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Sol–gel immobilization of Alcalase from *Bacillus licheniformis* for application in the synthesis of C-terminal peptide amides

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ABSTRACT

Alcalase 2.4L FG, a commercial preparation of Subtilisin A, was physically entrapped in glass sol–gel matrices using alkoxysilanes of different types mixed with tetramethoxysilane (TMOS). The materials were used for catalyzing C-terminal amidation of Z-Ala-Phe-OMe in a mixture of *tert*-butanol/DMF. From the screening of silane monomers in the sol–gel coating process, it was concluded that dimethyldimethoxysilane (DMDMOS) gave the best performance, and Alcalase immobilized therein exhibited the highest activity in the ammonolysis of Z-Ala-Phe-OMe. The percentage of protein immobilization was in the range of 68–98%, and total amidation activity of the immobilized Alcalase was up to 1.76 μmol/h/mg gel. We investigated the immobilization efficiency for a protein mass range of 2.8–9.7 mg per mmol total silanes, to determine the immobilization capacity of the biosilica support. The optimum enzyme loading capacity in the silica matrix was 115 mg/g dry silica xerogel (11.5%, w/w). The amount of the DMDMOS silicate was optimized by adjusting the molar ratio of silane mixture (DMDMOS and TMOS at 1:1). Biocatalyst sol–gel particles prepared at optimum immobilization conditions retained 100% of the original activity even after 14 cycles of repeated use. Reproducibility of the immobilization technique was also investigated by evaluating the catalytic efficiency of the obtained preparations. The thermal stability of the protease at 70 °C increased threefold upon entrapment in sol–gel materials, and twofold under storage for 50 days at ambient temperature.

1. Introduction

Peptides exhibit a broad range of biological and functional properties. In the living body, they play the role of mediators and regulators of vital biological functions. It is for this reason that there is a commercial interest in the production of enantiopure short peptides for applications in the food and pharmaceutical industries. In the past years, much attention has been given to the use of enzymes in peptide synthesis, to improve peptide manufacture, productivity and costs, according to the green chemistry principles. Proteases, such as Subtilisin A from *Bacillus licheniformis*, proved to be attractive catalysts for the synthesis of biologically active peptides containing a C-terminal primary amide function. Recently, alcaline protease Subtilisin, free or immobilized as cross-linked enzyme aggregates, proved to be an excellent catalyst for selective deprotection of the C-terminal tert-butyl esters of peptides and synthesis of peptide derivatives via modification of the carboxy-terminus of peptides by C-terminal amidation or synthesis of various esters by transesterification [1–3]. Subtilisin can be used to synthesize C-terminal amides of peptides by ammonolysis following two different approaches: thermodynamically controlled (equilibrium controlled) and kinetically controlled synthesis [4]. Kinetically controlled synthesis, starting from an α-amino ester, is usually preferred in amide synthesis because the energy barrier for product formation is low. The ester reacts with the enzyme to form an acyl enzyme intermediate, which then reacts either with an amine to form the desired amide, or with water leading

Abbreviations: MeTMOS, methyltrimethoxysilane; PrTMOS, propyltrimethoxysilane; OcTMOS, octyltrimethoxysilane; PhTMOS, phenyltrimethoxysilane; VTMOS, vinyltrimethoxysilane; TMOS, tetramethoxysilane; APrTMOS, (3-aminopropyl)trimethoxysilane; *i*-BuTMOS, *i*-butyltrimethoxysilane; DMDMOS, dimethyldimethoxysialne; MPDMOS, methylphenyldimethoxysilane; DMF, N,N-dimethylformamide; ^{t}BuOH, *tert*-butanol; DP-NH2, dipeptide amide; DP-OH, dipeptide free acid; DP-OMe, dipeptide methyl ester; Z, benzyloxycarbonyl protecting group; A/H, ratio of amidation and hydrolysis yields.

