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Application of a chemoenzymatic glycosylation method to α-chymotrypsin and *Candida rugosa* lipase surface modifications

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Abstract

A recently developed chemoenzymatic glycosylation procedure has been successfully applied on two hydrolytic enzymes, α-chymotrypsin and *Candida rugosa* lipase. First, a number of sucrose molecules have been bound to the surface lysine residues and then, lengthening of the glycosidic chains has been carried out by the action of a levansucrase from *Bacillus subtilis*. For both steps, reaction conditions have been studied in order to obtain a range of glycosylation degrees. The influence of glycoside binding on biocatalyst surface characteristics has been assessed and a progressive increase in global enzyme hydrophilic character with glycosylation has been observed. Besides, the study of hydrolytic activity and kinetic constants showed that the performed modifications brought about a certain decrease in enzyme hydrolytic activity and very slight variations in enzyme–substrate affinity.

Keywords: α-Chymotrypsin; Lipase; Glycosylation; Hydrophilicity; Activity; Kinetic constants

1. Introduction

Much enzymological research has focused on the development of strategies for optimizing enzymatic behaviour for industrial applications. Special interest has been paid to the improvement of thermal [1–4], pH or proteolytic stability [5–7], physico–chemical properties [8–12] and, lately, to the development of new activities (i.e. application of hydrolytic enzymes to synthetic reactions) [9,13–16].

Since protein surface characteristics, namely hydrophobicity and surface charge distribution, appear to play a major role in enzyme properties [2,17,18] it should be possible to alter enzyme function by modifying the catalyst surface and/or the micro-environment. Several strategies have been proposed [19], but the most popular one seems to be the covalent modification of protein surface residues by attaching compounds (i.e. glycosides) that can help modify enzyme hydrophobic–hydrophilic character or surface charge. The idea of using sugars as enzyme surface modifiers is inspired by natural proteins, most of which are glycoproteins in which the glycosidic moiety plays an important role in increasing solution viscosity, resistance to proteolytic attack, drug life-time in blood, etc. [20].

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Enzyme modification has been mainly achieved by chemical means: many methods have been used, most of them based on the binding of sugar aldehyde groups to protein lysine amino residues [1,3,21]. A novel chemoenzymatic glycosylation technique was recently developed [22], which involves chemical binding of sucrose molecules to the enzyme surface in a first step, followed by a second step in which a glycosyltransferase transfers fructose residues from a donor to the enzyme-bound glycosidic moieties, lengthening them in a progressive and easy-to-control way. The glycosyltransferase used in this second step acts on a non-conventional substrate, whose particular size and structure could provoke specificity and steric problems. Nevertheless, this technique had produced excellent glycosylation results when applied to a small enzyme, lysozyme (MW = 14.4 kDa).

The aim of the present work has been to assess the applicability of the previously developed chemoenzymatic glycosylation method to new substrates, namely some enzymes with structures and sizes different from lysozyme, in an attempt to validate its general application to protein glycosylation. Two hydrolytic enzymes, α-chymotrypsin and *Candida rugosa* lipase have been glycosylated using this technique. In both cases, reaction conditions have been thoroughly studied and several modification degrees have been achieved. The effects of increasing amount of enzyme-bound sugar on surface characteristics, such as hydrophilicity and on enzyme hydrolytic activity and kinetics have also been studied.

2. Experimental

2.1. Materials

Bovine pancreas α-chymotrypsin (EC 3.4.21.1), *Candida rugosa* lipase (EC 3.1.1.3), cyanogen bromide, *N*-acetyltyrosine ethyl ester (ATEE) and tributyrin were obtained from SIGMA. Radioactive labeled sucrose was obtained from NEN Research Products. Levansucrase from *Bacillus subtilis* was kindly provided by Eridania Beghin-Say company. All other reagents were analytical grade.

2.2. Assay of α-chymotrypsin hydrolytic activity

Controls and samples were diluted in osmosed water to 5 mg of protein/ml. Then, 25 μl of diluted controls and samples were added to 10 ml of substrate, at an initial pH value of 7 and a temperature of 30°C. The substrate was 50 mM *N*-acetyl tyrosine ester (ATEE) and 20 mM $CaCl_2$ in a mixture of 30% ethanol and 70% 5 mM Tris/HCl buffer (pH 7.0). The pH of the mixture was kept at a value of 7.0 by addition of 50 mM sodium hydroxide in a pH-stat Metrohm. The profile of NaOH addition was recorded during 5 min and the enzyme activity was calculated from the slope of the straight lines obtained. The added NaOH indicated the amount of acid produced during the enzymatic hydrolysis of the amino-acid ester (ATEE). One α-chymotrypsin activity unit was defined as the amount of enzyme which hydrolyzed 1 μmol of substrate per min at pH 7.0 and 30°C.

