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Influence of different silica derivatives in the immobilization and stabilization of a *Bacillus licheniformis* protease (Subtilisin Carlsberg)

L. Ferreira [a,*], M.A. Ramos [b], J.S. Dordick [c], M.H. Gil [a]

[a] *Departamento de Engenharia Quimica, Universidade de Coimbra, Pinhal de Marrocos, Coimbra 3030-290, Portugal*
[b] *Instituto Superior de Engenharia, Institute Politecnico de Coimbra, Coimbra 3000, Portugal*
[c] *Department of Chemical Engineering, Rensselaer Polytechnic Institute, 102 Ricketts Building, Troy, NY 12180, USA*

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Abstract

Alcalase 2T, a commercial preparation of Subtilisin Carlsberg, was covalent immobilized onto physiochemically characterized silica supports. The effect of mean pore diameter and surface chemistry on enzyme activity in the hydrolysis of casein has been examined. Two sets of chemically distinct silica supports were used presenting terminal amino (S_{APTES}) or hydroxyl groups ($S_{TESPM\text{-}pHEMA}$). The percentage of immobilized protein was smaller in S_{APTES} (31–39%) than in $S_{TESPM\text{-}pHEMA}$ (62–71%), but presented higher total and specific activity. Silicas with large pores (S_{1000}, 130/1200 Å) presented higher specific activities relative to those with smaller pore sizes (S_{300}, 130/550 Å). The influence of glutaraldehyde concentration and the time of enzyme coupling to the $S_{1000}S_{APTES}$ supports was examined. The apparent K_m value for the $S_{1000}S_{APTES}$ immobilized enzyme is lower than the soluble one which may be explained by the partitioning effects of the substrate. No intraparticle diffusion limitations were observed for the immobilized enzyme and therefore the substrate diffusion does not influence the observable kinetics. Finally, the optimum pH, optimum temperature, thermal stability, operational stability, and storage stability of the immobilized and freely soluble enzymes were compared.

Keywords: Powder characterization; Silica derivatization; Protease; Immobilized enzyme; Enzyme stabilization

1. Introduction

Proteases, such as Alcalase 2T (commercial preparation of Subtilisin Carlsberg), have attracted much attention because of their use in the cleavage of proteins [1,2] and enzymatic analysis of protein sequences [3], as well as their applications in organic synthesis, particularly in non-aqueous media involving complex substrates [4–6]. The immobilization of proteases on solid support by covalent attachment can offer several advantages over the free enzyme including easy handling, recovery from the reaction medium and reuse and/or operation in continuous reactors.

Proteases have been immobilized using a wide range of methods including deposition or precipitation onto porous supports [7,8] and covalent attachment to activated preexisting supports [9–14]. In all of these cases, a wide range of materials have been studied, and in some cases a relationship between catalyst

* Corresponding author. Tel.: +351-239-798700; fax: +351-239-798703.
E-mail address: linofer3@eq.uc.pt (L. Ferreira).

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activity and the key physical and chemical characteristics of the support has been undertaken. However, very often, the physical and chemical data refers to the original support (based on the manufacturer information) without being updated along the chemical modification process of the solid surfaces. Furthermore, one must be aware for the adequate choice of techniques used to characterize the supports besides their accuracy [15]. The well understanding of the physicochemical behavior of immobilization supports is important to evaluate the effect of mean pore size, surface area and the immobilization methodology on enzyme activity, to determine the intraparticle mass transfer of substrates in immobilized systems, and finally, to estimate the attachment points between support and the enzyme which is crucial for the stabilization of the biocatalyst.

The rationale of this work was to immobilize Alcalase 2T onto silica supports for which full physico-chemical characterization has been previously reported [15], and to obtain a correlation between the properties of different silica derivatives and the resulting immobilized enzyme activity. Furthermore, the effect of several reaction parameters (pH, temperature, and substrate concentration) on the catalytic activity of the immobilized and freely soluble enzyme were studied, as well, their stability.

2. Materials and methods

2.1. Materials

The two porous silicas used in this work were supplied by Macherey-Nagel (Duren, Germany) specified by the manufacturer as: Nucleosil 300 (S_{300}) and Nucleosil 1000 (S_{1000}). 3-(Trimethoxysilyl)propyl methacrylate (S_{TESPM}), 3-aminopropyltriethoxysilane (S_{APTES}), sodium cyanoborohydride (95%) and 2-hydroxyethylmethacrylate (HEMA) were purchased from Aldrich (Bornem, Belgium). Glutaraldehyde (25%), 1,1′-carbonyldiimidazole (CDI) and *N*-transcinnamoylimidazole were obtained from Sigma. Casein (M_w of 30,000 Da) was purchased from Fluka AG (Buchs, Switzerland). Alcalase 2T was a gift by Novo-Nordisk (Bagsvaerd, Denmark). Other chemicals and solvents were of the highest grade commercially available.