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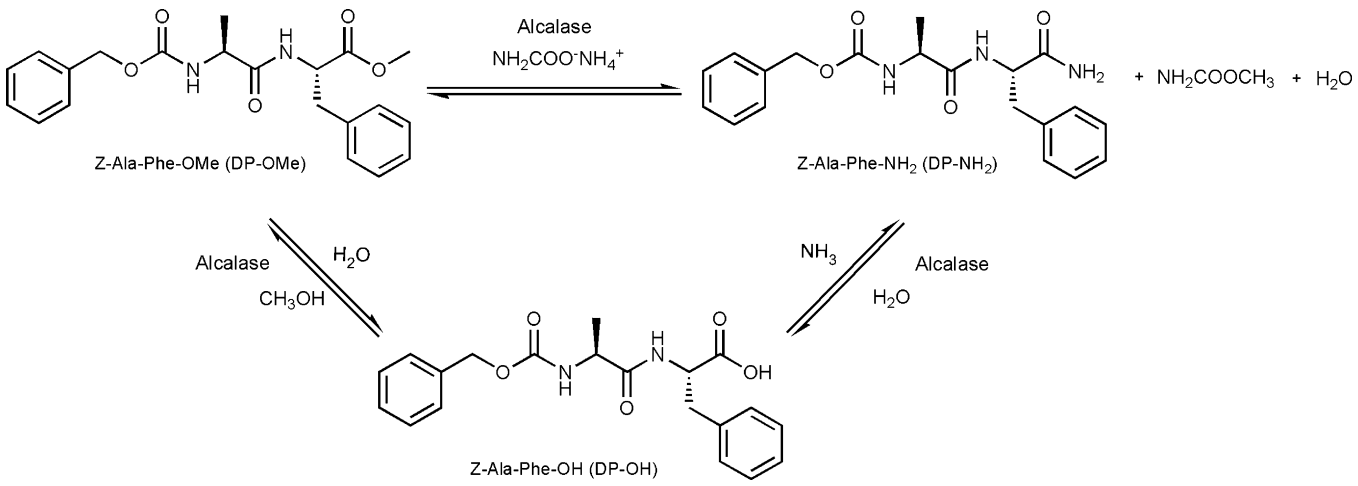

Fig. 1. Scheme of enzymatic synthesis of Z-Ala-Phe-NH_2 by Alcalase immobilized in sol–gel matrices.

to undesired hydrolysis. The obtained yield depends on the balance of three different reactions catalyzed by the same enzyme [5]. In our case, the process involves the synthesis of Z-Ala-Phe-NH_2, the hydrolysis of the activated substrate Z-Ala-Phe-OMe, and the hydrolysis of the newly formed amide Z-Ala-Phe-NH_2 (Fig. 1). The maximum yield depends on the enzyme properties and the type of substrate activation. Hydrolases with high ratios of transferase to hydrolase activity are favorable for use. Using an immobilized protease, due to the mass transfer influences, different yields may be obtained compared to the native or aggregated enzyme. The use of an activated acyl donor (ester) allows higher yields than in case of equilibrium controlled synthesis [6]. The enzymatic synthesis of C-terminal arylamides has been described by Nuijens et al. [5], by converting N-terminally protected amino acids and peptides using Alcalase, an industrial serine protease. Several proteases, as α-chymotrypsin, papain, and Alcalase catalyzed the synthesis of dipeptides in organic media [7].

Proteases, the most important category of industrial enzymes, are becoming increasingly popular catalysts for peptide bond formation [8]. The utilization of microbial proteases for peptide synthesis was comprehensively reviewed by Kumar and Bhalla [9]. Unfortunately, their industrial application is often hampered by the lack of operational stability. In organic solvents or at high temperatures, many enzymes are instable and often show low activity. Therefore, enzyme stabilization will continue to be a key issue in biotechnology [10]. Immobilization on a solid support can overcome these drawbacks, allowing them to be recycled, facilitating their separation from the reaction medium, and increasing their stability [11,12].