2.3. Assay of lipase hydrolytic activity

The substrate was composed of 15 ml of tributyrin, 50 ml of emulsifying agent and 235 ml of water; the emulsifying agent was prepared by mixing 17.9 g of NaCl, 0.41 g of KH_2PO_4, 540 ml of glycerol, 6 g of arabic gum and enough osmosed water to obtain a total volume of 1000 ml.

Controls and samples were diluted in osmosed water to 3 mg of protein/ml. Then, 0.5 ml of diluted controls and samples were added to 10 ml of substrate, at an initial pH value of 6.8–6.9 and a temperature of 30°C. The assay solution was kept at pH 6.8 by addition of 50 mM sodium hydroxide in a pH-stat Metrohm. The added NaOH neutralized the butyric acid

produced by enzymatic hydrolysis of the tributyrin substrate. The profile of NaOH addition was recorded during 8 min and the enzyme activity was calculated from the slope of the straight lines obtained. One lipase activity unit was defined as the amount of enzyme which produced 1 μmol of butyric acid per min at pH 6.8 and 30°C.

2.4. Chemoenzymatic glycosylation of enzymes [22]

2.4.1. Chemical binding of sucrose

Sucrose was activated by treatment with cyanogen bromide [23], after which sufficient enzyme to achieve the desired sucrose/enzyme molar ratios in the reaction mixture was added at pH 9.0. The coupling reaction was allowed to occur for 16 h at 4°C and stopped by addition of glycine. The modified enzymes were purified and freeze-dried. Control solutions were prepared without addition of cyanogen bromide.

2.4.2. Enzymatic lengthening of the glycosidic chains by levansucrase from bacillus subtilis

A 0.14 mM solution of the previously prepared sucrose-modified enzymes (around 9 mol of sucrose/mol of enzyme) was prepared in 50 mM potassium phosphate buffer (pH 6.0). Suitable amounts of this solution were treated with 0.13 mg of levansucrase/ml (5 units/ml) at 37°C in the presence of several sucrose concentrations for different reaction times. Product and reagents were finally separated and the modified enzymes were freeze-dried. Control solutions were prepared without addition of levansucrase.

2.4.3. Purification of glycosylated enzymes

α-Chymotrypsin and lipase in control solutions and samples were separated from residual reagents by anion exchange chromatography, using a Mono-Q HR 5/5 column connected to an FPLC (fast protein liquid chromatography) system, both from Pharmacia LKB. The column was eluted at 1 ml/min with a gradient of 20 mM piperazine buffer pH 9.5 as eluent A and the same buffer containing 1 M NaCl as eluent B. Protein fractions were collected, dialyzed and freeze-dried.

2.4.4. Determination of the extent of modification

Sugar fixed onto the enzyme surface was detected by addition of a certain amount of suitable radioactive sucrose to the reaction mixture and measurement of the radioactivity associated with the enzyme after purification, using a liquid scintillation system Beckman LS 1801. Uniformly ^{14}C-labeled sucrose was used for the chemical binding step and sucrose labeled with ^{14}C on fructose for the enzymatic lengthening step.

2.5. Determination of surface hydrophilic character of the enzymes

Native and modified enzyme hydrophilicity was evaluated from the reversed phase chromatography profiles obtained in an HPLC Hewlett Packard 1090 equipped with a Nucleosil C18 5 μm column (220 × 2.1, SFCC). Following sample injection, the column was eluted at 0.5 ml/min with a gradient of H_2O/0.1% TFA as eluent A and ethanol/0.1% TFA as eluent B. After a 5 min step of 100% A, a linear gradient from 40% to 100% B in 15 min was applied. Eluted proteins were evaluated from the on line measurement of the optical density at 280 nm.

2.6. Determination of kinetic constants for hydrolysis reactions

Kinetic parameters of the enzyme catalysed hydrolysis reactions were calculated from the study of the hydrolytic activities at several substrate concentrations, using Lineweaver-Burk plots.

3. Results and discussion

3.1. Enzyme glycosylation procedure: applicability and optimization of the reaction conditions

Glycosylation of both α-chymotrypsin and *Candida rugosa* lipase has been carried out by a two step chemoenzymatic method previously described [22]: first, sucrose molecules were chemically affixed to the enzyme surface; then, the glycosidic chains were lengthened by sequential transfer of fructose residues from sucrose, in a reaction catalysed by levansucrase from *Bacillus subtilis*.