2.2. Functionalization and chemico-physical characterization of silica supports

Two methods were used for the modification of silicas. In the first method, the silicas were silanized with S_{APTES} and S_{TESPM} according to the methodology reported by Ramos et al. [15]. In the second method, the modified silicas containing vinyl groups were grafted with HEMA by X-ray irradiation, using a 15 MeV 20 kW linear electron accelerator. In all experiments, 1 g of silica was suspended in 10 ml of methanol containing 0.822 M HEMA and the mixture was irradiated at 0.95 Gy s^{-1}, for 16 h at room temperature, in the presence of air. After irradiation, the silica was filtered and extensively washed with methanol, extracted with methanol using a soxhlet during 6 h, and dried at 70 °C under reduced pressure until constant weight was achieved. Silicas were characterized regarding particle size, surface area, mean pore diameter, true density, porosity, yield of grafting and water vapor sorption, as previously described [15]. The silanized samples are designated by S_xS_y, where S_x denotes the original silica (S_{300} or S_{1000}) and S_y specifies the silane compound used (S_{TESPM} or S_{APTES}). The methacrylated silicas grafted with HEMA were denoted by $S_xS_{TESPM\text{-}pHEMA}$.

2.3. Methods of immobilization

2.3.1. Immobilization on S_xS_{APTES} supports

Glutaraldehyde was used as the activating agent for the coupling of the protease to amine groups of the modified silicas. Typically, 50 mg of the S_xS_{APTES}-silica was added to 4.9 ml of 0.1 M phosphate buffer pH 8.0 and 0.1 ml of glutaraldehyde solution (25% (v/v)) (in some experiments, the glutaraldehyde concentration was changed to study its influence on enzymatic activity). Support activation was carried out at 25 °C, without stirring, for 15 min. The activated silicas were then removed by filtration and thoroughly rinsed with distilled water (3× 10 ml). Enzyme solutions (0.1 ml with a protein content of 4.49 mg ml^{-1}) in 0.1 M phosphate buffer pH 8.0 were added to 4.9 ml of the same buffer with the activated support (≅50 mg of S_xS_{APTES}-silica). In some cases, sodium cyanoborohydride reductions were performed to convert unstable Schiff's bases (formed between the aldehyde groups of the glutaraldehyde molecule and

the terminal amino groups of the enzyme) into stable secondary amines. Therefore, 80 μl of an aqueous solution of sodium cyanoborohydride, 0.08% (w/v) in 0.1 M phosphate buffer pH 8.0, which represented an excess, was added after 30 min of enzyme solution addition. The coupling reactions (in the presence or absence of sodium cyanoborohydride) were performed over 18 h (or at different coupling times if stated in the text) at 25 °C without stirring, after which the solids were filtered and rinsed with 0.1 M Tris–HCl buffer pH 8.5 until the filtrate was totally free of protein (determined by Sedmak method). At this point, it was assumed that the protein that was not removed was either covalently bound or physically entrapped within the silica matrix. All washing solutions were analyzed for protein using the Sedmak method (see below) and the activity of immobilized enzymes were determined using casein as the substrate.

2.3.2. Immobilization on $S_xS_{TESPM\text{-}pHEMA}$ supports

CDI was used as the activating agent for coupling the protease to the hydroxyl groups of HEMA grafted onto silica. Typically, 50 mg of $S_xS_{TESPM\text{-}pHEMA}$ silica was added to 5.0 ml of anhydrous DMSO containing 150 mg of CDI. The activation reaction was carried out at 25 °C without stirring for 2 h. The activated silica was then removed by filtration and washed with water (3× 10 ml). The coupling reactions were performed in the same way as described for the S_xS_{APTES} supports for 18 h at 25 °C, but in the absence of sodium cyanoborohydride.

2.4. Proteolytic activity assay

The proteolytic assays were performed either in the presence of 0.1 ml of enzyme solution (with a protein content of 0.449 mg ml^{-1}), or ∼50 mg of immobilized enzyme (see above), in the case of soluble and immobilized enzyme, respectively. Enzymes were added to the reaction media formed by a mixture of 1 ml of 0.1 M phosphate buffer pH 8.0 with 5 ml of 1.0% (w/v) casein solution. The mixture was incubated for a desired time (5 and 10 min for soluble and immobilized Alcalase, respectively) at 50 °C with magnetic stirring (200 rpm) and a 0.5 ml aliquot was taken and added to an equal volume of 0.4 M trichloroacetic acid (for the immobilized enzymes a preceding centrifugation step at 5000 rpm for 1 min was necessary). The resulting precipitate was removed by centrifugation (5000 rpm, 2 min) after standing for 25 min at 25 °C. The supernatant (0.5 ml) was placed in a test tube containing 5 ml of 0.4 M sodium carbonate and 0.5 ml of five-fold diluted Folin's reagent. After thorough mixing, the solution was allowed to stand for 20 min at 37 °C, and the absorbance measured spectrophotometrically at 660 nm. The absorbance values were then converted to equivalent tyrosine concentrations using a tyrosine calibration curve. One unit of protease activity (U) is defined as the quantity of enzyme needed to produce the amino acid equivalent of 1 μg of tyrosine per min. In parallel with the enzyme assays, blank reactions without enzymes (with or without modified silicas) were performed.

2.5. Active sites, kinetic constants and protein content determinations

The soluble and immobilized enzymes were titrated in aqueous solution prior to use to determine the fraction of active centers present in a given sample, and hence, enable accurate measurement of k_{cat} values. These titrations were performed according to the method of Schonbaum [16] via the spectrophotometric determination (at 335 nm) of enzyme acylation by *N*-transcinnamoylimidazole.

The effect of substrate concentration on enzyme activity was studied to determine the kinetic parameters (K_m, V_{max}, k_{cat} and k_{cat}/K_m). Several concentrations of casein were used ranging from 6 to 50 μM. The K_m and V_{max} values were estimated from Eadie–Hofstee plots. The k_{cat} was calculated using the active enzyme concentrations as determined by active sites titration.