Numerous methods for immobilization of biocatalysts on rigid supports, such as covalent immobilization [13], ionic immobilization [14,15], hydrophobic adsorption [16], and encapsulation in polymer and inorganic matrices [17] have been explored to achieve a high-yield, reproducible and robust immobilization technique. In the case of multimeric enzymes, immobilization can produce enzyme rigidification if multipoint or multisubunit attachment to the support is achieved [18]. Glyoxyl-agarose supports represented a good substrate for the immobilization/stabilization of Alcalase via multipoint covalent attachment [19,20]. Covalent attachment of Alcalase on silica supports by multipoint attachment, using glutaraldehyde as bifunctional reagent, also resulted in better operational and storage stability compared to the soluble enzyme [21].

Stability of enzymes in non-aqueous environment, as well as thermostability, was often reported to be enhanced by immobilization [22]. Immobilization of α-chymotrypsin to polyacrylamide gel covalently protects α-chymotrypsin from irreversible inactivation by organic solvents at room temperature and by elevated temperatures, up to 70 °C [23].

The incorporation of enzymes in silica matrices has proved to be a good strategy to improve the catalytic efficiency of many enzymes. Sol–gel matrices are porous and thermally stable and they prevent leaching of the enzyme from the support [24]. A sol–gel process involves the hydrolysis of silane precursors, tetra-alkoxysilanes mixed with alkoxysilanes followed by polycondensation to form ceramic materials. The obtained gels are allowed to mature and dry at ambient temperature [25]. Biomolecules encapsulated in sol–gel matrices typically exhibit improved resistance to thermal and chemical denaturation and increased storage and operational stability [21].

Immobilization of enzymes inside a porous silica matrix allows the enzyme molecules to become fully dispersed without the possibility of interacting with any external interface. Therefore, the stabilization will be achieved by dispersion of the protease on the support preventing aggregation, autolysis or proteolysis. The structural rigidity of the sol–gel matrix protects the integrity of encapsulated enzyme molecules and prevents their leaching; the mesoporous structures and high pore volume of sol–gel polymer afford the free diffusion of small substrate molecules and their effective interactions with the enzyme [26].

Sangeetha et al. reported 80% activity retention of Subtilisin encapsulated in sol–gel matrices obtained from vinyltrimethoxysilane precursor, as well as the possibility to use this biocatalyst for synthesis of peptides [8]. Several serine proteases (Alcalase, α-chymotrypsin, trypsin, and subtilisin Carlsberg) were successfully encapsulated in sol–gel matrices [27,28]. To the best of our knowledge, immobilization of the serine protease Alcalase in dimethyldimethoxysilane (DMDMOS)-containing sol–gel system was not yet reported.

In the present work we investigated several precursor systems, to obtain the highest enzyme loading and activity. The stability of encapsulated Alcalase was an important objective of our research. The model reaction for testing the sol–gel biocatalysts was the enzymatic amidation of Z-Ala-Phe-OMe, using ammonium carbamate as the source of nucleophilic ammonia (Fig. 1). Hydrolysis of the ester group occurred as secondary reaction. The reproducibility of the immobilization method, in terms of protein loading and catalytic efficiency has been studied. The enzyme with the highest encapsulation efficiency was scrutinized for operational, thermal and storage stability, compared to the free enzyme.

2. Materials and methods

2.1. Materials

Alcalase 2.4L FG (PLN05354) was obtained from Novozymes. The protein content, assayed in our laboratory, was 58 mg per ml enzyme solution. Silane precursors tetramethoxysilane (TMOS, 98%), methyl-(MeTMOS, 98%), propyl-(PrTMOS, 98%), vinyl-(VTMOS, 98%), octyl-(OcTMOS, 97%), 3-aminopropyl-(APrTMOS, 97%), *i*-butyltrimethoxysilane (*i*-BuTMOS, 97%) and phenyltrimethoxysilane (PhTMOS, 97%) were purchased from Alfa Aesar, Brunschwig Chemie, The Netherlands. Dimethyldimethoxysilane (DMDMOS, 96%) was from Fluka and methylphenyldimethoxysilane (MPDMOS, 95%) was from Aldrich. The C-terminal methyl ester of dipeptide, Z-Ala-Phe-OMe (HPLC purity ≥98%), was purchased from Bachem AG, Switzerland. The reference peptides Z-Ala-Phe-NH_2 and Z-Ala-Phe-OH, as well as Z-Ala-Phe-OBu^t, were provided by MSD, The Netherlands. The solvents were stored over 4 Å molecular sieves and used without further purification. All other chemicals used were of analytical grade.

