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In Silico Analysis of Enzyme Surface and Glycosylation Effect as a Tool for Efficient Covalent Immobilisation of CalB and PGA on Sepabeads®

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Abstract: This study presents a computational analysis of the structures of lipase B from *Candida antarctica* (CalB) and two penicillin G acylases (PGAs), from eukaryotic and prokaryotic sources, respectively. Molecular simulations were used to point out the regions of the enzymes that are prone to interact with immobilisation supports. In order to evaluate the accessibility of the active site, the location of the amino acid residues involved in the formation of covalent bonds with the polymers was visualised. The mapping of the distribution of hydrophobic and hydrophilic regions on the enzyme surface provided a view of the areas of the protein that can establish either hydrophobic or hydrophilic interactions with the carriers. Experimental data obtained from the immobilisation of the enzymes on supports bearing different chemical functionalities suggest the involvement of the glycan moiety in enzyme–polymer interactions. In the case of PGA the glycan moiety can constitute an extra site for the covalent linkage of the enzyme on the polymer.

Keywords: covalent immobilisation; GRID; lipase B from *Candida antarctica*; molecular modelling; penicillin G amidase; Sepabeads®

Introduction

During the last years, numerous efforts have been devoted to the development of insoluble immobilised enzymes for various applications, due to the many benefits of using immobilised enzymes rather than their soluble counterparts.

A wide array of polymeric carriers has been developed to meet most of the technological requirements of the various biocatalysed processes. Novel carriers offer the possibility of selecting porosity, chemical functionalisation, particle size and also to balance the hydrophobic/hydrophilic nature of the microenvironment surrounding the enzyme.[1]

Immobilisation protocols for enzymes of interest and especially for new enzymes are generally developed on empirical basis. Most often the choice of a suitable carrier is guided by the observation that a certain polymer has proven its efficiency with the broadest number of enzymes. This may result in the non-optimal exploitation of enzyme catalytic potential.

The present study follows our recent report on the production of a PGA from *Providencia rettgeri* (PGA*p*) expressed in *Pichia pastoris*,[2] a eukaryotic host that determines the co-translational glycosylation of the protein.[3]

That study pointed out how the glycosylated PGA exhibits higher stability and activity upon immobilisation on amino-functionalised methacrylic polymers. This behaviour differentiates PGA*p* from the more known and used PGA from *Escherichia coli*, which is

not glycosylated and that is commonly immobilised efficiently on epoxy supports.[4]

Since the sequence identity is higher than 90% and the 3-D structures of the two enzymes present negligible differences,[2,5,6] we started to consider the glycan moiety as the possible cause of the different behaviour of the two PGAs. This is a factor that has been often neglected in similar investigations. It is widely recognised that glycosylation alters the behaviour of the native protein, by increasing thermal stability, improving solubility and preventing aggregation as well as proteolitic attack.[7] Halling and co-workers suggested how different glycosylation patterns may induce a variation of the activity/a_w profiles of enzymes in organic solvent.[8] Finally, mutagenesis studies demonstrated that the glycan moiety is crucial for the stabilisation of the open and active conformation of the lipase from *Candida rugosa*.[9]

Although eukaryotic hosts are more and more often chosen for the expression of many industrially used recombinant enzymes,[10] scarce attention has been paid so far to the understanding of how the glycan moiety can affect the efficiency of an immobilisation process.

Here we present a study that, by starting from the computational analysis of enzyme structure, aims at shedding light on the factors that determine the enzyme-carrier interaction and ultimately the enzyme efficiency.

The attention was focused on the two PGAs previously investigated and on the recombinant lipase B from *Candida antarctica* (CalB), which is expressed in *Aspergillus oryzae* and, consequently, undergoes glycosylation.[11]

Finally, the activity and stability of the immobilised enzymes were studied and data analysed in the light of structural information coming from the computational investigation.

Results and Discussion

Computational Analysis of the Structure of Glycosylated PGA*p*

N-Glycosylation of proteins expressed in *P. pastoris* takes place in the correspondence of the Asn-X-Ser/Thr sequence[7] through the linking of the Asn to two GlcNAc residues.[12]

Little has been reported in the literature concerning the *O*-glycans of the organism, presumably due to the small percentage of *O*-linked material in most glycoproteins tested to date.[13–15]

Each glycosylated site may contain many different glycan structures leading to pronounced heterogeneity and it is also well known that the composition of the sugar moiety at the glycosylation sites is dependent not only on the host used for heterologous expression but also on the culture conditions. In a study concerning the glycosylation of *C. rugosa* lipase expressed in *P. pastoris*, the MALDI-MS analysis revealed that the glycan linked to the GlcNAc residues was composed by mannoses ranging from 8 to 25 moieties, the most abundant species contained a total of 11 mannose residues.[16]

In principle, it is reasonable to expect that one or more highly polar glycans presenting a large number of nucleophylic hydroxy groups must determine important non-covalent interactions and will also cause a different chemical reactivity of the glycosylated enzyme.