The protein content in the crude enzyme or immobilized enzyme preparations was determined by the Sedmak method [17], using bovine serum albumin (BSA) as the standard (the protein is, therefore, expressed in BSA equivalents). The amount of protein bounded onto silica supports was determined indirectly from the difference between the initial total protein exposed to the supports and the amount of protein recovered in the wash.

2.6. Effect of pH and temperature on enzyme activity

The effect of pH on enzyme activity was studied in 0.2 M phosphate buffer for pH 6.5–8.0 and 0.2 M

Tris–HCl buffer for pH 8.5–9.2, using casein (1% (w/v)) as substrate. Enzyme activity was also studied at temperatures ranging from 35 to 70 °C. The reaction mixtures without casein were maintained at the desired temperature (2 min) and then soluble and immobilized enzymes were added.

2.7. Stability measurements

The thermal stability of the enzymes was studied by measuring the residual activity after incubation of the soluble or immobilized enzymes in 0.1 M phosphate buffer pH 8.0, in the absence of substrate, at various temperatures (50 and 60 °C) for different incubation times. The enzyme thermal deactivation process was fitted to the series model proposed by Henley and Sadana [18] which involves several enzymatic states (E, E_1 and E_2) and first-order deactivation rate coefficients (k_1 and k_2), and is illustrated in the following general scheme:

$$E \xrightarrow{k_1} E_1 \xrightarrow{k_2} E_2$$

This model assumes that at time 0, $[E_1] = 0$ and $[E_2] = 0$, the transition state between E, E_1 and E_2 is irreversible, and there are no parallel deactivations. The first-order deactivation rate constants (k_i) were calculated from Eq. (1), after experimental plots of relative activity versus incubation time were adjusted to exponential decays (single or double)

$$\tilde{\tilde{a}} = D_1 e^{-k_1 t} + D_2 e^{-k_2 t} + D_3 \quad (1)$$

where $\tilde{\tilde{a}}$ is the normalized activity D_1, D_2 and D_3 are the parameters of the fitted exponential decay, and k_1, k_2, the first-order deactivation rate coefficients. Pseudo half-lives $pt_{1/2}$ (time necessary to reach 50% of relative activity) were calculated directly from experimental time courses of inactivation for each enzyme preparation. The stabilization factor (SF) was calculated as the ratio between the half-life of the immobilized enzyme and that of the corresponding soluble enzyme.

In the operational stability studies, two sets of each immobilized enzyme were used, either with or without reduction by sodium cyanoborohydride. After determining the proteolytic activity of the immobilized enzymes, the supernatant from the suspension was decanted and the supports washed with 2 ml of 0.1 M phosphate buffer pH 8.0. The supports were then centrifuged, the supernatant was decanted and the supports subjected to the proteolytic assay for the second cycle and so on.

The storage stability of the enzymes was evaluated by incubating them in 0.1 M phosphate buffer pH 8.0, with 0.020% (w/v) of sodium azide, at 25 °C, for various times and then assaying the enzymes.

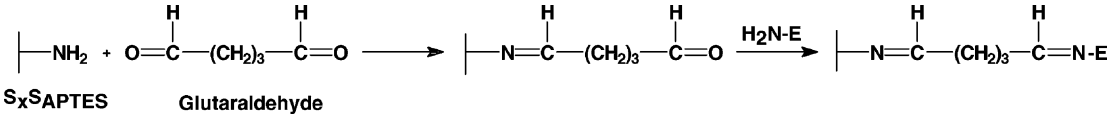
2.8. Electrophoresis assay

SDS-PAGE of Alcalase preparation was performed in poly(acrylamide-co-bisacrylamide) gels (15%), using a Phastsystem unit [19]. The buffer system in the gel was 0.625 M Tris–HCl pH 6.8 and the buffer for electrophoresis consisted of 0.1 M Tris–HCl pH 6.8, 0.1 M Bicine and 0.1% SDS. Protein standards were run on a lane parallel to that of crude proteases, and consisted of myosin (188 kDa), β-galactosidase (108 kDa), BSA (68 kDa), ovalbumin (48.5 kDa), carbonic anhydrase (33.4 kDa), soybean trypsin inhibitor (28.5 kDa), lysozyme (20.8 kDa), and aprotinin (7.3 kDa). After electrical resolution of the sample proteins, the gels were silver stained.

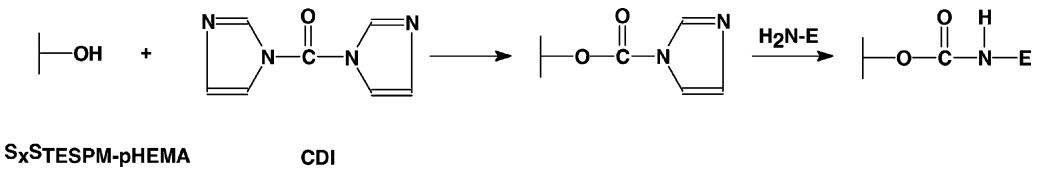
3. Results and discussion

3.1. Crude enzyme characterization

Since Alcalase is a commercial crude preparation produced by a selected strain of *Bacillus licheniformis*, with the main enzyme being a serine protease (Subtilisin Carlsberg) with a molecular weight of 27.3 kDa [20], it was important for this study to characterize its purity by SDS-PAGE. The electrophoretogram of Alcalase preparation (data not shown) shows several protein bands (up to ca. 55 KDa) besides the one corresponding to Subtilisin Carlsberg enzyme (<20% of the overall proteins), mainly at low molecular weight (ca. 30–40%, below 20.8 KDa). This means that the enzyme preparation has a significant content of protein contaminants including, probably, hydrolyzate products of the enzyme (proteins with low molecular weight).



