCM-41 for adsorption of lipase B from *C. antarctica*.

Another possibility is that immobilisation of native CalB onto MCM-41 occurred mostly on the outside of the particles; this could explain its relatively low half-life of only 24 min compared with 119 min for EGNHS-lipase immobilised onto CNS (Table 2). It is clear that the lipase favoured the presence of the nitrile group on the CNS. This is plausible, considering the distribution of charges on the CalB based on its isoelectric point of 6.0 [15]. Since the CNS particle possesses an overall negative charge [42], this would attract the positively charged residues on the CalB at pH values up to 6, facilitating the immobilisation process. Studies by Brigida et al. [64] have confirmed this with the immobilisation of CalB onto negatively charged coconut fibres: the best immobilisation yield and recovery occurred at pH 4, 5 and 6, yet the biocatalyst immobilised at pH 7 was fourfold more stable. This may be due to the fact that CalB has a pH optimum of pH 7.0–8.0 [65], at which pH the enzyme would possess a favourable molecular conformation. Based on these and other observations [42], the present immobilisations were performed at CalB's isoelectric point (pH 6.0) for optimum yield and stability.

Calcium alginate beads also had a notable stabilising effect on CalB (Fig. 4) but these must be kept in liquid to avoid swelling/shrinkage effects and are not as mechanically robust as MPS particles.

5. Conclusion

This study demonstrates that CalB may be stabilised by up to 60-fold by treatment with EGNHS and subsequent immobilisation on CNS-type mesoporous silicate. Promising results were also obtained with a number of different systems involving glutaraldehyde and other MPS such as SBA-15. The suitability of these MPS for use as immobilisation matrices for CalB is due to their ability to accommodate (at least some of) the enzyme within their long channel pores, protecting it from external shear forces, or from extremes of temperature or pH. Such conditions are often encountered in industrial processes and can lead to destruction of the enzyme. Immobilisation of the enzyme on the surface of the immobilisation matrix, as occurs in the case of many commercial carriers, will be less protective of enzyme activity than immobilisation within the channels of MPS particles. The added benefits of storage stability and reusability suggest that the immobilisation systems presented may have potential for application in biocatalysis and bioreactors.

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