In order to study these phenomena in detail, we compared the 3-D models of the non-glycosylated penicillin G amidase from *E. coli* (PGA*e*) and the amidase from *P. rettgeri* (PGA*p*). The latter model was constructed by using homology modelling methods (see Experimental Section).[17] Of the five glycosylation sites present on the unprocessed PGA*p* precursor, four glycosylation sites are present in the catalytically active protein. For two of them (Asn B:241 and Asn B:388) the probability of being glycosylated appears negligible since they are located in the core of the protein and their glycosylation would induce a striking change in the folding of the protein with, as consequence, a loss of activity. This is especially true for Asn B:241 that is located in the oxyanionic hole. Since *N*-glycosylation takes place in the endoplasmatic reticulum and is a co-traslational modification, the protein glycosylation precedes folding. This means that the inaccessibility of these two Asn in the folded protein does not mean that they are inaccessible to glycosylation. However, it has been demonstrated that glycans are virtually absent in inaccessible sites. This can be ascribed to misfolding and degradation of the corresponding glycosylated proteins.[7]

The other two *N*-glycosylation sites (Asn A:20 = green residue and Asn B:428 = orange residue in Figure 1) are located on the surface of the protein and are accessible from the outer environment.

By using the GRID computational method[18] we calculated the molecular interaction field (MIF) generated by a hydroxy probe interacting with the atoms of the Asn residues. The study pointed out that the size of the MIF generated by Asn A:20 is at least three times larger as compared to that generated by Asn B:428 (data not shown). This observation suggests that Asn A:20 is more exposed to the solvent and that the presence of the glycan moiety would not determine a high conformational perturbation in the protein folding.

The data are in agreement with previous experimental evidence (electrophoretic analysis) of a preferential glycosylation on the α-subunit and a far lower incidence of glycosylation of the β-chain.[3]

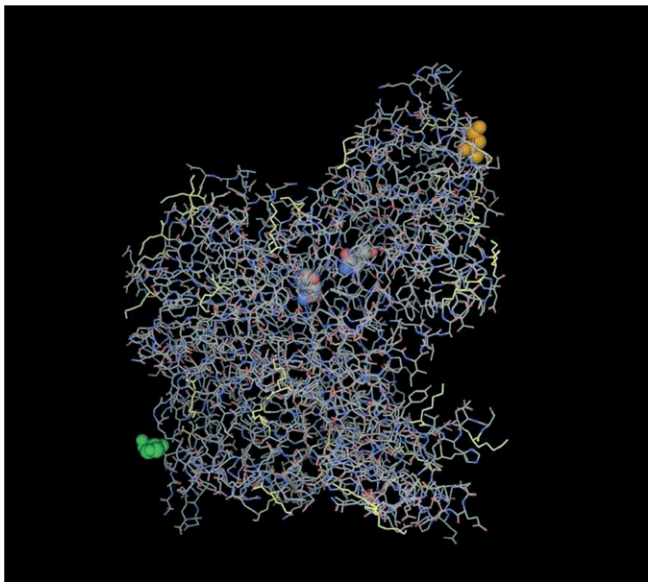


Figure 1. Three-dimensional structure of PGA from *P. rettgeri* (PGA*p*) expressed in *P. pastoris* obtained by homology modelling. The green residue corresponds to the glycosylation site (Asn A:20) on the α-chain, whereas the orange residue (Asn B:428) represents the glycosylation site on the β-chain. Yellow residues correspond to the Lys present on the surface of the enzyme and that are more prone to react with the functionalised carrier.

The distance of Asn A:20 from the active site is more than 35 Å and as can be noted in Figure 1 the glycan is expected to be diametrically opposed to the opening of the active site. This observation induced us to explore the possibility of taking advantage of the location of the glycan moiety to improve the efficiency of the immobilised enzyme. It can be speculated that, by promoting the interaction between the glycan and the carrier, the activity of the immobilised enzyme should be affected positively. Firstly, by promoting hydrophilic interactions with the carrier, an optimum orientation of the enzyme could be achieved by leaving the active site freely accessible. Secondly, the presence of the undesired binding between the carrier and Lys residues positioned in proximity of the active site might be reduced. As a matter of fact, 11 out of the 37 Lys residues distributed on the PGA surface (nearly 30% of the total) are located around the active site (Figure 1, yellow residues) so that their involvement in the binding to the carrier would compromise the access of the substrate to the active site.

In order to enable the formation of covalent bonds between the glycan and the carrier we thought to use amino-functionalised polymers, which must be pre-activated with glutaraldehyde. Since the mannoses of the glycans present *cis* vicinal hydroxy groups, the formation of cyclic acetals with the aldehyde groups of the carrier can be hypothesised.

For instance, it has been described that the cross-linking of a mannose-based polysaccharide (guar-gum) with glutaraldehyde occurs due to the formation of cyclic acetals with the involvement of the *cis*-vicinal hydroxy groups of the mannoses.[19,20]

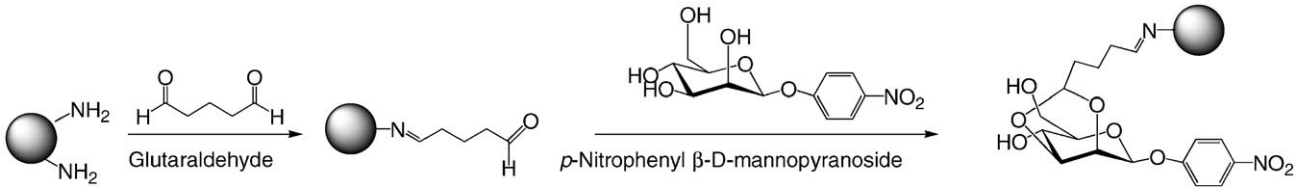
Indeed, we verified experimentally that mannose residues can effectively bind covalently the carrier (about 0.4 mmol per gram of support) by reacting the *p*-nitrophenyl β-D-mannoside with the pre-activated amino resin (Scheme 1). A further factor that is expected to drive the reaction toward the formation of the covalent bond is represented by the fact that the reaction takes place on a solid phase, so that the equilibrium between the synthetic and hydrolytic reactions is shifted toward the bond formation, as previously demonstrated.[21]

The formation of the five-membered cyclic acetals is thermodynamically favoured and, unlike linear acetals, they are also reported to be stable in a reasonable broad range of pH.[22] In our case, it was also verified that the bond formed between the carrier and the mannoside is quite stable since no appreciable leakage was observed after five days of incubation at slightly acidic pH value (Kpi 0.1 M, pH 6.0) that is most often chosen for carrying out the enzymatic synthesis of semi-synthetic β-lactam antibiotics.