Scheme 1. Immobilization on S_xS_{APTES} supports using glutaraldehyde as the activating agent.



Scheme 2. Immobilization on $S_xS_{TESPM\text{-}pHEMA}$ supports using CDI as the activating agent.

3.2. Screening of silica supports for enzyme immobilization

Two sets of chemically distinct silica supports were used for the immobilization of Alcalase. In one set, the original silicas were silanized with S_{APTES}, providing terminal NH_2 groups (denoted as S_xS_{APTES} silicas). Upon treatment by glutaraldehyde, the enzyme (via the ε-amino group of lysine residues) was immobilized to these supports (Scheme 1). In a second set, the silicas were silanized with S_{TESPM} and then grafted with HEMA by using X-ray irradiation (denoted as $S_xS_{TESPM\text{-}pHEMA}$ silicas). Upon treatment by CDI, the enzyme (via the ε-amino group of lysine residues) was immobilized in these supports (Scheme 2). The characterization of the original silicas and silanized silicas was previously reported [15,21]. The overall results are presented in Table 1.

The influence of the initial enzyme concentration in the immobilization reaction was evaluated for protein concentrations ranging from 0.299 to 89.8 mg g^{-1} of support, when $S_{300}S_{APTES}$ supports were used (Fig. 1). The graphic representation of enzyme activity versus protein concentration showed that the best loading result of the empirically chosen protein concentrations was 8.98 mg of protein per g of support, since a proportionality between activity and added protein is observed. Furthermore, the results obtained suggest that steric hindrance effects occurred for protein concentrations above 8.98 mg g^{-1} of support, as con-

Table 1
Physical and chemical properties of the silica derivatives [15,21]

Sample	Particle size (μm)[a]	Surface area ($m^2\ g^{-1}$)[b]	Mean pore diameter (Å)[c]	True density ($g\ ml^{-1}$)[d]	Porosity (%)[e]	Yield of grafting (%)[f]	Water vapor sorption (%)[g]
$S_{300}S_{TESPM\text{-}pHEMA}$	30	46	130/550	2.14	75.9	2.3/12.9	15.6
$S_{300}S_{APTES}$	30	58	130/550	2.27	81.1	1.5/–	21.9
$S_{1000}S_{TESPM\text{-}pHEMA}$	29	35	130/1200	2.23	79.6	0.4/6.3	6.5
$S_{1000}S_{APTES}$	29	40	130/1200	2.34	83.1	1.2/–	6.9

[a] Determined by laser diffraction (d_{50} results).
[b] Evaluated by BET (gas adsorption analysis).
[c] Mode of first peak/mode of second peak, determined by mercury porosimetry.
[d] Determined by helium pycnometry.
[e] Calculated as ((true density − bulk density)/true density) × 100.
[f] The first value is related to the yield of silanization process and the second value to the yield of the HEMA grafting. The values in each case were calculated from TGA assays. The ratio S_xS_{APTES}/S_x (for $S_{300}S_{APTES}$ and $S_{1000}S_{APTES}$) or $S_xS_{TESPM\text{-}pHEMA}/S_xS_{TESPM}$ taking in account S_xS_{TESPM}/S_x (for $S_{300}S_{TESPM\text{-}pHEMA}$ and $S_{1000}S_{TESPM\text{-}pHEMA}$) were calculated.
[g] %Sorption = 100 $(M_w - M_d)/M_d$, where M_w is the wet mass and M_d the dry mass.

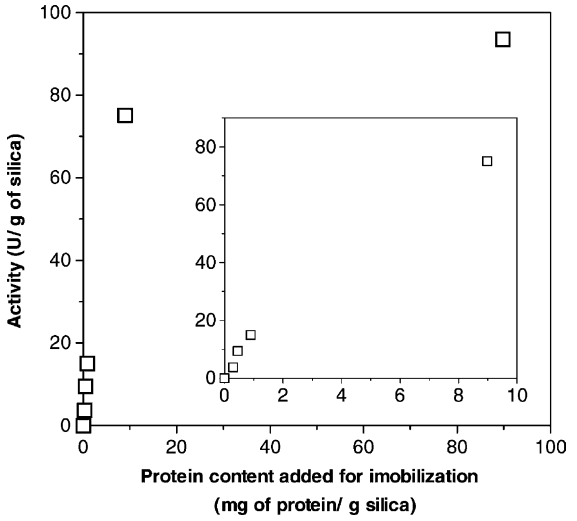


Fig. 1. Effect of the initial protein loading on $S_{300}S_{APTES}$-Alcalase activity.

firmed by the small increase in the activity. Therefore, in subsequent studies a protein loading of 8.98 mg g^{-1} of support was used.

The results of Alcalase immobilization on different silica supports are shown in Table 2 and some conclusions may be taken. First, the percentage of immobilized protein is higher on $S_xS_{TESPM\text{-}pHEMA}$ than in S_xS_{APTES} supports as result of the high content of attachment points for enzyme immobilization (see yield of grafting, Table 1). Furthermore, in the supports with the same surface chemistry, higher percentages of protein immobilized were achieved for supports with lower mean pore diameter (Table 2) and consequently higher superficial area (i.e. high content of attachment points).