During the chemical glycosylation step, sugar is preferentially bound to free primary amino groups (lysine and/or terminal α-amino groups) [24]. Several sucrose/free enzyme amino groups molar ratios were used in the reaction mixture and a range of modification degrees were achieved, as can be seen in Fig. 1. Differing extents of glycosylation could be very useful for evaluating the effects of increasing bound sugar content on enzyme properties. The modification degrees mentioned are average values: anion exchange and reversed phase chromatography profiles of the enzyme solutions after modification show a remarkable homogeneity, but the possibility of product mixtures cannot be discounted, since this is a major problem encountered with chemical modification techniques.

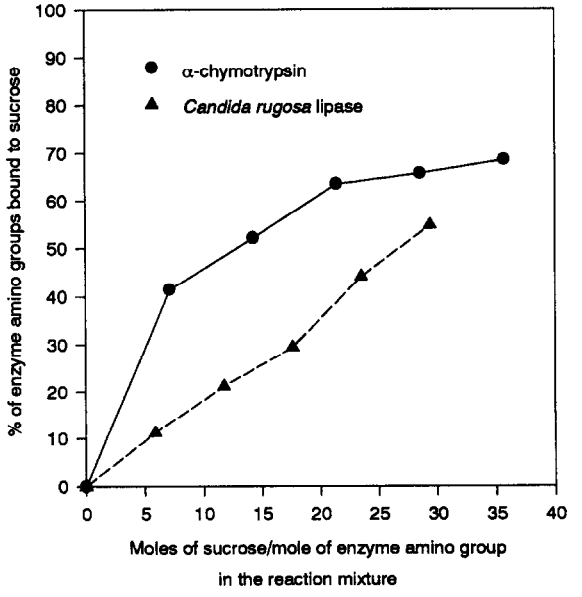

Fig. 1. Degrees of chemical binding of sucrose to α-chymotrypsin and *Candida rugosa* lipase surface lysines.

High modification degrees (nearly 70% of modified amino groups for α-chymotrypsin and above 50% for lipase) were obtained using sucrose/enzyme ratios in the reaction mixture of around 30 mol of sucrose/mol of protein lysine. Obtaining high sucrose binding levels was important, since these molecules were the starting point for the enzymatic lengthening step. The amount of bound sugar increased with increasing sucrose/enzyme ratio in the reaction mixture, but this trend was more pronounced for α-chymotrypsin, in which 6 amino groups (41%) were modified with 7 mol of sucrose/mol of NH_2 residue, while in the case of lipase no more than 3 groups (17%) were modified under same reaction conditions. This fact might be explained by local microenvironment effects, modifying the reactivity of specific amino groups. As a consequence, a stationary sucrose binding level was reached for α-chymotrypsin, meaning that all the accessible and reactive amino groups had been used. In the case of lipase, the chemical glycosylation profile pointed out that there were some accessible amino groups lost. This hypothesis is supported by previously published results [22] on another small enzyme, lysozyme (14.4 kDa), which showed a sucrose binding pattern similar to that of α-chymotrypsin. Furthermore, the chemical glycosylation profiles indicate that, in the case of α-chymotrypsin a stationary binding level was attained, meaning that all the accessible amino groups had reacted. In the case of lipase, the profile suggests that some accessible amino groups remain even at the highest sucrose levels. This difference between α-chymotrypsin and lipase is not unexpected, considering that the latter initially had 17 free amino groups while the former only had 14. Again supporting

this explanation, chemical modification of lysozyme, which possesses only 6 amino groups, reached a stationary level at 95% modification, for high sucrose/amino groups ratios in the reaction mixture [22].

In all cases, it was difficult to generate very low degrees of modification by chemical means under the tested reaction conditions (the minimum values obtained were 2 amino groups for the lipase and 6 for the α-chymotrypsin). It could be interesting to test lower sucrose concentrations in the reaction mixture, but the risk of obtaining complex product mixtures would be unavoidable.

The high degree of modification obtained with α-chymotrypsin agree with previous reports, in which derivatization of 10 [25] and 12 [2] amino groups of α-chymotrypsin with anhydrides is described. When bulky reagents are involved, the extent of modification is usually quite low, as reported by Lenders and Crichton [1] who were able to derivatize only 2 amino groups in amylolytic enzymes with large dextrane molecules.

