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β-Glucosidase immobilized and stabilized on agarose matrix functionalized with distinct reactive groups

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ABSTRACT

The bioconversion of lignocellulosic biomass to fermentable sugars for production of ethanol requires a multienzyme system named cellulase. This system contains enzymes that act synergistically in the hydrolysis of cellulose: endoglucanase, cellobiohydrolase and β-glucosidase. The first two enzymes act directly on cellulose, yielding mainly glucose and cellobiose, which is hydrolyzed into glucose by β-glucosidase. An industrial process would be more economical by using immobilized systems that allow the reuse of the enzyme and improve the enzyme stability against different inactivation agents. Particularly, the hydrolysis of cellobiose would be performed using immobilized enzyme because cellobiose molecules are soluble in the reaction medium. In this work, β-glucosidase was immobilized on agarose matrix derivatized with different reactive groups, e.g. polyethylenimine (PEI), glyoxyl (linear aliphatic aldehydes) and amine-epoxy, trying to optimize the stability and activity of the immobilized enzyme. Using reversible attachment (immobilization by anion exchange), the derivatives were active, but with poor thermal stability, e.g. PEI agarose derivative was approximately 7 times more stable than the soluble β-glucosidase. However, these derivatives have important characteristics for an industrial process: reuse of the enzyme and/or the application of continuous systems. Among the activated supports with irreversible attachment (covalent immobilization), glyoxyl agarose did not reach a good thermal stability; it seems that the enzyme surface is very poor in amino groups from lysine residues. Better results were obtained with amine-epoxy agarose supports. β-Glucosidase immobilized on that support kept 80% of its activity and was ca. 200 times more stable than the soluble enzyme.

1. Introduction

Lignocellulosic biomass, which includes agricultural residues, paper wastes and wood chips, is an ideal inexpensive, renewable, abundantly available resource. Cellulose is the most abundant and renewable biopolymer on Earth [1]. The enzymatic modification of cellulose is a challenge in all applications using cellulose-based fibers due to the potential benefits. Enzymatic hydrolysis of cellulose has been an important topic of scientific and practical interest from 1950s. Most of the appropriate scientific literature deals with the mechanism and kinetics of this degradation reaction catalyzed by a multienzyme system, cellulase [2–6].

Extracellular microbial enzymes have the potential to be powerful tools for modifying cellulose. Cellulases are enzymes that hydrolyze the β-(1,4)-linkages in cellulose. Cellulases are produced as a multicomponent enzyme system comprised usually of three enzymes that act synergistically in the hydrolysis of cellulose: endoglucanase (EC 3.2.1.4), cellobiohydrolase (EC 3.2.1.91) and cellobiase (β-glucosidase, EC 3.2.1.21). The first two enzymes act directly on cellulose, yielding mainly cellobiose and glucose as the reaction products. The cellobiose is then hydrolyzed into glucose by cellobiase. Endoglucanases and cellobiohydrolases degrade soluble cellodextrins and amorphous cellulose. However, it is the cellobiohydrolases that degrade crystalline cellulose most efficiently [1,3,4,7].

The industrial process would be more economical by using the immobilized systems since they allow the reuse of the enzyme and improve the enzyme stability against different inactivation agents [8]. However, the immobilization of an enzyme does not guarantee its structure stabilization which is a target biocatalyst industrial application. For this, multipoint covalent immobilization of enzymes is the recommended technique that may promote a very interesting rigidification of protein molecules, resulting in high thermal stabilized derivatives [8–11].

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An immobilization system should meet certain characteristics to allow the enzyme stabilization via multipoint covalent attachment: the selection of the best support characteristics (offering large internal surfaces and a high density of reactive groups), the reactive group's features (low steric hindrance for the reaction with the enzyme, high stability under immobilization conditions) and proper immobilization conditions (those favoring both the reactivity of the groups and the flexibility of the enzyme molecule). Among the available immobilization techniques, immobilization on glyoxyl agarose and epoxy agarose seem to fulfill these requirements [12–14]. Glyoxyl agarose permits the immobilization of the enzyme through the area with the highest density of lysine residues, that is, where the highest likelihood of multipoint covalent attachment may be achieved. This could have a significant impact in the enzyme stability [15–20]. Epoxy-activated supports seem to be almost-ideal systems to develop many protocols for enzyme immobilization. Epoxy groups are very stable at neutral pH values and are able to react with different nucleophilic groups on the protein surface (e.g. amino, hydroxyl, or thiol moieties). However, epoxy groups are hardly reactive for enzyme immobilization under mild experimental conditions (neutral pH and low ionic strength). In the case of epoxy supports, immobilization occurs through a two step mechanism which involves a first rapid physical adsorption followed by chemical reaction between the enzyme and the support [13,14,21]. Taking advantage of this, a new generation of heterofunctional supports has been developed with different groups able to promote the first adsorption of the enzyme together to the epoxy-groups, which may allow the enzyme immobilization via different areas [13]. This could increase the possibilities of finding an area of the enzyme where the highest possibilities of multipoint covalent attachment are possible or an enzyme orientation which could have a significant impact in the enzyme stability (e.g. immobilization by an unstable loop or by labile structural regions, prevention of the inactivation by chemical reagents [22]).