Second, regarding enzyme activities, S_xS_{APTES}-immobilized Alcalase present higher total and specific activities relatively to $S_xS_{TESPM\text{-}pHEMA}$-immobilized ones (Table 2). The lower enzyme activities on $S_xS_{TESPM\text{-}pHEMA}$ supports is likely ascribed to steric impediments (as result of the high content of immobilized enzyme or p(HEMA) network complexity) and to enzyme conformation changes (as result of the multipoint attachment of the enzyme to support). Moreover, the high content of immobilized enzyme without expressing activity may reflect the immobilization of low molecular weight contaminant proteins from Alcalase preparation (see above). Finally, it should be noted that previously [21] we have shown a similar hydrolytic profile for another protease from *Bacillus subtillis* (similar molecular weight) immobilized into the same silica derivatives, using casein as substrate. In both cases, $S_{1000}S_y$ and S_xS_{APTES} silica derivatives presented an higher enzyme activity than $S_{300}S_y$ and $S_xS_{TESPM\text{-}pHEMA}$ ones, respectively. In that work [21], it was found by active sites titration that the concentration of active enzyme immobilized into $S_{300}S_{APTES}$ supports was higher than in $S_{1000}S_{APTES}$ supports and lower than on $S_{300}S_{TESPM\text{-}pHEMA}$ and $S_{1000}S_{TESPM\text{-}pHEMA}$. One would expect similar

Table 2
Influence of different silica derivatives on the Alcalase activity[a]

Support	Immobilized protein (mg of protein per g of silica)	Percentage of immobilized protein (%)	Activity (U g^{-1} silica)	Specific activity (U mg^{-1} protein)	Relative activity (%)[b]
$S_{300}S_{APTES}$[c]	3.5 ± 0.3	39.1 ± 2.8	62.9 ± 2.1	17.9 ± 0.6	14.1
$S_{1000}S_{APTES}$[c]	2.8 ± 0.2	31.1 ± 1.8	112.4 ± 7.0	40.3 ± 2.5	31.6
$S_{300}S_{TESPM\text{-}pHEMA}$[d]	6.3 ± 0.3	70.6 ± 3.5	30.4 ± 1.5	4.8 ± 0.2	3.8
$S_{1000}S_{TESPM\text{-}pHEMA}$[d]	5.6 ± 0.2	62.1 ± 2.3	37.4 ± 1.9	6.7 ± 0.3	5.3
S_{1000}[e]	2.4 ± 0.4	27.1 ± 4.9	5.3 ± 0.4	2.2 ± 0.2	1.7

[a] The values reflect the mean and standard deviation of three different measurements.

[b] The relative activity was calculated from the ratio: (specific activity for immobilized enzyme/specific activity for soluble enzyme) × 100. The specific activity for soluble enzyme was 127.4 ± 4.8 U (mg protein)$^{-1}$ (average ± S.D., $n = 3$).

[c] Silicas silanized with 3-aminopropyltriethoxysilane containing NH_2-terminal groups. The enzymes were immobilized on these supports via glutaraldehyde coupling.

[d] Silicas silanised with 3-(trimethoxysilyl) propyl methacrylate and then grafted with hydroxyethyl methacrylate (containing OH-terminal groups). The enzymes were immobilized on these supports via CDI coupling.

[e] Original silica, without any chemical modification. The enzyme was immobilized on these supports via CDI coupling.

results for Alcalase immobilized into the different silica derivatives. Therefore, this means that the low values of enzyme activity for Alcalase immobilized into $S_{300}S_{APTES}$ and $S_xS_{TESPM\text{-}pHEMA}$ derivatives as compared to $S_{1000}S_{APTES}$ ones are likely ascribed to steric hindrances and spatial restrictions effects in those supports and not to protein inactivation.

Third, silicas with large mean pore diameter (S_{1000}) present higher total and specific activities relative to those with smaller pore sizes (S_{300}). This reflects the lower steric impediments and lower spatial restrictions on S_{1000} over S_{300} silicas.

Fourth, the immobilization of Alcalase directly onto non-silanized S_{1000}-silicas supports (using CDI activation) yielded a biocatalyst with low activity, yet, the percentage of immobilized protein was similar to the values achieved in the immobilization process in S_xS_{APTES} supports. The inactivation of the enzyme promoted by the direct linkage to the support may explain the relatively low activity.

Finally, the activity of all immobilized enzymes into S_xS_{APTES} and $S_xS_{TESPM\text{-}pHEMA}$ supports ranged from 4 to 32% of the native enzyme activities in the hydrolysis of casein. This is similar to activities observed with other proteases immobilized into different supports [10,13].