The second glycosylation step consisted of an enzymatic lengthening of the chemically bound glycosides by a levansucrase from *Bacillus subtilis*. This enzyme transfers fructose from sucrose to acceptors (mainly sugars) containing a terminal fructose, forming levan-like polymeric structures [26] and it has been shown to efficiently work on a small enzyme, lysozyme, which had been chemically modified with sucrose molecules [22]. The ability of levansucrase to catalyse the fructose transfer onto larger protein-like substrates, such as α-chymotrypsin and lipase has been assessed.

The efficiency of levansucrase at lengthening glycosidic chains attached to α-chymotrypsin and lipase was evaluated at different sucrose concentrations in the reaction mixture (Fig. 2A) and for several reaction times (Fig. 2B).

Enzymatic glycosylation proceeded successfully for both enzymes, but it appeared more efficient to α-chymotrypsin (maximum value of around 70 mol of fructose transferred per mol of enzyme) than for lipase (around 40 mol of fructose transferred per mol of enzyme). The degree of lengthening increased with sucrose concentration in the medium, in a pattern that seemed to be similar for α-chymotrypsin and lipase up to 0.75 M of sucrose; beyond this point, lengthening of lipase-bound sugar chains reached a stationary level, while α-chymotrypsin chains kept increasing in size, at least up to 1 M sucrose concentration in the reaction mix-

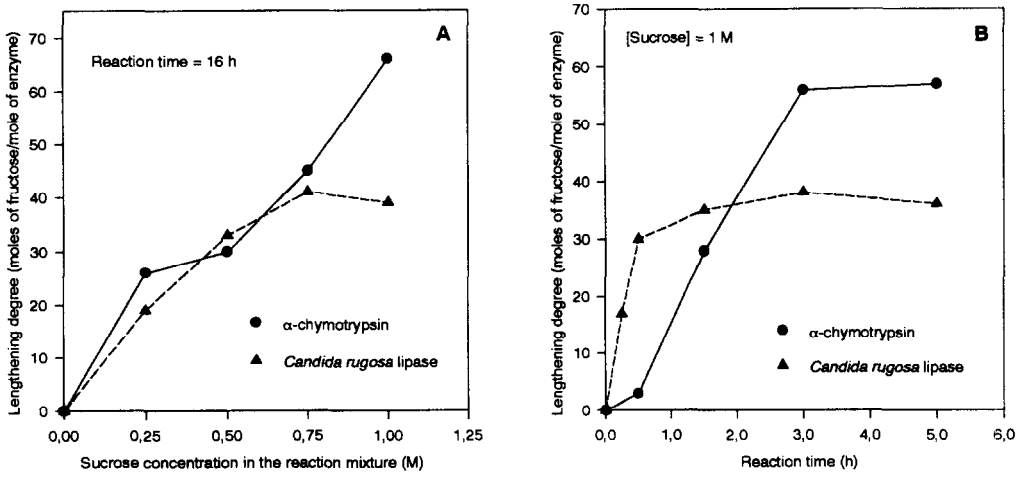

Fig. 2. Lengthening of enzyme-bound glycosidic chains by levansucrase from *Bacillus subtilis*. (A) Lengthening degree as a function of sucrose concentration in the reaction mixture (37°C, pH 6.0, 16 h reaction time). (B) Lengthening degree as a function of reaction time (37°C, pH 6.0, 1 M sucrose).

ture. The different behaviour of levansucrase when acting on different substrates could be due to the sizes of the modified enzymes tested: for bulkier substrates (lipase, MW = 60 kDa), levansucrase action may be affected by steric hindrance; this result agrees with those obtained for chemoenzymatic glycosylation of a smaller enzyme, lysozyme (MW = 14.4 kDa), where longer polysaccharide chains (up to 150 mol of fructose/mol of enzyme) were obtained [22].

As for the influence of reaction time on enzymatic glycosylation, it was studied at 1 M sucrose concentration. Maximum polymerization was achieved after 1.5 h reaction time for lipase and after around 3 h for α-chymotrypsin. It is noteworthy that a maximum chain length was achieved in both cases and this value was clearly higher for the smaller protein (α-chymotrypsin), as had been inferred from the results obtained while studying the influence of sucrose concentration.

3.2. *Effects of glycosylation on surface hydrophilicity*

The influence of enzyme surface characteristics (i.e. charge and hydrophilic/hydrophobic balance) on physico–chemical and catalytic properties has been widely investigated [2,17,18].