Immobilisation of Glycosylated and Non-Glycosylated PGAs

The two PGAs were immobilised on a class of chemically homogeneous methacrylic carriers, Sepabeads®, presenting amino or epoxy reacting groups.[23,24]

Sepabeads® are methacrylic polymers formed by a monomer bearing the reactive functional group and a monomer acting as a cross-linker. They are mechanically stable resins prepared through a suspension



Scheme 1. Formation of a cyclic acetal between the *cis* hydroxy groups on C-2 and C-3 of the mannoside and the aldehyde groups on the carrier.

polymerisation technique, where a porogenic agent is used to generate different degrees of porosity. This ensures that Sepabeads® have an internal geometry with large flat surfaces that favour mass transfer processes.

As depicted in Scheme 2, Sepabeads® with amino groups were used after pre-activation with glutaraldehyde and the results were compared with those obtained with the corresponding epoxy Sepabeads®.

The classical epoxy supports are almost ideal matrices since very stable covalent bonds can be formed. The high density of oxirane groups on the bead surface[23] is the cause of multipoint attachment of the enzyme onto the resin, which is believed to constitute the major origin of enzyme operational stability.[4] As already reported, epoxy Sepabeads® have proven to give stabilising multipoint immobilisation of different proteins, including penicillin G amidase from *E. coli*.[25]

The immobilisation of PGA on pre-activated amino carriers has also been reported although less frequently.[26–28] In some cases sodium borohydride was suggested as a route to extend the operational life-time of the biocatalyst. However, this chemical treatment causes a partial loss of enzymatic activity.[28]

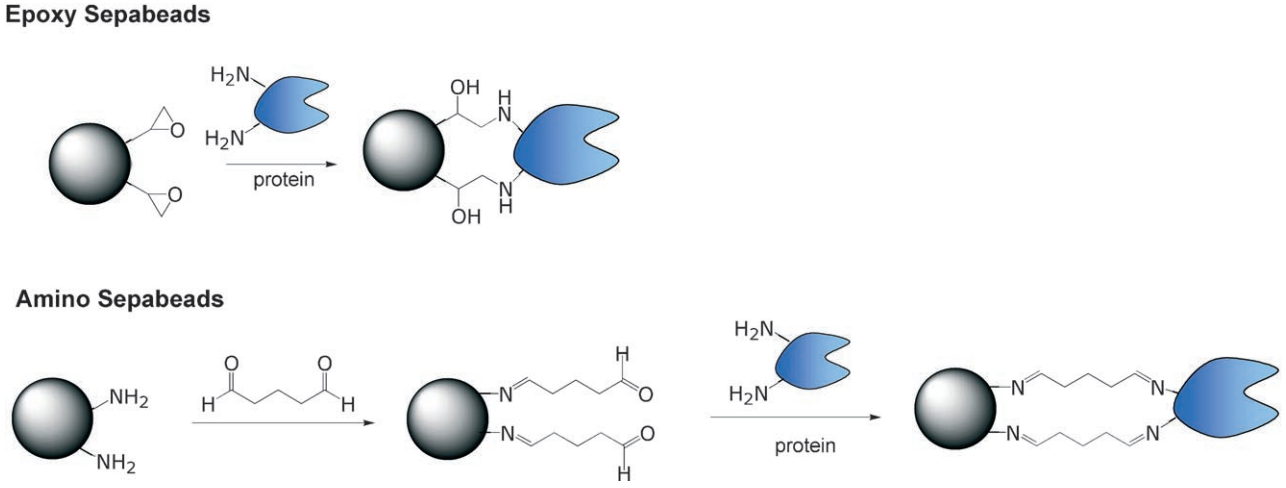
A further major difference between the epoxy and amino supports lies in the lower hydrophobicity of the amino support that is better solvated by water as compared to the epoxy polymer (Resindion technical information). Since the covalent immobilisation is preceded by the physical interaction between the enzymes and the carrier, proteins can be immobilised on the more hydrophilic aminic structure more rapidly even at low ionic strength (about 5–50 mM), whereas immobilisation on epoxy supports proceeds at higher ionic strength (buffers at >1 M concentration).

Table 1 reports the results obtained with immobilisation of PGA*e* and PGA*p* on both classes of Sepabeads®.

In all cases more than 90% of initial proteins were loaded on the polymers. Therefore, the differences emerging from Table 1 can be reasonably ascribed to the effect of the structural differences of the resins on the enzyme efficiency.

The higher activity of PGAp immobilised on the amino support suggests that interactions occurring between the amino carrier and the glycan favours a more correct orientation of the protein making it accessible to substrates.

Figure 2 reports the thermal stability (60 °C) of both the immobilised and native amidases. The native PGA*p* shows a much higher stability in solution as compared to the PGA*e*, which is primarily ascribable to glycosylation since the two protein structures pres-



Scheme 2. Covalent immobilisation of enzymes on epoxy and amino Sepabeads®.