In the present study, cellobiase has been immobilized onto very different supports, including glyoxyl-agarose and also some conventional ones such as DEAE-Sepharose and cyanogen bromide (CNBr) activated supports, trying to optimize the stability/activity of the immobilized enzyme.

2. Materials and methods

2.1. Materials

Cellobiase was donated by Novozymes A/S (Bagsvaerd, Denmark). Glucose oxidase (47,200 units/g solid) type II-S from *Aspergillus niger* and peroxidase (113 Purpurogalin units/mg solid) type I from horseradish was purchased from Sigma Chem. Co. (St. Louis, MO). D(+) Cellobiose was purchased from Fluka Chemie AG (Buchs, Switzerland). Cross-linked agarose beads 4%, 6% and 10% (w/v) were donated by Hispanagar S.A. (Madrid). CNBr-activated Sepharose™ 4B was purchased from Amersham Biosciences (Uppsala, Sweden). DEAE-Sepharose 4B was purchased from GE-Healthcare Bio-Sci AB (Uppsala, Sweden). Polyethylenimine 25 kDa (PEI 25) and 600–1000 Da (PEI 600–1000), 2,3-epoxy-1-propanol (glycydol), ethylenediamine (EDA), 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), hydrochloride N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (CDI), aspartic acid and sodium borohydride were purchased from Sigma Chem. Co. (St. Louis, MO, USA). Sodium periodate, epychloridrine and glutaraldehyde 25% were purchased from Fluka (Neu Ulm, Germany). Organic solvents and all other reagents were of analytical grade.

2.2. Enzymatic activity assays

Enzymatic activity of soluble and immobilized cellobiase was measured at 25 °C by assaying the initial reaction rate of glucose production using 2 g/l D(+) cellobiose as substrate prepared in 50 mM acetate buffer pH 4.8.

The concentration of glucose was measured by colorimetric method using glucose oxidase-peroxidase-ABTS system prepared in 50 mM phosphate buffer pH 6. The increasing absorbance was measured at 405 nm. The mixture of reaction was composed by 0.5 ml of glucose oxidase (2.5 g/l), 0.5 ml of peroxidase (2.5 g/l), 0.4 ml of ABTS (1 mM) and 1 ml of cellobiose 2 g/l. The reaction was initiated by adding of 25–200 μl of soluble enzyme solution or immobilized enzyme suspension.

Briefly, D-glucose is oxidized by glucose oxidase to D-glucanolactona (which in turn hydrolyzes spontaneously to gluconic acid) and hydrogen peroxide, using molecular oxygen as electron acceptor. ABTS in presence of hydrogen peroxide is oxidized by peroxidase to its radical cation. The ABTS oxidized exhibits maximum absorbance at approximately 405 nm. The change in the solution absorbance is proportional to glucose concentration since the reaction is equimolar [28].

The catalytic activity was expressed as International Units (1 μmol of glucose released per minute).

2.3. Preparation of immobilization supports

2.3.1. Preparation of CNBr supports

One gram of the gel (CNBr-activated Sepharose 4B) was suspended in 100 ml of water and the pH solution was adjusted between 2 and 3, using diluted HCl solution. The suspension was stirred during 30 min and dried by filtration under vacuum.

2.3.2. Preparation of glyoxyl supports

Glyoxyl supports were prepared activating the agarose matrix with glycidol and subsequent oxidation with periodate [12]. Under gentle agitation and in an ice bath, 105 g of agarose, previously washed with distilled water, were mixed with 30 ml of distilled water, 50 ml of NaOH 1.7 N, containing 1.425 g sodium borohydride (previously cold prepared), and 36 ml of glycidol, which was added very slowly to avoid raising the temperature above 25 °C. The suspension formed was agitated for 15 h. The etherified gel (glyceryl-agarose) was washed with distilled water over a sintered glass filter under vacuum. After the last washing, the gel was thoroughly sucked dry to remove the interstitial humidity. Then, 105 g of the gel was resuspended in 895 ml of water (support to suspension ratio of 1:10) and 3.21 g of sodium periodate was added. After 2 h of gentle agitation at room temperature, the glyoxyl-agarose support was washed with water, filtered under vacuum and suck dried.

2.3.3. Preparation of monoaminoethyl-N-ethyl-agarose (MANAE-agarose) supports

Monoaminoethyl-N-ethyl-agarose (MANAE-agarose) was prepared according to Fernandez-Lafuente et al. [23]. This support was prepared from glyoxyl-agarose support, by adding 200 ml of 2 M ethylenediamine (EDA) solution at pH 10 to a 35 g of glyoxyl-agarose support. After 2 h of gentle agitation, sodium borohydride was added to a final concentration of 10 mg/ml. Again, after 2 h of gentle agitation, the MANAE-agarose support was washed successively with 100 mM acetate buffer pH 4, 100 mM borate buffer pH 9 and finally distilled water.