Sugars are hydrophilic compounds and therefore their binding to the protein surface should bring about an increase in its global hydrophilic character. In order to evaluate the influence of glycosylation on this property, the chromatographic profiles obtained after passage of the modified enzymes through a reversed phase C18 HPLC column have been studied. This technique requires enzyme elution by organic solvents, which usually involves protein unfolding; hydrophobicity data would correspond then to overall hydrophilic nature, and not only to surface characteristics. Anyway, taking into account that the modified groups are lysines, which are generally found on the protein surface, it could be assumed that the variations in hydrophilicity determined corresponded to changes in surface hydrophobic/hydrophilic balance.

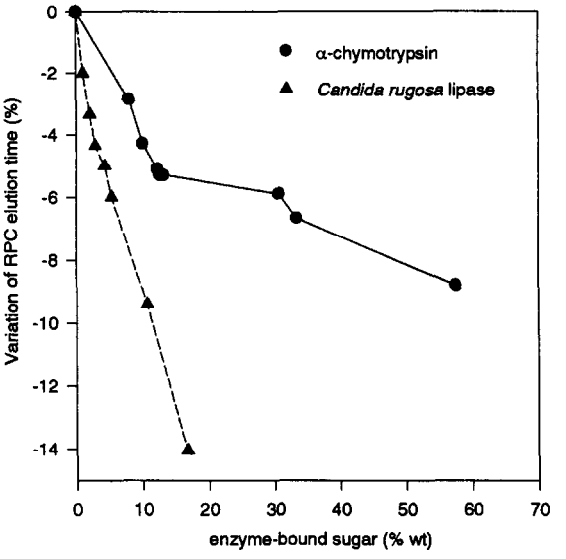

Fig. 3. Variation of α-chymotrypsin and *Candida rugosa* lipase surface hydrophilicity after chemoenzymatic glycosylation: elution times from a C18 HPLC column (eluents: water, 0.1% TFA/ethanol, 0.1% TFA).

As can be seen in Fig. 3, a decrease in elution time was observed after modification, which could be related to a global increase in the hydrophilic character of the biocatalysts. For the two studied enzymes, the greater the extent of glycosylation the stronger were the effects on enzyme surface hydrophobic/hydrophilic balance, although the decrease in elution times was more dramatic for *Candida rugosa* lipase than for α-chymotrypsin. This could be explained by the fact that the chosen lipase is initially a more hydrophilic protein than α-chymotrypsin, as shown by the RPC elution times of native enzymes (6.0 min for lipase and 9.8 min for α-chymotrypsin).

Regarding protein surface charge, both α-chymotrypsin and lipase have undergone a decrease in positive charge, since the modified surface residues were amino groups. Nevertheless, nearly no change in ion exchange chromatography profiles was observed during purification of the modified enzymes, presumably because an anion exchange resin was used

(Mono-Q). Protein binding to the resin takes place through negatively charged residues, which amount remained unaltered after glycosylation.

3.3. Effects of glycosylation on hydrolytic activity

The effects of chemoenzymatic glycosylation on the ability of α-chymotrypsin and lipase to catalyse typical hydrolytic reactions have been studied: hydrolysis of an aminoacid ester (ATEE) was chosen for α-chymotrypsin and hydrolysis of a triacylglycerol (tributyrin) for lipase. In both cases, a decrease in hydrolytic activity was observed, although the effects were greater for lipase than for α-chymotrypsin. Activity of the latter decreased with increasing glycosylation as shown in Fig. 4, until it reached a stationary level (around 30% residual activity) which remained constant even for quite high amounts of fixed glycoside (from 20 to 60 wt%).

More dramatic effects of surface modifications were observed on *Candida rugosa* lipase hydrolytic activity: at low bound-sugar content (up to 5 wt%) it retained around 60% of its initial activity, but at higher glycosylation levels (after the enzymatic lengthening of glycosidic chains) nearly complete loss of activity was observed. This phenomenon could be attributed to the influence of the bound glycosidic moieties, which may hinder the specific binding of substrate (in the case of lipase, the reaction mechanism includes a step in which the lifting of a enzyme surface flap is needed and this could be blocked by the bulky sugar chains). Additionally, the reaction and purification conditions could have been more damaging for lipase than α-chymotrypsin. Activity loss has been described in many cases as a common side-effect in chemical modification of enzymes [1,4,6,21,23,27–30].