2.2. Entrapment of Alcalase in sol–gel matrices

The previously described immobilization method [29] based on the Reetz procedure for the entrapment of lipases [30] has been used. Alcalase 2.4L FG solution was used for immobilization. In a 4 ml glass vial, Alcalase 2.4L FG solution (0.3–1 ml), PEG 20000 (200 μl), 1 M NaF (100 μl), and isopropyl alcohol (200 μl) were mixed (magnetic stirring, 600 rot/min). By continuous stirring, 6 mmol silane precursors were added. The resulting mixture was vigorously stirred at ambient temperature until the gel formation started. The gel was kept for 24 h at 4 °C in the refrigerator to complete polymerization. The bulk gel was washed with isopropyl alcohol (7 ml), milli Q water (5 ml), isopropyl alcohol (5 ml) and n-hexane (5 ml) and dried at ambient temperature for 48 h. Finally, it was crushed in a mortar and kept in the refrigerator. The water content of the immobilized enzyme preparations was between 4.6 and 6.9 wt.%, as determined by Karl–Fischer titration. The washing solutions were analyzed for protein content and the activity of immobilized enzyme was determined using the indicated model reaction.

2.3. Enzymatic hydrolysis of Z-Ala-Phe-OBu^t

The optimum reaction conditions for the C-terminal hydrolysis of tert-butyl peptide esters were used as previously described [2]. Hydrolysis reactions of Z-Ala-Phe-OBu^t were performed in batch mode in a 24-tubes carrousel (GreenHouse PlusTM Parallel Synthesizer; Radleys Discovery Technologies) with controlled temperature, cooling system and magnetic stirring. 10 mg of sol–gel preparation or 10 μl of Alcalase 2.4L FG solution (40 mg protein/ml) were added to 5 ml 0.1 M sodium phosphate buffer, pH 7.0, containing 2.5 ml Z-Ala-Phe-OBu^t 20 mM in DMF at 40 °C. Samples taken at different time intervals were diluted by the addition of an equal volume of acetonitrile and analyzed by RP-HPLC. The identity of the compounds was determined with the use of reference compounds. The hydrolysis yields (molar conversion) were calculated from the peak area integration (1), while specific activities were expressed as the amount of formed Z-Ala-Phe-OH (in micromole) in 1 h interval by 1 mg of protein (2). All reactions were performed in duplicate.

$$\text{Hydrolysis yield}\,(\%) = \left(\frac{A_{\text{DP-OH}}}{A_{\text{DP-OH}} + A_{\text{DP-OBu}^t}}\right) \times 100 \quad (1)$$

$$\text{Activity} = \left(\frac{\mu\text{mol DP-OH}}{\text{time (h)} \times \text{enzyme amount (mg)}}\right) \quad (2)$$