2.3.4. Preparation of glutaraldehyde supports

Glutaraldehyde support was obtained by activation of the primary amino groups of MANAE-agarose, according to Fernández-

Lafuente et al. [24] with slight modifications. 20 g of MANAE-agarose was suspended in 22.4 ml of 200 mM sodium phosphate buffer pH 7. Afterwards, 33.6 ml of a 25% glutaraldehyde solution were added and the system was kept under gentle stirring for 16 h at room temperature. Finally, the activated support was washed with water and vacuum dried.

2.3.5. Preparation of polyethylenimine supports

Polyethylenimine (PEI) agarose support was prepared from glyoxyl-agarose support according to Mateo et al. [29]. 100 g of polyethylenimine was dissolved in 900 ml of 100 mM carbonate buffer pH 10.05 and 100 ml of glyoxyl-agarose were added. The suspension was gently stirred for 3 h at room temperature. Afterwards, sodium borohydride was added to a final concentration of 10 mg/ml and the system was kept under gentle stirring for 2 h. The PEI-agarose support was successively washed with 100 mM acetate buffer pH 4, 100 mM borate buffer, pH 9, 1 M NaCl solution and finally distilled water.

2.3.6. Preparation of amino-epoxy supports

Activation was performed with epichlorohydrin as previously described by Armisén et al. [25]. 10 ml of 4 BCL agarose were washed thoroughly with distilled water and the moist gel was suspended in 30 ml of 0.8 M NaOH containing 340 mg of $NaBH_4$, 11.4 ml of acetone and two additions of 5.7 ml of epichlorohydrin (after 2 and 4 h of reaction). The suspension was stirred for 8 h at 25 °C and finally washed thoroughly with distilled water.

Controlled amination of the support was performed as previously described by Mateo et al. [26]. 10 g of epoxy agarose was incubated in 60 ml of 2% (v/v) ethylenediamine at pH 7.0 for different times (from 15 min to 24 h) under very gently stirring. Then, the supports were washed with distilled water, 1 M NaCl and finally with distilled water.

2.4. Cellobiase immobilization

The immobilization course was monitored measuring the enzyme activity in the supernatant and in the whole suspension at different time intervals. Additionally, controls with soluble enzyme were used to determine the possible inactivating effect of the pH, temperature, or dilution on the enzyme during the immobilization. In all cases, the suspensions were prepared using relation 1:5 or 1:10 (volume of support:volume of suspension) and gently stirred at 25 °C at different times. The load of the supports was from 10 to 50 μl of commercial enzyme per gram of support to avoid mass transfer limitations.

For the immobilization on CNBr-activated Sepharose 4B, the enzyme was diluted in 25 mM sodium phosphate buffer pH 7.0, the suspension was gently stirred during 1 h. After that, the gel was filtered and washed with 0.1 M sodium bicarbonate at pH 8.0. Afterwards, the gel was blocked with 1 M ethanolamine at pH 8.0 during 2 h with gentle stirring, and washed with 25 mM potassium phosphate buffer at pH 7 and storage at 4 °C.

For the immobilization on highly activated glyoxyl agarose support the enzyme was diluted in 100 mM sodium bicarbonate buffer pH 10.05 and incubated with the support [26]. The enzyme–support interaction was ended by addition of sodium borohydride to a final concentration of 1 mg/ml, and incubated for further 30 min under stirring [27]. Afterwards, the immobilized preparation was washed with an excess of 25 mM sodium phosphate pH 7 and stored at 4 °C until further use.

Immobilization on DEAE and PEI (25 kDa or 600–1000 Da) agarose supports was carried out in 5 mM sodium phosphate buffer pH 7.0. The enzyme–support interaction was ended by washing the preparations with excess 25 mM sodium phosphate buffer pH 7 and stored at 4 °C until further use.

Immobilization on glutaraldehyde supports was carried out in 25 mM sodium phosphate buffer pH 7.0. The enzyme–support interaction was ended by washing the preparations with excess 25 mM sodium phosphate buffer pH 7 and stored at 4 °C until further use.

Immobilization on amino-epoxy supports was carried out incubating the enzyme solution in 5 mM sodium phosphate buffer pH 7.0 for 12 h (overnight) [13]. Afterwards, the gel was filtered and incubated with 0.1 M sodium bicarbonate at pH 10 for 12 h (overnight). The enzyme–support interaction was ended by blocking the remaining epoxy groups with 3 M glycine or aspartic acid pH 8.0 for 12 h (overnight). The preparation was then thoroughly washed with 25 mM sodium phosphate buffer pH 7 and stored at 4 °C until further use.