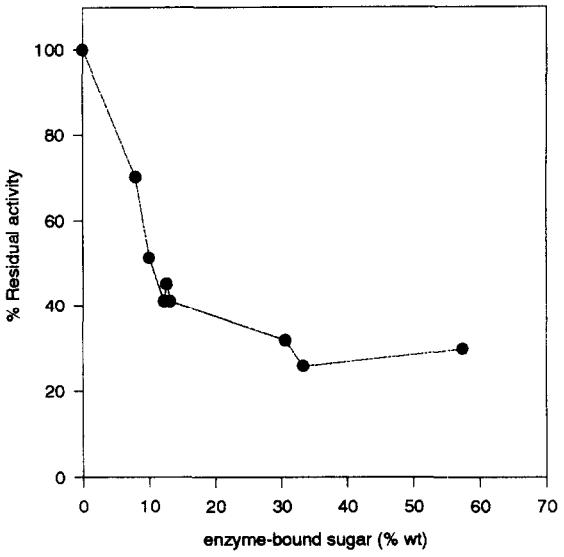

Fig. 4. Hydrolytic activity of chemoenzymatically glycosylated α-chymotrypsin.

In the present work, kinetic parameters were calculated for modified α-chymotrypsin and lipase and compared to those obtained for native enzymes for the same hydrolytic reactions (Table 1).

Many authors have studied chemical modification of enzymes, but most of them have focused on its effects on thermostability or

Table 1
Hydrolysis kinetic constants of native and glycosylated α-chymotrypsin and *Candida rugosa* lipase

α-Chymotrypsin			*Candida rugosa* lipase		
enzyme-bound sugar (wt%)	Vm (U/mg protein)	K_M (mM)	enzyme-bound sugar (wt%)	Vm (U/mg protein)	K_M (mM)
0	500	53	0	125	84
7.9	267	52	1.1	83	69
10.0	250	46	2.0	77	65
12.6	133	44	2.9	83	56
30.6	82	30	4.3	100	60
57.5	82	29	5.3	77	59

physico–chemical characteristics and very few have provided relevant kinetic constants. Consequences of enzyme modification depend on the treated enzyme, the reagent and the derivatization procedure and it is very difficult to propose general rules. Some researchers have modified enzymes with polysaccharides or other modifiers without observing any remarkable variation in kinetic constants [29–32], while others have reported more or less relevant changes in maximum velocities and/or Michaelis constants.

The data presented in Table 1 indicate a decrease in maximum velocity values for glycosylated α-chymotrypsin and lipase, which confirm the already mentioned losses of enzymatic activity as a consequence of glycosylation, and agreed with some of the results found in the literature [33,34]. This decrease can been explained by the binding of molecules to positions near the active site, by changes in enzyme conformation or by non-specific steric hindrance.

On the other hand, a very slight increase in enzyme–substrate affinity was detected for glycosylated enzymes, since the K_M values decreased as higher amounts of sugar were bound to enzyme surface. The existence of non-covalent stabilizing interactions (hydrogen bonding or van der Waals interactions) between the hydrophilic compounds linked to enzyme surface and the substrates could be one of the factors contributing to this decrease. Nevertheless, the significance of the variations in K_M is not very clear, since the effects were less than a factor of two in all cases.

Authors who previously observed some increase in enzyme–substrate affinity after modification [1,35] proposed no explanation for this phenomenon. The opposite effect (decrease in enzyme–substrate affinity after modification) for enzyme binding to polysaccharides [33,36] has also been reported and it was mainly explained by unfavorable steric effects. In the present case, the progressive binding of sugar onto the enzymes was not associated to lower enzyme–substrate affinities; this would imply either a negligible influence of steric hindrances on this property, or the occurrence of simultaneous stabilizing effects. It has to be noted that the chemoenzymatic glycosylation technique developed here permits manipulation of enzyme-bound glycosidic chain length, by acting on reaction conditions; therefore, the optimal glycoside size could be chosen in order to improve enzyme functionality.

The presented work has shown how a chemoenzymatic glycosylation approach could be successfully applied to several biocatalysts, which differ in their sizes and structures. The general applicability of this method to enzyme surface modification can therefore be inferred. Quite high glycosylation levels have been obtained, even for large proteins and the technique can control the amount of bound glycoside by proper choice of the reaction conditions. The study of some enzyme properties showed a loss of hydrolytic activity and very slight improvements in enzyme–substrate affinities after modification. Nevertheless, the influence of biocatalysts surface modifications on their properties were strongly dependent on protein structure and catalytic mechanism. Thus, it would be interesting to assess the effects of the described glycosylation on a variety of enzyme properties and to compare them with the effects of other surface modifications.

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