Table 1. Activity of PGA*e* (non-glycosylated) and PGA*p* (glycosylated) immobilised on epoxy and amino Sepabeads®.

Support	Water content (% w/w)	Specific activity[a] (U/g_{dry})
PGA*e*		
Epoxy Sepabeads®	62	287
Amino Sepabeads® *(short spacer)*	55	267
PGA*p*		
Epoxy Sepabeads®	63	197
Amino Sepabeads® *(short spacer)*	62	341

[a] The specific activity was determined by hydrolysis of benzylpenicillin.

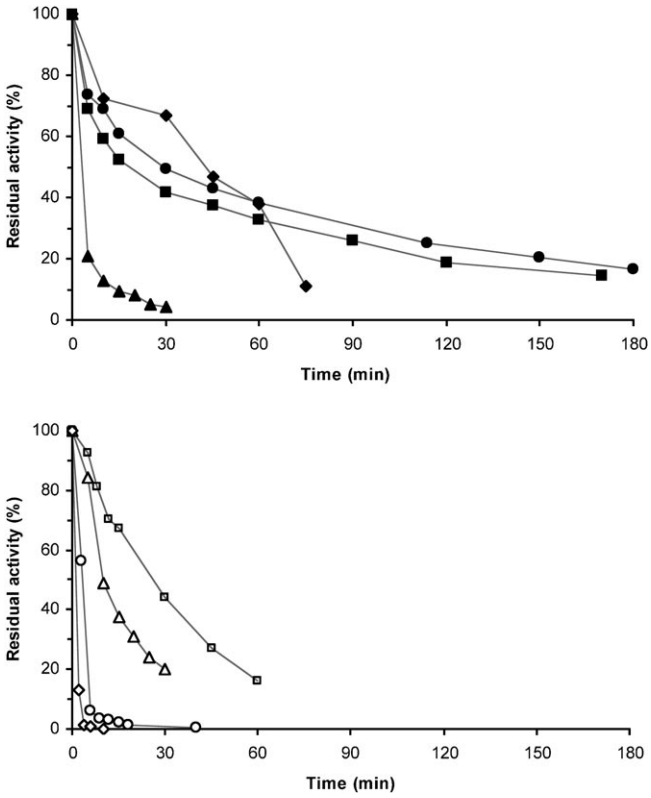


Figure 2. Thermal stability at 60 °C of PGA from *P. rettgeri* (black symbols) and PGA from *E. coli* (empty symbols) immobilised on different Sepabeads® carriers. Legend: triangles = epoxy polymer; squares = amino polymers with short spacer arms; circles = amino polymers with long spacer arms; diamonds = native enzyme in solution.

ent negligible differences.[17] To further investigate the effect of glycosylation on the stabilisation of PGA*p*, an amino Sepabeads® polymer was prepared bearing the aminic functionality on a longer spacer arm, namely by inserting four additional methylene residues between the polymer backbone and the reactive functionality. Longer spacers can affect enzyme stability since they allow for higher thermal vibration. However, at the same time the enzyme molecules can distribute and accommodate themselves sufficiently distant from the polymer surface, thus minimising steric hindrance and also maintaining a higher conformational flexibility.

PGA*p* shows a striking preference towards the amino carrier, which indicates that different mechanisms of anchoring and stabilisation occur on the carriers bearing the two different functionalities.

The stability of PGA*p* on the epoxy polymer is the lowest of the series and this suggests either an improper formation of multipoint attachment or a repulsion between the hydrophilic glycan and the hydrophobic resin, which leads to a faster denaturation of the protein.

The analysis of the thermal inactivation of PGA*e* points out a significantly different situation. The enzyme is less stable when immobilised on the long spacer arm amino polymer due to the conformational freedom of the long arms, which cannot confer structural rigidity to the protein, whereas immobilisation on shorter arms polymers leads to higher stability.

Structural Analysis and Immobilisation of CalB

The influence of protein glycosylation on immobilisation efficiency was then investigated also for lipase B from *C. antarctica*, an enzyme expressed in *Aspergillus oryzae*, a eukaryotic host that determines its glycosylation. Interestingly, Sheldon and co-workers have recently reported that CalB undergoes self-cross-linking upon oxidation of the glycan moiety with sodium periodate.[11]

The analysis of the primary sequence of CalB indicated that there is one single *N*-glycosylation site, namely the Asn 74. Figure 3 presents a frontal view of the enzyme, where the green residues on the left are two *N*-acetylglucosamine molecules, as present in the crystal structure, bound to the Asn 74 and they represent the initial part of the glycan that decorates the protein.

The Lys residues on the surface of the enzyme are visualised in the space filling notation and it can be noted how Lys 290, (pointed out by the arrow) might