Immobilization on MANAE agarose support followed by crosslinking using glutaraldehyde was carried out in 5 mM sodium phosphate buffer pH 7.0. The suspension was maintained under gently stirred for 30 min. After that commercial glutaraldehyde 25% was added to a final concentration of 0.5% (v/v). This suspension was gently stirred for 1 h. The enzyme–support interaction was ended by washing the preparation with excess 25 mM sodium phosphate buffer pH 7 and stored at 4 °C until further use.

The parameters of immobilization procedure were defined as: yield of immobilization (YI) is the ratio between the amount of immobilized enzyme and the amount of enzyme offered to immobilization. The activity recovery (RA) is the ratio between the measured derivative activity and the theoretical immobilized activity (difference between the initial activity and the activity measured in the final supernatant).

2.5. Enzyme desorption from DEAE and PEI supports assay

Desorption course was monitored measuring the enzyme activity in the supernatant and in the whole suspension (initially prepared in 5 mM sodium phosphate solution, pH 7, 1:10) for different ionic strength adjusted with 5 M NaCl solution. For each ionic straight used, the suspension was gently stirred for 30 min. The end of the assay was admitted when the enzyme activity in the supernatant and whole suspension were the same.

2.6. Purification of the commercial cellobiase

The commercial cellobiase was purified by adsorption/desorption on DEAE-agarose, followed by dialysis against excess of sodium phosphate buffer, pH 7.0 at 4 °C under gently stirred by 24 h and using 10–12 kDa cut-off membrane.

2.7. Inactivation assays

Immobilized enzyme preparations were incubated under the temperature, ionic strength, enzyme concentrations, organic solvents and pH conditions detailed in each case in the corresponding figure legend. Sampling was performing periodically and the residual enzymatic activity was calculated as the ratio of the activity of each sample at defined time and the initial activities before the inactivation conditions.

The single-step non-first-order model, proposed by Sadana and Henley [30], was fitted to the experimental data. This model considers that a single-step inactivation leads to a final state that exhibits a residual activity, which is very stable and does not unfold or inactivate. The activity–time expression is:

$$\frac{A}{A_0} = (1-\alpha)\exp(-kt) + \alpha \quad (1)$$

where A/A_0 is the activity (dimensionless); α is the ratio between specific activity of the final state and specific activity of the initial

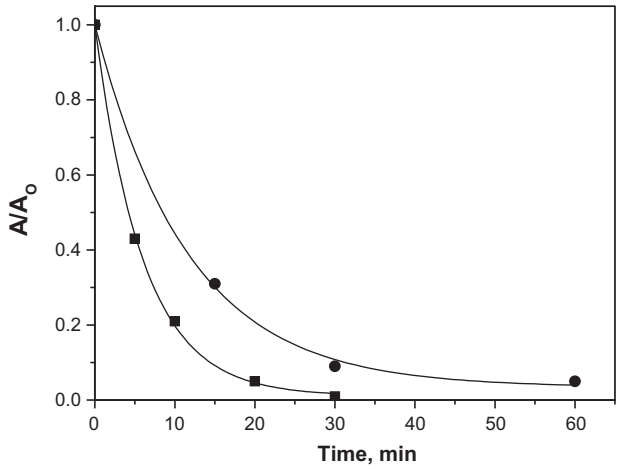

Fig. 1. Thermal inactivation at 65 °C, pH 4.8 of (●) commercial cellobiase and (■) purified enzyme. The initial activities (A_0) were set as the unit. The curves were obtained by fitting the single-step non-first-order model (Eq. (1)) to the deactivation data.

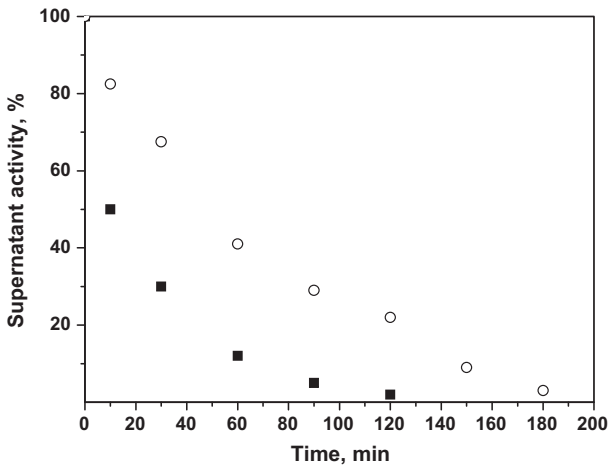

Fig. 2. Immobilization course of (○) commercial and (■) purified cellobiase on PEI (600–1000 Da) agarose support. Experimental conditions: 25 °C, pH 7.0 (5 mM sodium phosphate buffer).

state, and k is the first-order deactivation rate constant (time^{-1}). The parameter k should describe the unfolding or the inactivation process, and the parameter α the long-term level of activity [30].