2.4. Enzymatic ammonolysis of Z-Ala-Phe-OMe

The optimum reaction conditions for maximum amide yield and minimum secondary hydrolysis were used as previously described [4]. Amidation reactions of model dipeptide were performed in batch mode in a 24-tubes carrousel (GreenHouse PlusTM Parallel Synthesizer; Radleys Discovery Technologies) with controlled temperature, cooling system and magnetic stirring. All the solvents were dried using molecular sieves 4 Å (10%, w/w) for 24 h and the ammonium carbamate was dried overnight over P_2O_5 prior to reaction, and the water content was determined by Karl–Fischer titration. The test tubes were charged with a mixture of Z-Ala-Phe-OMe substrate (10 mM), ammonium carbamate (1:10 molar ratio) and 5 ml of a solvent mixture composed of DMF and ^{t}BuOH 17.5:82.5 (v/v). The mixture was kept at 30 °C for 30 min. The reaction was initiated by the addition of 10 mg of sol–gel preparation or 10 μl of Alcalase 2.4L FG solution. To have a proper comparison of the immobilized and soluble enzyme, the same activity units (2.6 U/ml) were introduced in all test reactions for kinetic and stability studies. The reaction mixture was incubated at 30 °C. Samples taken at different time intervals were diluted by the addition of an equal volume of acetonitrile and analyzed by RP-HPLC. Control reactions without enzyme were performed under identical conditions, without enzyme. Instead of enzyme solution, 10 μl of the 0.1 M phosphate buffer pH 7.0 was added to the reaction mixture. The identity of the compounds was determined with the use of reference compounds. The relative percentage yield (molar conversion) was calculated from the peak area integration (Eqs. (3) and (4)). Amidation specific activities were expressed as the amount of formed Z-Ala-Phe-NH_2 (in micromole) in 1 h interval by 1 mg of protein (Eq. (5)). The reaction without enzyme did not give any product in the same condition. All the reactions were performed in duplicate.

$$\text{Amide yield}(\%) = \left(\frac{A_{\text{DP-NH}_2}}{A_{\text{DP-NH}_2} + A_{\text{DP-OH}} + A_{\text{DP-OMe}}}\right) \times 100 \quad (3)$$

$$\text{Free acid peptide yield}(\%) = \left(\frac{A_{\text{DP-OH}}}{A_{\text{DP-NH}_2} + A_{\text{DP-OH}} + A_{\text{DP-OMe}}}\right) \times 100 \quad (4)$$

$$\text{Activity} = \left(\frac{\mu\text{mol DP-NH}_2}{\text{time (h)} \times \text{enzyme amount (mg)}}\right) \quad (5)$$

2.5. Protein content and protein loading determination

The Bradford method [31] was used to measure the protein concentration of solutions with dissolved protein at 595 nm with a SAFIRE spectrophotometer (Tecan Benelux BVBA, Giessen, The Netherlands), using bovine serum albumin (BSA) as a standard. The amount of encapsulated protein onto silica supports was determined indirectly as the difference between the initial total protein subjected to immobilization and the amount of protein recovered in the washing solutions after immobilization. The entrapment yields were calculated as the ratio of encapsulated protein amount to the amount of protein subjected to immobilization. The protein loading was calculated as a ratio of encapsulated protein to the amount of obtained dry xerogel.

2.6. HPLC analysis

The amidation reaction mixtures were analyzed by reverse phase (RP) on a Atlantis T3 3 μm, 2.1 mm × 100 mm column

(Waters) thermostated at a temperature of 30 °C using a Waters HPLC system equipped with UV dual wavelength detector and autosampler. The eluting components were detected at 220 nm. The mobile phase A was 0.1% trifluoroacetic acid in water, while mobile phase B was 0.1% trifluoroacetic acid in acetonitrile. The mobile phase flow rate was 0.5 ml/min. A linear gradient from 2 to 98% acetonitrile was applied from 0 to 17.5 min. From 17.5 to 22 min, the mobile phase composition was constant 98% acetonitrile and 2% water.

2.7. Enzyme stability experiments

2.7.1. Thermal stability studies

For determination of thermal stability, free and sol–gel immobilized protease were incubated in a mixture of ^{t}BuOH/DMF 82.5:17.5 (v/v) at various temperatures (30, 40, 50, 60 and 70 °C) for 10 min, and their residual amidation activity was measured. The concentration of biocatalyst was kept at 2.6 U/ml.

2.7.2. Storage stability studies

The residual activity of encapsulated and free protease in the amidation of Z-Ala-Phe-OMe was measured during storage at ambient temperature for 50 days, while keeping the concentration of biocatalyst at 2.6 U/ml.