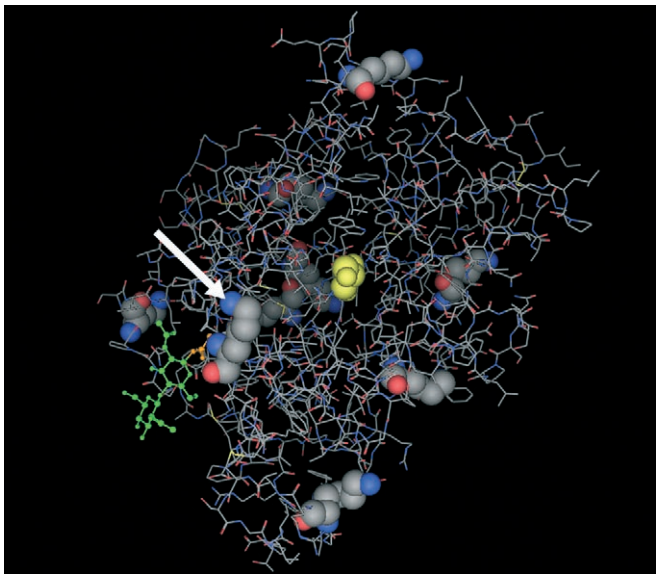


Figure 3. Crystal structure of CalB (PDB id: 1TCA).[29] The two GlcNAc unities linked to Asn 74 are evidenced in green. The catalytic Ser is shown in yellow in the centre of the active site. The superficial Lys residues are given in grey, blue and red (space filling notation) and it can be noted how Lys 290 (white arrow) is the closest to the opening of the active site.

constitute an undesired site for the formation of covalent bonds with the carrier due to its location close to the entrance of the active site.

As in the case of PGA the immobilisation of CalB was carried out on both amino and epoxy Sepabeads®. All enzymatic preparations were rinsed thoroughly with NaCl 0.5 M in Kpi buffer in order to desorb proteins that were non-covalently bound to the polymer. However, it was verified that a negligible amount of enzyme (<0.2%) was released upon this treatment indicating that all the protein was effectively linked to the carrier covalently.

The activity of CalB (*p*-nitrophenyl palmitate hydrolysis) immobilised on epoxy Sepabeads® is more than 40-fold higher as compared to the commercial preparation Novozym 435® (2.1 μmol/min·g_{dry}), where the CalB is physically adsorbed on a macroporous methacrylic polymer (Table 2).[33] It must be noted that all the enzymatic preparations retained their activity for at least 2 months when stored at 4 °C.

As shown in Figure 4, native CalB is a thermostable enzyme. The immobilised CalB does not demonstrate a clear preference for amino carriers but rather undergoes a severe decrease of stability when immobilised on amino polymers bearing longer spacer arms.

Moreover, the destabilising effect of the long spacer arm on CalB indicates that the stability of PGA*p* on amino polymers cannot simply be ascribed to an intrinsic higher stability of the glycosylated native protein.

Graphics reported in Figure 4 also suggest the contribution of different kinetics and mechanisms of inactivation.

In order to gain more information on the factors that determine the different behaviour of the glycosylated PGA*p* and CalB when immobilised on the amino Sepabeads®, we analysed and compared the chemical nature of the surfaces of the two enzymes.

Although in the case of CalB the interfacial activation through the "lid" opening is absent,[29] nevertheless the enzyme has evolved for acting at the lipid/water interface. This is clearly demonstrated by the distribution of the hydrophilic and hydrophobic regions on the protein surface as shown in Figure 5. The visualisation was obtained by means of the GRID computational method (see Experimental Section) that allows the description of the regions of the protein able to establish either hydrophobic or hydrophilic interactions.

The GRID program calculated the molecular fields that are generated by the interaction of the enzyme with two different chemical probes, aliphatic carbon (yellow, on the left) and water (blue, on the right). The blue areas correspond to hydrophilic zones that are more prone to be hydrated, whereas the yellow

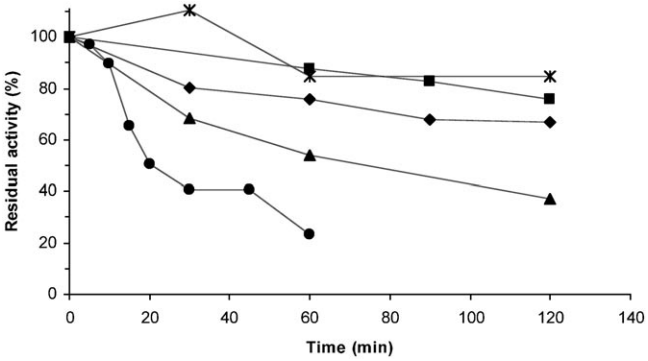


Figure 4. Thermal stability at 50 °C of Novozym 435® and CalB immobilised on different Sepabeads® carriers. Legend: Stars = Novozym 435®; triangles = epoxy polymer; squares = amino polymers with short spacer arms; circles = amino polymers with long spacer arms; diamonds = native enzyme in solution.

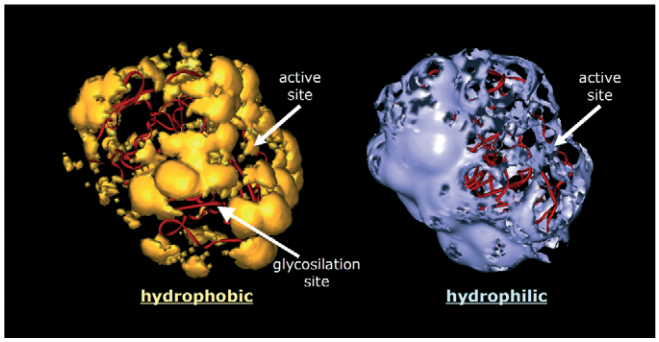


Figure 5. Comparison of the molecular interaction fields generated by interaction of CalB with the aliphatic carbon probe (yellow, on the left) and with the water probe (blue, on the right), calculated by the GRID program and visualised at the energy level of 1 kcal mol^{-1} and 3 kcal mol^{-1}, respectively.

Table 2. Activity of CalB[a] (glycosylated) immobilised on epoxy and amino Sepabeads®.