The two-parameter model was fitted to the deactivation data using the Levenberg–Marquardt method of iterative convergence, at 0.95 confidence level. The biocatalyst half-life was then calculated using the fitted model. Factors of stability were defined as the ratio between the half-lives of each derivative and the half-life of the purified enzyme or CNBr-agarose derivative (derivative of reference).

3. Results and discussion

3.1. Purification of the commercial cellobiase

The commercial cellobiase was purified by adsorption/desorption on DEAE-agarose. The adsorption step was carried out under low ionic strength (5 mM) and the desorption step was carried out under high ionic strength (200 mM). Afterwards, the desorbed enzyme was dialyzed against sodium phosphate buffer 5 mM, pH 7.0, in excess. After all purification steps the recovered activity was approximately 50% of the initial activity and the enzyme solution changed from dark brown to dark yellow.

The purification procedure influenced the commercial cellobiase stability and its immobilization velocity. The purified soluble enzyme was approximately 2-fold less stable than the commercial soluble enzyme (see Fig. 1 and Table 1), probably due to the additives (glycerol, PEG, etc.) used to protect and preserve the commercial enzyme. The commercial enzyme presents good stability in a large range of pH, from 5 to 10, so it is expected that the enzyme could be immobilized on any kind of support.

It was decided to prepare the biocatalysts using purified enzyme to have the real stabilization of the enzyme by immobilization procedure. Besides, some immobilization procedures were carried out using commercial enzyme to verify the influence of the additives in the immobilization of enzyme (time of immobilization, yield of immobilization, activity recovery and thermal stability).

Fig. 2 shows that the commercial enzyme was almost totally immobilized on PEI (600–1000 Da) agarose support after 3 h of reaction, while the purified soluble enzyme was completely immobilized after only 2 h of reaction.

Table 2
Immobilization parameters of commercial cellobiase by ion exchange on DEAE and PEI-agarose supports. Immobilization conditions: 25 °C, pH 7.0 (sodium phosphate buffer, 5 mM) and 2 h of reaction.

Support	YI (%)	RA (%)
DEAE-agarose 6 BCL	97.6	51
PEI (600–1000 Da) agarose 6 BCL	99.2	92

3.2. Reversible immobilization of commercial and purified cellobiase

Commercial and purified cellobiase was immobilized by ion exchange on supports highly activated with positive charges (DEAE-agarose and PEI (600–1000 Da)-agarose). These supports permit the orientation of the enzyme by the region containing high concentration of negative charges (aspartic and glutamic residues).

The adsorption on that supports was quick. After 2 h of reaction almost all enzyme was immobilized. A significant result was observed in the recuperation of activity in the immobilized enzyme. Cellobiase immobilized on PEI-agarose (600–1000 Da) (see Table 2) recovered approximately 2-fold more activity than cellobiase immobilized on DEAE-agarose. This result can be explicated by high concentration of reactive groups (forming a flexible polymeric bed) on the PEI-agarose support that better accommodates the enzyme molecule without significant changes in its tridimensional structure.

Fig. 3 shows the profile immobilization of cellobiase on those supports. We can see that immobilization on PEI-agarose is faster than on DEAE-agarose, because PEI support contains more amino groups compared to the DEAE support. However, for both supports

Table 1
Parameters of deactivation models (65 °C, pH 4.8): soluble purified and commercial cellobiase.

Enzyme	$\alpha \pm \sigma$	$k \pm \sigma$	R^2	Half-life[a], min	Stabilization factor
Purified cellobiase	0.01064 ± 0.00901	0.16644 ± 0.00522	0.9994	4.2	1
Commercial cellobiase	0.0343 ± 0.00602	0.08579 ± 0.00345	0.9991	8.5	2

[a] Half-lives were calculated from Eq. 1, replacing A/A_0 by 0.5.

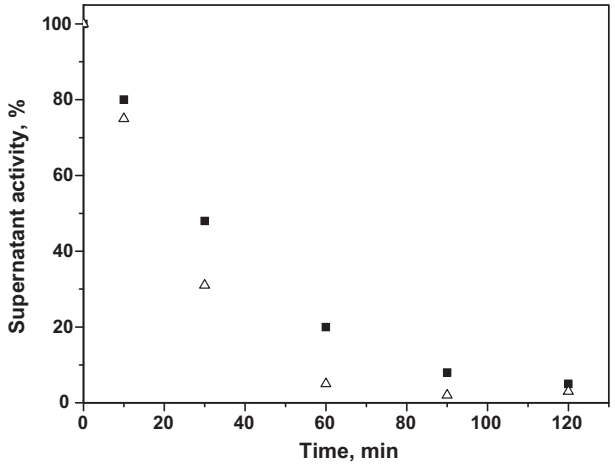

Fig. 3. Immobilization course of commercial cellobiase on (△) PEI (600–1000 Da)-agarose 6 BCL and (■) DEAE-agarose 6 BCL at 25 °C, pH 7.0 (sodium phosphate buffer 5 mM). Loading of 50 μl of commercial cellobiase per gram of support.