2.7.3. Operational stability of the immobilized protease

Reactions were performed in batch mode in a Memmert Modell 500 incubator with controlled temperature, using a rotator (Stuart SB2) for parallel reactions. The solvents and the ammonium source were dried prior to reaction, as described in Section 2.4. The amidation reactions have been performed in 4 ml capacity glass vials charged with a mixture of 16 mg sol–gel preparation (5.2 U/ml), Z-Ala-Phe-OMe substrate (10 mM), ammonium carbamate (1:10 molar ratio) and 4 ml of a solvent mixture composed of DMF and ^{t}BuOH (17.5:82.5, v/v). The reaction was stopped at 22 h and samples taken were diluted by the addition of an equal volume of acetonitrile and analyzed by RP-HPLC. At the end of the reaction period, the immobilized enzyme was separated from the reaction mixture, washed with 2 ml of solvent mixture DMF/^{t}BuOH (17.5:82.5, v/v) and resuspended in freshly prepared substrate to start a new run. The relative percentage yield (molar conversion) was calculated from the peak area integration. The catalytic efficiency was expressed as the percentage yield of formed Z-Ala-Phe-NH_2 in 1 h interval by 1 mg of immobilized enzyme. All the reactions were performed in duplicate.

2.8. Electrophoresis assay

Acrylamide gel electrophoresis was performed using the XC II™ mini-cell electrophoresis system (Novex) and precast 10% Bis–Tris NuPAGE gels according to the manufacturer's protocols (Novex). The proteins were visualized by staining the gels with Coomassie Blue dye or by silver staining [32].

2.9. Kinetics of free and immobilized enzyme

Kinetics of soluble and entrapped enzyme was accomplished for amidation of Z-Ala-Phe-OMe in a mixture of DMF/^{t}BuOH (17.5:82.5, v/v) at 30 °C, at different substrate concentrations, while keeping the concentration of biocatalyst at 2.6 U/ml. The kinetic studies were assayed at substrate concentrations from 5 to 350 mM. The Michaelis–Menten constant (K_M) and maximum velocity (V_M) values were obtained automatically from Hanes Woolf plots using Enzyme Kinetics Software (add on module of Sigma plot), and used for calculation of the kinetic constant k_{cat} and catalytic efficiency (k_{cat}/K_M).

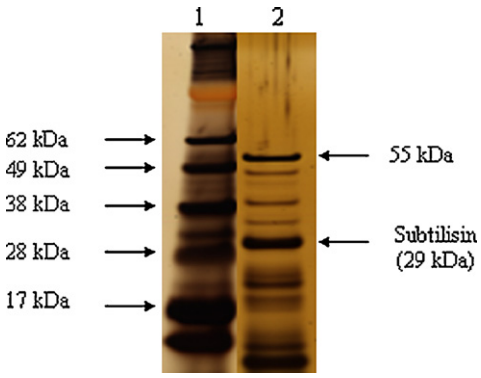

Fig. 2. SDS-polyacrylamide gel electrophoresis of Alcalase. Lane 1, standard molecular weight markers; lane 2, Alcalase 2.4L FG.

3. Results and discussion

3.1. Characterization of crude Alcalase

Since Alcalase 2.4L FG is a commercial crude preparation produced by *B. licheniformis* strain, with the main enzyme being Subtilisin A (an alkaline serine protease), it was important to study its purity by SDS-PAGE electrophoresis. The protein content was 58 mg per ml enzyme solution, and the specific activity of the soluble protease for the amidation of Z-Ala-Phe-OMe was 33.8 μmol/h/mg protein after 2 h of incubation. The electrophoretogram of Alcalase preparation presented several protein bands up to ca. 55 kDa, the most intense at 29 kDa corresponding to Subtilisin A (Fig. 2). There were also several protein bands at lower molecular weight (below ca. 22 kDa), indicating that the enzyme preparation had a high content of protein contamination, probably autolysis products of the enzyme. The presence of contaminant proteins could influence the reproducibility of immobilization batches, but it was not the case for our sol–gel experiments (*cf.* Section 3.4), and purification of the commercial enzyme was not necessary. Enzyme loading capacity could be also reduced by the contaminant proteins and it is presumable that a purified Subtilisin A would have a higher specific activity following immobilization. However, the commercial enzyme was suitable for our purpose, and purification seems to not be a feasible solution for possible industrial application, considering the additional costs.