Loss of native activity could be as result of mass transfer limitations and therefore the observable modulus (Φ) was calculated according to Eq. (2) [22]:

$$\Phi = \left(\frac{R}{3}\right)^2 \left(\frac{\nu_{obs}\tau}{D_{AB}\varepsilon S_0}\right) \quad (2)$$

where R is the particle radius (obtained from Table 1), ν_{obs} is the observed reaction rate per unit volume of catalyst (calculated from Tables 1 and 2), τ is the tortuosity of the pores (the maximum value (7.0) was used), D_{AB} is the bulk substrate diffusivity ($2.1 \times 10^{-6}\,cm^2\,s^{-1}$ for casein, calculated from the Stokes–Einstein equation [22]), ε is the porosity of the support (obtained from Table 1) and S_0 is the bulk substrate concentration. The observable modulus calculated for immobilized Alcalase into the different silicas were below 0.1 which corresponds to an internal effectiveness factor (η_I, which is the ratio between the actual observed activity and the activity that would be obtained in the absence of intraparticle diffusion limitations) of ca. 1 [22]. This means that no intraparticle mass limitations were observed. In fact, this is not surprising given the low particle size of the different silicas and the slow enzyme activity in the proteolysis of casein. Hence, we can conclude that the loss of enzyme activity in the immobilized Alcalase, relatively to the free counterpart, was not influenced by mass transfer limitations.

From these results and within the experimental ranges used, we can conclude that $S_{1000}S_{APTES}$ support is the best support for enzyme immobilization and thus was selected for further experiments.

3.3. Effective factors in the enzyme immobilization on $S_{1000}S_{APTES}$-silica supports

A variety of factors can influence enzyme activity on S_xS_{APTES}-silica supports, therefore, we examined systematically the influence of glutaraldehyde concentration and the time of enzyme coupling to the support. As shown in Fig. 2, a glutaraldehyde concentration of 0.5% (v/v) is optimal for both total and specific activity of the immobilized Alcalase preparation. At higher glutaraldehyde concentrations, more protein is bound, however, the activity of enzyme bound to the silica support decreases. This may be due to the

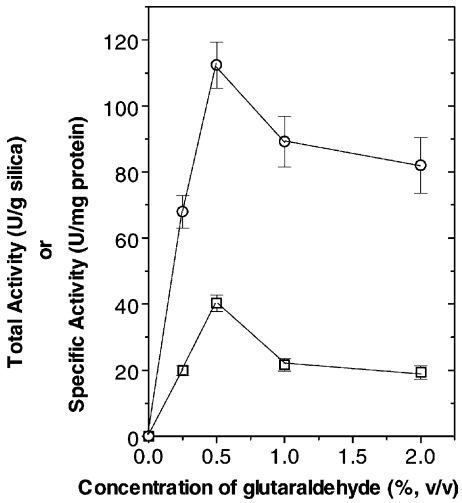


Fig. 2. Effect of glutaraldehyde concentration on the total (○) and specific (□) activities of Alcalase immobilized onto $S_{1000}S_{APTES}$-silica supports (average ± S.D., $n = 3$). The percentage of immobilized protein was 38.1, 37.2, 45.8 and 47.3% for 0.25, 0.5, 1 and 2% (v/v) of glutaraldehyde concentration, respectively.

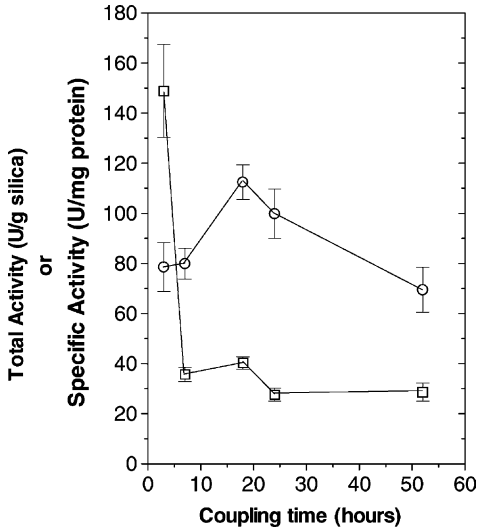


Fig. 3. Effect of coupling time reaction, between the activated support (a glutaraldehyde concentration of 0.5% (v/v) was used) and the enzyme, on the total (○) and specific (□) activities of Alcalase immobilized onto $S_{1000}S_{APTES}$-silica supports (average ± S.D., $n = 3$). The percentage of immobilized protein was 5.9, 24.9, 37.2, 40.3 and 27.1% for 3, 7, 18, 24 and 52 h of coupling time, respectively.

reticulation among enzyme molecules favored by the use of a bifunctional molecule and therefore affecting the overall activity. Hence for further studies, 0.5% (v/v) glutaraldehyde was used.

The influence of the coupling time on the catalytic properties of the $S_{1000}S_{APTES}$-immobilized Alcalase is depicted in Fig. 3. While the total activity remained relatively constant as a function of coupling time (although a slight maximum is evident at 18 h), the specific activity dropped precipitously up to 7 h of coupling, after which it remained almost unchanged up to 52 h. The high specific activity achieved in the first 3 h of immobilization strongly suggests that the immobilized enzyme has high intrinsic activity when only a small fraction of the total protein is immobilized. Beyond this point, proteolysis during the immobilization process or glutaraldehyde damage effect may result in lower specific activity for longer immobilization times. By storage stability tests of different Alcalase 2T concentrations in 0.1 M phosphate buffer pH 8 (see below) a small proteolysis effect was observed upon 52 h (this may explain the decreasing of immobilized protein percentage from 40.3% after 24 h to 27.1% after 52 h). However, one may expect that the results achieved are mainly due to the damage effect promoted by glutaraldehyde to the enzyme. It is noteworthy that similar decrease in the specific activity for longer times when silica carriers were activated by glutaraldehyde were described in the literature [23]. A trade-off exists between increased total activity and increased coupling time such that a maximum in total activity is achieved after 18 h, and this was the coupling time chosen for the further studies.