Support	Bound protein %	Water content (% w/w)	Specific activity[a] (U/g_{dry})
Epoxy Sepabeads®	85	64	92
Amino Sepabeads® *(short spacer)*	98	57	25
Amino Sepabeads® *(long spacer)*	89	73	44
Novozym 435®	-	3	2.1

[a] Hydrolysis of *p*-nitrophenyl palmitate.

spots represent the hydrophobic parts of the enzyme surface. The difference between CalB and PGA*p* (Figure 6) is striking: while the PGA surface presents a homogeneous distribution of both hydrophilic and hydrophobic areas, CalB is characterised by regions that have an exclusive hydrophobic or hydrophilic nature.

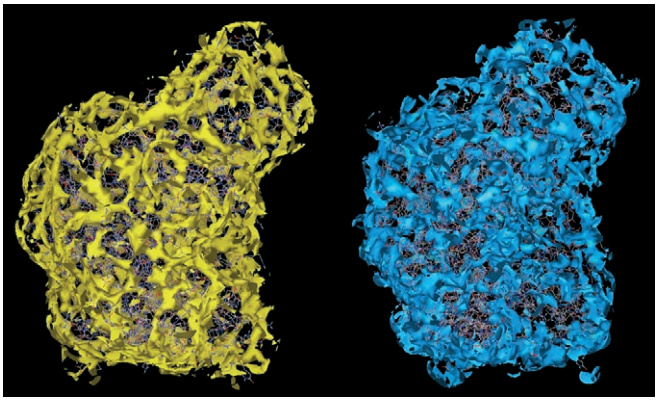


Figure 6. Comparison of the molecular interaction fields generated by interaction of PGA*p* with the aliphatic carbon probe (yellow, on the left) and with the water probe (blue, on the right), calculated by the GRID program and visualised at the energy level of 1 kcal mol^{-1} and 3 kcal mol^{-1}, respectively. A uniform distribution of the polar and hydrophobic zones on the surface of the enzyme can be noted.

The insertion of a glycan moiety in the PGA*p* structure can determine the creation of a strongly hydrophilic area and a major change in the balance of hydrophilic/hydrophobic interactions with the enzyme surface.

In the case of CalB it is interesting to note how a broad hydrophilic area is present opposite to the active site. This would suggest that the orientation and even the conformation of CalB can change significantly when adsorbed on carriers that differ in hydrophilicity. Indeed, it has already been reported that the enzyme changes its behaviour upon moving from hydrophobic to hydrophilic carriers.[30,31]

While it is quite reasonable to expect that CalB adsorbs on a hydrophilic carrier by interacting with the broad polar area opposite to the active site, the situation, in the case of hydrophobic interactions, would be less clearly defined. Indeed, in a significant number of cases N-linked glycans are involved in covering/stabilising hydrophobic patches of the protein surface, similarly to what is depicted in Figure 5.

Conclusions

Computational simulation of the structures of PGA from *Providencia rettgeri* (PGA*p*) expressed in *Pichia pastoris* and lipase B from *Candida antarctica* (CalB) was used to visualise the glycosylation sites as well as regions of enzymes prone to interact and establish covalent bonds with immobilisation carriers. The description of the hydrophilic/hydrophobic balance of the surface of the two proteins clearly revealed the functional diversity of the lipase, which acts at the aqueous/oil interface. The experimental data obtained from the immobilisation of PGA*p* and CalB indicate that the two enzymes, although both glycosylated, show different preferences towards polymers bearing either amino or epoxy functionalities. The different behaviours suggest a possible involvement of the glycan moiety in the anchoring and stabilisation of the glycosylated GA on amino supports.

Experimental Section

Materials

Enzymes: Recombinant penicillin G amidase from *P. rettgeri* (PGA*p*) was expressed in *P. pastoris* and produced as described before.[2] It was used as lyophilised powder having an activity of 14 U/mg (benzylpenicillin units). Penicillin G amidase from *E. coli* (PGA*e*) was purchased from Fluka and used as lyophilised powder having an activity of 17 U/mg (benzylpenicillin units). Lipase B from *C. antarctica* (Chirazyme L-2) was obtained from Roche and contains 23% of protein (determined by the Pierce method)[32] and this is in accordance with what is stated by the producer (10–30% according to the Bradford assay). The enzymatic preparation has an activity of 45 U/mg (tributyrin hydrolysis). Novozym 435® was kindly donated by Novozymes. According to Secundo et al. it contains 5% w/w of protein and 2% of CalB.[33]

Immobilisation carriers: Sepabeads® are methacrylic polymers produced and commercialised by Resindion S.r.l. (Mitsubishi Chem. Corp, Milano, Italy), supplied with a water content of about 60% w/w. Sepabeads® with oxirane or amino groups were used in the present work. Two different amino polymers were used, which differ in the length of the spacer connecting the polymer backbone and the reactive functionality (the longer spacer presents four additional methylene groups).

Chemicals: All chemicals were purchased from Sigma–Aldrich and were used without any further purification.

Spectroscopy: UV measurements were performed with a Lambda 20 UV/Vis Perkin–Elmer spectrophotometer.

Enzyme Immobilisation on Epoxy Sepabeads®

The immobilisation of the three enzymes on the polymer was carried out at 25 °C and 40 rpm in a blood rotator in KH_2PO_4/K_2HPO_4 Kpi buffer (1.25 M, pH 8.0). 300 U of PGA per gram of polymer were used with a support/buffer ratio of 1/4 w/v. The immobilisation of CalB was carried out by dissolving 900 units of enzyme (tributyrin hydrolysis), in its lyophilised form, in buffer with a support/buffer ratio of 2/3 w/v. For both enzymes the immobilisation proceeded for 18 h, under constant stirring, then for 20 h without stirring.