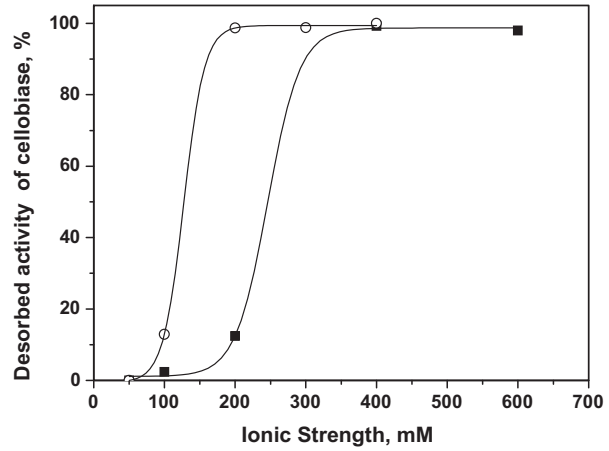

Fig. 4. Desorption profiles of cellobiase from (○) DEAE-agarose and (■) PEI-agarose supports. Initial suspensions were prepared in 5 mM sodium phosphate buffer pH 7 and the ionic strength was adjusted with 5 M NaCl solution.

almost all enzyme was immobilized after 2 h of reaction (above 97%).

Fig. 4 shows that the enzyme is more strongly adsorbed on PEI-agarose support than on DEAE-agarose support. Ionic strength of 200 mM was necessary to desorb all enzymes from PEI (600–1000 Da)-agarose support. This intense physical adsorption on PEI-agarose has important industrial implications considering that the enzyme will not elute from the support under gently reaction conditions. Besides, this immobilization method permits the regeneration of the support after complete inactivation of the enzyme. This fact would contribute to minimize the costs of the production.

The high strength of binding between the enzyme and PEI-agarose supports also contributed for a best stabilization of the enzyme. Fig. 5 and Table 3 show that cellobiase immobilized on

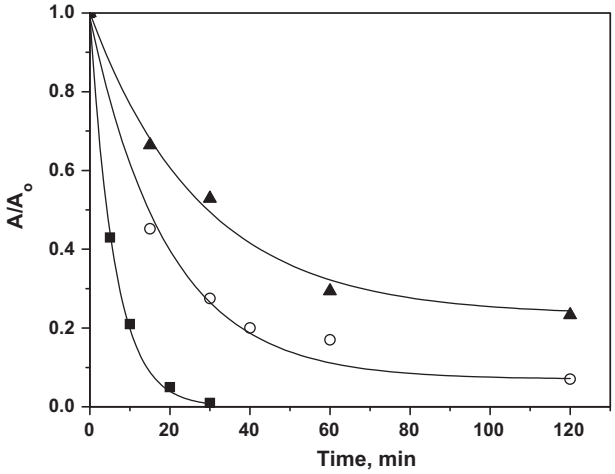

Fig. 5. Thermal inactivation at 65 °C, pH 4.8, of (■) purified cellobiase and purified enzyme immobilized on (○) DEAE-agarose and (▲) PEI (600–1000 Da)-agarose. All suspensions were prepared in 5 mM sodium acetate buffer. The initial activities (A_0) were set as the unit. The curves were obtained by fitting the single-step non-first-order model (Eq. (1)) to the deactivation data.

PEI-agarose support was approximately 2-fold more stable than cellobiase immobilized on DEAE-agarose. This result may be produced by the creation of a very hydrophilic microenvironment of the polymeric PEI surrounding each enzyme molecule. This high hydrophilization of the enzyme surface may difficult the exposition of some internal hydrophobic pockets to the external medium and this may promote a certain stabilization of the immobilized enzyme.

3.3. Irreversible immobilization of cellobiase

Commercial cellobiase was immobilized by covalent attachments on agarose activated with different functional groups, such as, amino, epoxy and aldehyde. A bifunctional support was tested, amino-epoxy-agarose. For linkage amino (enzyme)-aldehyde (support) two strategies were used: covalent attachment on agarose activated with glutaraldehyde (glutaraldehyde-agarose) and adsorption on amino-agarose followed by cross-linked with soluble glutaraldehyde (amino-glutaraldehyde-agarose). All derivatives were compared with the enzyme immobilized on CNBr-agarose (reference derivative). For this support, the immobilization time was kept constant (30 min) and the immobilization yield was around 15% and the recovered activity was 100%.

Fig. 6 shows the immobilization profiles for commercial and purified enzyme on different activated supports. Clearly we can see that the commercial cellobiase immobilizes more slowly than the purified enzyme. This result shows that, probably, the additives in the commercial enzyme preparation compete by the active sites of the supports, hindering the immobilization of the enzyme, or reduce the mobility of the enzyme molecule decreasing the immobilization rate.