3.4. Enzyme attachment on $S_{1000}S_{APTES}$ supports

It is well known that multipoint attachment can stabilize immobilized enzymes [9,14,24,25]. The combination of glutaraldehyde concentration, pH and time of enzyme coupling reaction on the activated support is likely to affect the total number of attachment sites that Alcalase will have with a support. Either a large excess of glutaraldehyde or long enzyme coupling time have been used, which would favor the multipoint attachment [25]. Furthermore, the enzyme coupling reaction to the support at pH 8.0 adopted in this work, although not ideal (pK of lysine is 10.7), has been described to yield multipoint attachment with other supports containing aldehyde functionality [25].

The total number of attachment points can be estimated by knowing the surface density of aldehyde groups on the activated silicas. The amino contents of $S_{1000}S_{APTES}$ silicas, before activation, was 140 μmol g^{-1}, as calculated from the yield of grafting via TGA analysis (Table 1). Assuming that all the amino-terminal groups in the supports will be activated by glutaraldehyde (the concentration of glutaraldehyde is present in a 30-fold molar excess) and taking into account the surface area of the support, we can calculate the surface density of activated groups on the silicas. The surface density is defined as the number of residues of active groups per 1000 $Å^2$ of silica support (surface density = 6.02 × active groups concentration/surface area (μmol g^{-1}/m^2 g^{-1})). Given the surface of $S_{1000}S_{APTES}$ to be 40 m^2 g^{-1}, the surface density of aldehyde groups is calculated to be 21 residues per 1000 $Å^2$. On the other hand, considering the Alcalase radius of ≅21 Å [11], the total external area of the protein must be 5542 $Å^2$. If we assume that no more than 10% of this external area can contact the support [24], there will be ca. 12 aldehyde groups in the $S_{1000}S_{APTES}$ supports, that are able to

react with the amino groups on the enzymes. According to the nucleotide sequence of Alcalase 2T enzyme (data from Brookhaven Protein Data Bank—entry 1SCA), there are nine lysine residues in one enzyme molecule, and from the three-dimensional structure, eight of them (exposed to the exterior of the enzyme) can react with the activated $S_{1000}S_{APTES}$-silica supports. Hence, multipoint attachment is expected. Furthermore, this assumption is further confirmed by the stability studies on $S_{1000}S_{APTES}$-Alcalase discussed below.

3.5. Determination of kinetic parameters in soluble and immobilized enzyme

The catalytic activity of soluble and immobilized Alcalase was assessed using casein as the substrate. In all cases, Michaelis–Menten kinetics were observed. From Table 3 the K_m value for the immobilized preparation is lower than the soluble one. This may be explained by the partitioning effects of the substrate in the immobilized enzyme relatively to the soluble one. Surprisingly, in the case of Alcalase, the immobilized derivative showed a higher k_{cat} and k_{cat}/K_m (1.4- and 1.7-fold higher, respectively) than the soluble Alcalase for casein hydrolysis. Possibly, the conformational changes of the Alcalase due to immobilization led to an improvement in enzyme performance.

3.6. Effect of pH and temperature on enzyme activity

The effect of reaction pH on the activity of immobilized enzyme was evaluated and optimum pH value of 8.0 was obtained for $S_{1000}S_{APTES}$-immobilized Alcalase. Since the activity profiles of free and immobilized enzyme were similar, including the range of optimal pH, no pH partition effects were observed. The optimum temperature, based on the initial rates, for both soluble and immobilized Alcalase was 60 °C. Temperature activation studies (35–70 °C) revealed that the activation energies (calculated by Arrhenius equation) for casein hydrolysis by soluble and immobilized enzyme preparations were 42.6 and 45.4 kJ mol^{-1} for soluble and immobilized Alcalase, respectively. These similar values of activation energies for each of the soluble and immobilized enzyme preparations imply that the activation enthalpy for casein hydrolysis is unaffected by immobilization. Moreover, these results confirm that substrate mass transfer is not limiting in the immobilized preparations.

3.7. Enzyme stability studies

The thermal stability of enzymes is an important feature for the application of the biocatalysts in a commercial point of view. Alcalase is stabilized against thermal deactivation at 50 °C upon immobilization onto $S_{1000}S_{APTES}$ (Fig. 4) with minimal deactivation after 2 h. This compares favorably to the soluble enzyme where nearly 50% activity is lost after the same period of time. The kinetics of irreversible thermal deactivation of both soluble and immobilized Alcalase at 50 °C appear to conform to single exponential decays (Fig. 4). However, at 60 °C, both soluble and immobilized Alcalase undergo a more complex deactivation that appears to be better fit to a double exponential decay (e.g. the deactivation occurs as two overlapping processes). The greater values of k_1 as compared to k_2

Table 3
Kinetic and temperature parameters for soluble and immobilized Alcalase

	Kinetic parameters			Deactivation rate constants (k_i)		$pt_{1/2}$[a] (min)	SF[b]
	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (μM^{-1} min^{-1})	k_1 (min^{-1})	k_2 (min^{-1})		
Soluble Alcalase	25.8	0.214	0.00829	0.0233 (50 °C)	–	–	–
				0.391 (60 °C)	0.0115 (60 °C)	11	1.0
Immobilized Alcalase	20.6	0.289	0.0140	0.00318 (50 °C)	–	–	–
				0.0573 (60 °C)	0.00708 (60 °C)	24	2.2

[a] Pseudo half-life times.
[b] Stabilization factor as a ratio of half-life times.