The solution was filtered and the liquid phase was recovered for protein determination. The immobilised protein was washed with Kpi buffer (0.02 M, pH 8.0, ratio support/buffer 1/4 w/v). Adsorbed proteins were desorbed by adding Kpi buffer (0.02 M, pH 8.0, ratio support/buffer 1/4 w/v) and then by stirring for 45 min. After each step the supernatant was recovered for protein determination.

In the case of CalB, due to its pronounced propensity to adsorb on hydrophobic surfaces, the immobilised CalB underwent a further treatment with NaCl 0.5 M in Kpi buffer (0.02 M, pH 8.0, ratio support/buffer 1/4 w/v) under stirring for 45 min to ensure the complete desorption of non-covalently bound proteins.

The preparations were finally rinsed with Kpi buffer (0.02 M, pH 8.0, ratio support/buffer 1/4 w/v) and the activity was checked. The immobilised enzymes were stored at 4 °C.

Enzyme Immobilisation on Amino Sepabeads®

The immobilisation was carried on the two polymers that were pre-activated with glutaraldehyde (2% v/v in Kpi buffer, 0.02 M, pH 8.0, support/buffer ratio of 1/4 w/v) for 60 min at 25 °C and then washed twice with the same buffer. The immobilisation of PGA was carried out by using 300 U of the lyophilised enzymatic preparation per gram of support (support/buffer ratio of 1/4 w/v). The immobilisation was carried out under stirring for 18 h in Kpi buffer (0.02 M, pH 8.0). The supernatant was then recovered for protein determination. The immobilisation of CalB was carried out according to the same procedure and using 900 U (tributyrin hydrolysis) of the lyophilised enzymatic preparation per gram of support (support/buffer ratio of 2/3 w/v).

After immobilisation all the enzymatic preparations were rinsed with Kpi buffer (0.02 M, pH 8.0, ratio support/buffer 1/4 w/v). Adsorbed proteins were desorbed by washing with NaCl 0.5 M in Kpi buffer (0.02 M, pH 8.0, ratio support/buffer 1/4 w/v) under stirring for 45 min. After each step the supernatant was recovered for protein determination. The preparations were finally rinsed with the same buffer and the activity was checked. The immobilised enzymes were stored at 4 °C.

It must be underlined that different batches of PGA*p* and amino Sepabeads® were used in order to obtain a more representative data set and to verify any effect due to the glycosylation microheterogeneity that can occur due to slight variations in fermentation conditions.

PGA Activity

Benzylpenicillin assay: The enzymatic activity was assayed by hydrolysis of benzylpenicillin potassium salt. A 10% solution of benzylpenicillin in Na_2HPO_4/NaH_2PO_4 buffer (0.02 M, pH 8.0) was prepared. About 30 mg_{wet} of immobilised preparation were suspended in 16 mL of Na_2HPO_4/NaH_2PO_4 buffer (0.02 M, pH 8.0) and 4 mL of benzylpenicillin solution were added. The released phenylacetic acid was titrated, under constant stirring, with NaOH 0.05 M by using an automatic titrator Mettler Toledo DL 50 Graphix.

One unit corresponds to the amount of preparation that hydrolyses 1 μmol of benzylpenicillin per min at 37 °C in phosphate buffer, pH 8.0.[34]

NIPAB assay: The PGA activity was determined using the chromogenic substrate NIPAB (2-nitro-5-phenylacetylaminobenzoic acid) by monitoring the release of 5-amino-2-nitrobenzoic acid (NABA). The assay mixture consisted of 2 mL Kpi buffer (0.02 M pH 8.0) and 1 mL of 0.015 M NIPAB solution in the same Kpi buffer and 20–25 mg_{wet} of immobilised preparation or 200–500 μL of a solution of the native enzyme (3.6 $mg\,mL^{-1}$) in Kpi buffer.

The samples were mixed for 10 min, 25 °C, at 150 rpm in an orbital shaker, then filtered (0.45 μm) and analysed spectrophotometrically. The conversion of NIPAB to NABA was detected at 405 nm ($\varepsilon_{405} = 9090$). One unit was defined as the amount of enzyme required to produce 1 μmol of NABA per min at 25 °C.[35]

CalB activity

p-*Nitrophenyl palmitate assay:* The activity was determined by following the hydrolysis of *p*-nitrophenyl palmitate to *p*-nitrophenol. The mixture for the assay of the immobilised preparation consisted of 1.8 mL Kpi buffer (0.025 M pH 7.5), 0.2 mL of a solution of *p*-nitrophenyl palmitate in acetonitrile (0.0032 M) and 20 mg of the wet immobilised preparation or 100 μL of a solution (9.7 $mg\,mL^{-1}$) of the native enzyme. The samples were mixed at 37 °C in a blood rotator. After filtration (0.45 μm), the pH was shifted to 10.0 by adding 2 mL of sodium hydroxide (0.025 M) and the samples were analysed spectrophotometrically at 410 nm.