Although the additives certainly present in the commercial enzymatic formulations increase the stability of the soluble enzyme, the same additives affect negatively the immobilization rates.

Table 3
Parameters of deactivation models (65 °C, pH 4.8): soluble and immobilized cellobiase. Suspensions prepared using 5 mM sodium acetate buffer.

Biocatalyst	$\alpha \pm \sigma$	$k \pm \sigma$	R^2	Half-life[a], min	Stabilization factor
Purified cellobiase	0.01064 ± 0.00901	0.16644 ± 0.00522	0.9994	4.2	1
Cellobiase-DEAE-Agarose	0.1073 ± 0.02329	0.05865 ± 0.00558	0.9934	14	3.3
Cellobiase-PEI-Agarose	0.2126 ± 0.02731	0.03391 ± 0.00323	0.9945	30	7.1

[a] Half-lives were calculated from Eq. 1, replacing A/A_0 by 0.5.

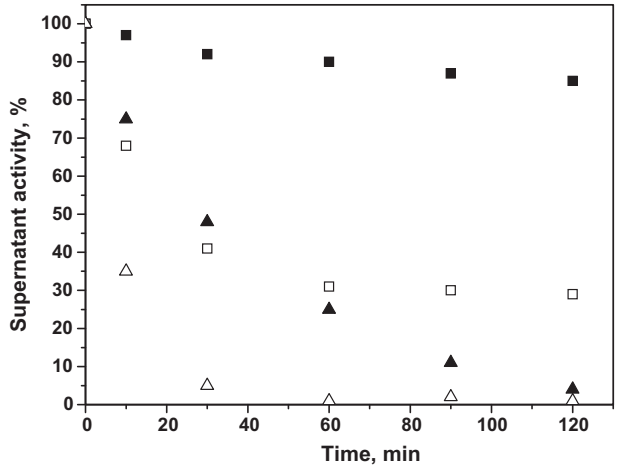

Fig. 6. Immobilization course of commercial (closed symbols) and purified (open symbols) cellobiase on different activated supports: amino-epoxy-agarose (triangles) and glyoxyl-agarose (squares).

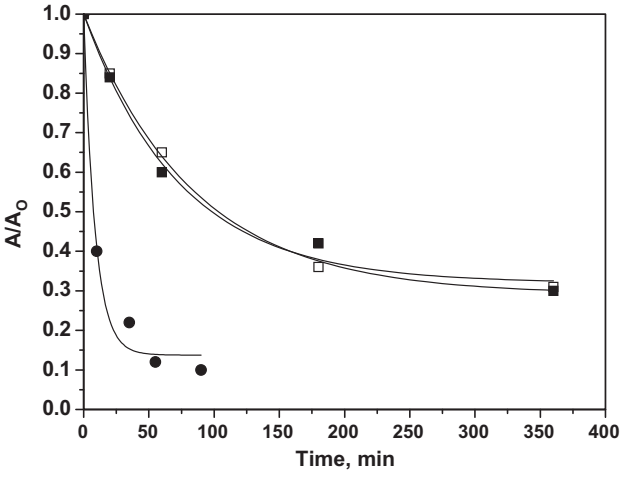

Fig. 7. Thermal inactivation at 65 °C and pH 4.8 of cellobiase purified immobilized on (●) CNBr-agarose, (■) glutaraldehyde-agarose, (□) amino-agarose and cross-linked with glutaraldehyde. The initial activities (A_0) were set as the unit. The curves were obtained by fitting the single-step non-first-order model (Eq. (1)) to the deactivation data.

Table 4
Immobilization parameters of cellobiase on different activated supports.

Support	Glyoxyl-agarose 4 BCL		Amino-epoxy-agarose	
cellobiase	Commercial	Purified	Commercial	Purified
YI (%)	15	71	96	99
RA (%)	3	22	53	68

Table 4 shows the immobilization yield and the recovered activities for all assayed supports after 2 h of immobilization reaction. The immobilization is more rapid using purified enzyme.

Fig. 7 and Table 5 show that derivatives prepared by linking the enzyme with aldehyde groups were more stable than cellobiase immobilized on CNBr-agarose (13–15-fold). Besides, the derivative immobilized on glutaraldehyde-agarose showed a recovered activity of 80% (Table 6). The cross-linked with soluble glutaraldehyde also presented a deflect effect over the enzyme. The activity recovery was only 67% and the thermal stability of the enzyme was not positively influenced.