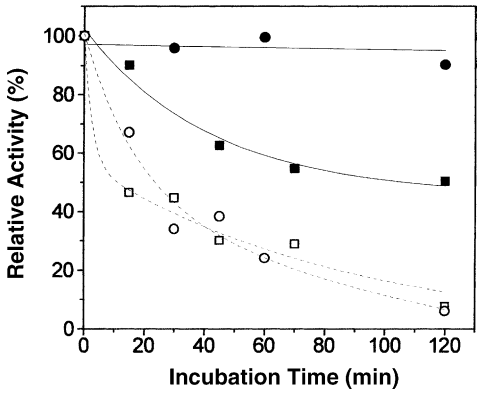


Fig. 4. Time dependence of thermal stability of soluble (■ and □) or $S_{1000}S_{APTES}$-immobilized (● and ○) Alcalase, at 50 °C (solid lines) and 60 °C (broken lines) in 0.1 M phosphate buffer pH 8.0. The starting activities were taken as 100%.

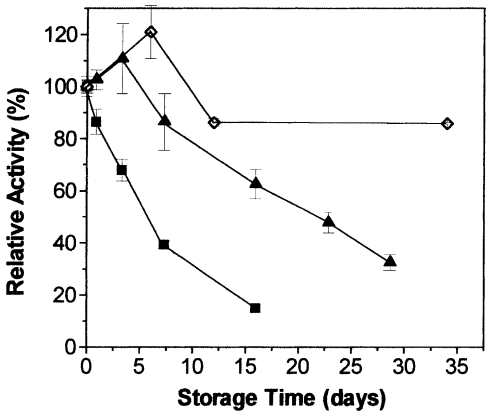


Fig. 5. Influence of the storage time in 0.1 M phosphate buffer pH 8.0 with 0.020% (w/v) of sodium azide on activity of soluble (▲ and ■) and $S_{1000}S_{APTES}$-immobilized (◇) Alcalase, at 25 °C. The immobilized enzyme was reduced with sodium cyanoborohydride. For soluble Alcalase two different protein concentrations were used: 4.49 mg ml^{-1} (■) and 0.0898 mg ml^{-1} (▲) of storage medium. The starting activities were taken as 100% (average ± S.D., $n = 3$).

(34- and 8.1-fold higher for soluble and immobilized Alcalase, respectively), indicates that the first deactivation process is faster than the second. In this case the immobilized Alcalase is just 2.2-fold more stable than the soluble enzyme (SF, Table 3).

The improvement in stability of the immobilized enzyme, mainly at 50 °C and in a small extent at 60 °C, may be attributed to several factors that stem from an immobilized preparation. These include the prevention of auto-proteolysis due to a restriction of the intermolecular contact in the immobilized enzymes and the protection of the enzymes from structural rearrangement, due to the likely multipoint attachments to the support [26].

Operational stability was carried out with immobilization conditions that either included or did not include the reducing agent sodium cyanoborohydride. As stated earlier, the coupling reaction between the silica support activated with glutaraldehyde and the enzyme produces imine bonds (Schiff's bases), which are unstable [9]. To stabilize this bond, several reducing agents, such as sodium borohydride [9], cyanoborohydride [27] or amine boranes [27], have been used. $S_{1000}S_{APTES}$-Alcalase either reduced or not by sodium cyanoborohydride could be used repeatedly with only a small loss in activity. Specifically, after five reaction cycles at 50 °C, Alcalase activity decreased only 15–25% (data not shown). Interestingly, no significant differences were observed when the immobilization of Alcalase was carried out in the presence or absence of sodium cyanoborohydride. Moreover, this confirms the thermal stability results obtained previously at 50 °C (see above). This suggests that the imine bond between the glutaraldehyde activated support and the enzyme is stable enough to confer good operational stability even in aqueous solutions. Finally, these results confirm our assumption of multipoint attachment between the enzyme and the support, since the enzymes do not desorbs significantly after five cycle reactions [14].

The immobilized enzyme was reduced with sodium cyanoborohydride before the storage stability studies. As shown in Fig. 5, the $S_{1000}S_{APTES}$-immobilized Alcalase retain about 86% of its original activity over a period of 1 month. Under the same storage conditions the soluble enzyme retain about 32% (30 days) and 15% (16 days) of its initial activity depending on the initial protein content. The improved stability of immobilized enzymes over their soluble counterparts may be related to the prevention of autolysis and thermal denaturation [12,28]. In conclusion, $S_{1000}S_{APTES}$-immobilized Alcalase present an improved stability over its soluble counterpart and may be attractive biocatalysts for industrial purposes.

4. Conclusions

For hydrolysis reactions, we have found that S_xS_{APTES}-silica derivatives are much better supports for enzyme immobilization than $S_xS_{TESPM\text{-}pHEMA}$ silica derivatives likely due to a lower surface complexity in the pores. Furthermore, the mean pore size of silica supports plays a major role in the enzyme catalytic performance. Silicas with large pores (S_{1000}) presented higher and total specific activities relative to those with smaller pore sizes (S_{300}). Finally, enzyme-support multipoint covalent attachment has proved to enhance the thermal, operational and storage stability of the immobilized enzyme compared to soluble one.

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