One unit of activity was defined as the amount of enzyme required to produce 1 μmol of *p*-nitrophenol per min at 37 °C. Blank experiments were carried out to exclude spontaneous hydrolysis in the experimental conditions used for the assay.[36]

Tributyrin assay: The activity of the native enzyme was assayed by following the hydrolysis of tributyrin and by measuring the liberation of butyric acid that was titrated with 0.1 M sodium hydroxide. 48.5 mL Kpi buffer (0.01 M, pH 7.0) were incubated in a thermostatted vessel at 25 °C, equipped with a mechanical stirrer. After addition of 1.47 mL tributyrin, the pH-stat system adjusted the pH at 7.0. After pH stabilisation, about 5–10 units of Chirazyme L-2 were added. The consumption of 0.1 M sodium hydroxide was monitored for 10–15 min. One unit of activity was defined as the amount of enzyme required to produce 1 μmol of butyric acid per min at 25 °C.

Protein Determination

The percentage of bound enzyme was determined by the difference between the concentration of the native protein before immobilisation and in the filtrates after immobilisation. The protein amount was determined by using bicinchoninic acid kit (SIGMA) for protein determination, using a PGA or CalB solution for calibration curve.[32]

Determination of Water Content

The water content of each preparation was evaluated by drying the samples at 110 °C for 6 h on aluminium dishes and by determining the difference in weight between the wet and the dried sample.

PGA Stability

Mixtures containing 1 mL of Kpi buffer (0.02 M, pH 8.0) and 20–25 mg of enzymatic wet preparation or 200–500 μL of a solution of the native enzyme (about 3 mg mL^{-1}) were incubated in 3 mL syringes, equipped with 0.45 μm filters, at 60 °C for variable periods of time and each experiment was repeated twice and the data presented are mean values (s.d. = 5.34). The residual activity was measured by NIPAB assay.

CalB Stability

Twenty milligrams of the wet immobilised preparation or 100 μL of a solution of native enzyme (about 9 mg mL^{-1}) were added to 1.8 mL of Kpi buffer (0.025 M pH 7.5) and incubated at 50 °C for variable periods of time. The residual activity was determined by following the hydrolysis of *p*-nitrophenyl palmitate. Each experiment was duplicated and data are presented as mean values.

Covalent Binding of β-D-Mannopyranoside onto Amino Sepabeads®

Amino Sepabeads® were pre-activated by mixing them with glutaraldehyde (2% v/v in Kpi buffer, 0.02 M, pH 8.0, support/buffer ratio 1/4 w/v) for 60 min at 25 °C. The resin was rinsed twice with the same buffer. A solution of *p*-nitrophenyl β-D-mannopyranoside (0.14 M in Kpi buffer/MeCN 60:40, equal to 0.9 mmol per gram of wet support) was added to the pre-activated amino support. The suspension was maintained at 4 °C without stirring for 5 days. The support was then rinsed with Kpi buffer (0.02 M, pH 8.0, ratio support/buffer 1/5 w/v). The carrier was rinsed extensively with NaCl 0.5 M in Kpi (pH 8.0, 0.02 M, ratio support/buffer 1/5 w/v) and maintained under stirring for 45 min to desorbe the non-covalently bound mannoside. After a final washing with Kpi buffer (0.02 M, pH 8.0, ratio support/buffer 1/5 w/v), the degree of immobilisation was determined by analysing the amount of *p*-nitrophenol released in the solution after the hydrolysis of the *p*-nitrophenyl β-D-mannopyranoside in solution with HCl 6 N (UV determination at 410 nm after adjusting the pH to 11 with NaOH 6N).

The stability of the acetal was studied by incubating 200 mg of the modified polymer in 1 mL of Kpi buffer 0.1 M, pH 6.0 for 5 days and by determining spectrophotometrically the *p*-nitrophenol in solution.

Computational Studies

All the calculations were performed on a dual Xeon Linux workstation. GRID version 22 (Moldiscovery, Inc., London) was used for the calculation of the MIFs. Molecular Operating Environment (MOE) version 2004.03 (Chemical Computing Group, Montreal) was used for molecular visualisation and manipulation.

Molecular Modelling and Structural Analysis of Enzymes

The protein sequence of PGA from *P. rettgeri* was taken from UniProtKB/TrEMBL (accession number: Q7WZI9) database by using the EMBL-EBI query instruments.

The three-dimensional structure of the PGA from *P. rettgeri* was calculated by means of a homology modelling algorithm, implemented in MOE (Molecular Operating Environment, Chempcomp Inc.), using the structure of a mutant of PGA from *P. rettgeri*, which was retrieved from PDB (PGA id: 1CP9), as a template. The identity between the two sequences is higher than 90% and the overall quality of the homology model, evaluated by the PROTEIN REPORT TOOL of MOE is comparable to that of the crystallographic template.

The *N*-glycosylation sites in the protein sequence were identified by using the protein motifs searching tool FUZZ-PRO as available in the EBI SRS server.[37] The standard "N-any-S/T" pattern was used as a query.

The impact of the presence of a glycan moiety, in terms of electrostatic interactions and steric hindrance, has been evaluated by using the GRID computational method, version 22a. The hydroxy probe was selected to construct the isoenergy surface.[6,38,39]

The sequence of lipase B from *C. antarctica* was retrieved from SwissProt database (accession number: P41365), by using the search tools provided by EMBL-EBI. The FUZZ-PRO tool, as provided by the EBI server, was used for the identification of *N*-glycosylation sites, by applying the same approach used in the case of PGA*p*. The three-dimensional structures of lipase B from *C. antarctica* was retrieved from PDB (CalB id: 1TCA).

The chemical nature of PGA and CalB surface was studied by using the GRID program that calculated the molecular fields that are generated by the interaction of the enzyme with water and aliphatic carbon probes, respectively.

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