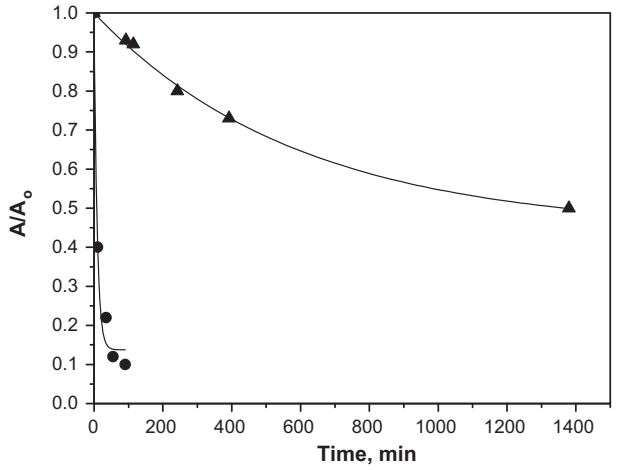

Fig. 8. Thermal inactivation at 65 °C and pH 4.8 of purified cellobiase immobilized on (●) CNBr-agarose, and (▲) amino-epoxy-agarose. The initial activities (A_0) were set as the unit. The curves were obtained by fitting the single-step non-first-order model (Eq. (1)) to the deactivation data.

Table 6
Parameters of immobilization of commercial cellobiase on agarose activated with different reactive groups and CNBr as standard. Immobilization conditions: 25 °C, pH 7 (5 mM sodium phosphate buffer).

Support	YI (%)	RA (%)
CNBr-agarose	11.4	100
Glutaraldehyde-agarose	99.2	80
Amino-glutaraldehyde-agarose	98.4	67

Purified cellobiase was immobilized by covalent attachments on amino-epoxy-agarose support and compared with one immobilized on CNBr-agarose (reference support). Using amino-epoxy support was possible to prepare a derivative ca. 200-fold more stable than one prepared on CNBr-agarose support (see Fig. 8 and Table 7). The derivative prepared on amino-epoxy-agarose presented 68% of recovered activity. The high stability of the enzyme immobilized on amino-epoxy support due to the formation of multiple covalent bonds between active groups from the support (epoxy) and active groups from the enzyme (–OH and –NH_2, for example). The first step of the immobilization on this support is a rapid and weak adsorption by ion exchange. After this first adsorption process and even at neutral pH an intramolecular covalent attachment can be produced. However, when the pH is increased for an alkaline value (pH 10) the covalent bonds are greatly favored. The tri-dimensional structure of the enzyme linked to the support by multiple points becomes more rigid and hardly unfolds due to the increased vibrational movement caused by raising temperatures.

Table 5
Parameters of deactivation models (65 °C, pH 4.8) of cellobiase immobilized on agarose activated with different reactive groups.

Support	$\alpha \pm \sigma$	$k \pm \sigma$	R^2	Half-life[a], min	Stabilization factor
CNBr-agarose	0.13758 ± 0.02844	0.11426 ± 0.01881	0.9885	7	1
Glutaraldehyde-agarose	0.31999 ± 0.02849	0.01353 ± 0.00179	0.9988	103	15
Amino-glutaraldehyde (MANAE + glutaraldehyde)	0.29112 ± 0.01222	0.01182 ± 0.01222	0.9917	98	13

[a] Half-lives were calculated from Eq. 1, replacing A/A_0 by 0.5.

Table 7
Parameters of deactivation models (65 °C, pH 4.8) of purified cellobiase immobilized on CNBr-agarose (reference support) and amino-epoxy-agarose support.

Support	$\alpha \pm \sigma$	$k \pm \sigma$	R^2	Half-life[a], min	Stabilization factor
CNBr-agarose	0.13758 ± 0.02844	0.11426 ± 0.01881	0.9885	7	1
Amino-epoxy-agarose	0.44483 ± 0.02182	0.00169 ± 0.00014	0.9965	1366	196

[a] Half-lives were calculated from Eq. 1, replacing A/A_0 by 0.5.

4. Conclusions

Using different strategies of immobilization was possible to prepare a derivative with high activity and thermal stability. The additives of the commercial enzymatic formulation increase the soluble cellobiase stability, around 2-fold, but also difficult its immobilization on the supports tested in this work. The commercial cellobiase stability was almost the same presented by cellobiase purified, but when the enzyme was purified the immobilization process presented better results. It was tested by two reversible immobilization methods (DEAE and PEI supports). The best reversible derivative was prepared using PEI-agarose support. That derivative presented a recovered activity of 92%, high ionic strength for enzyme desorption and thermal stability 6 times higher than that of the soluble and purified enzyme. The biocatalysts prepared by reversible linkage should be a good choice if the process requires only a reasonable stability, because after enzyme inactivation the support can be recovered and reused. The immobilization yield on glyoxyl agarose support was of 71%, but the immobilized enzyme was not stabilized. Probably, the enzyme molecule not contains a rich region in amino groups from lysine residues. The best derivative was prepared immobilizing cellobiase on amino-epoxy agarose: the recovered activity was 68% and the immobilized enzyme was ca. 200 times more stable than the purified cellobiase immobilized on CNBr-agarose support.

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