3.2. Hydrolytic activity of sol–gel entrapped Alcalase

In our experiments, organic–inorganic hybrid sol–gel matrices were obtained from TMOS and a trimethoxysilane or dimethoxysilane derivative. The mass ratio of protein used for immobilization was 9.7 mg per mmol silanes. Trimethylsiloxane derivatives carrying a methyl (MeTMOS), propyl (PrTMOS), *i*-butyl (*i*-BuTMOS), phenyl (PhTMOS), vinyl (VTMOS), 3-aminopropyl (APrTMOS), or octyl (OcTMOS) functional group have been used, as well as dimethoxysilane derivatives with dimethyl (DMDMOS) or methylphenyl (MPDMOS) groups. The influence of these alkyl or aryl groups on the immobilization efficiency and catalytic properties of sol–gel immobilized Alcalase was investigated in both hydrolysis and synthesis reactions. Such an assessment of the catalytic efficiency is important for comprehensive characterization of the immobilized enzyme in different environments, compared with the native enzyme. Enzymes like proteases usually perform well in aqueous media, but their insolubility in organic solvents can result in the aggregation of the enzyme molecules and decrease

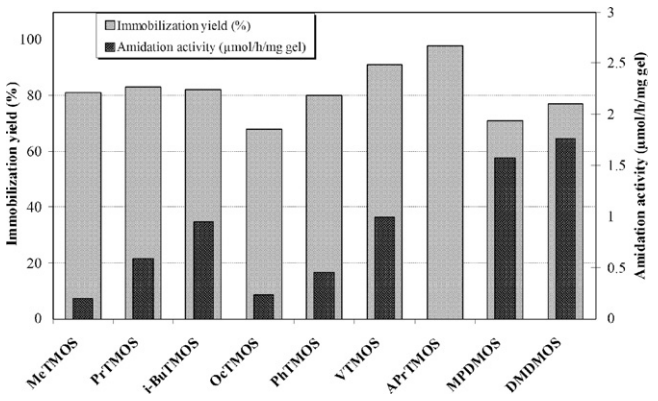

Fig. 3. Effect of alkyl and aryl groups in organic silanes (see Section 2 for explanation of abbreviations) mixed with tetramethoxysilane (TMOS) on the initial activity of hybrid gel-entrapped protease, in the amidation reaction of Z-Ala-Phe-OMe at 30 °C. Molar ratio of organic silane to TMOS was 1:1.

of catalytic activity. Immobilization might resolve this problem, since in the immobilized biocatalyst the enzyme is located in a solid insoluble phase as well, but aggregation is prevented.

The hydrolytic activity of the immobilized Alcalase was assayed using Z-Ala-Phe-OBu^t, a dipeptide ester, as substrate. The hydrolysis yields and activities, at 2 h incubation time and 40 °C, are listed in Table 1 for Alcalase encapsulated in different sol–gels, compared to the soluble enzyme values. Using silane precursors with different non-polar functional groups, did not result in significant differences concerning the yields and hydrolytic activity values, excepting the biocatalyst prepared with APrTMOS and TMOS, which revealed low hydrolytic activity. Maximum retention of specific activity related to the soluble enzyme, 36.2%, was obtained with VTMOS and TMOS as silane precursors. Previously, Sangeetha et al. [8], reported similar results for the hydrolysis of casein, demonstrating that subtilisin encapsulated in sol–gel matrices derived from VTMOS was a better precursor for sol–gel encapsulation of subtilisin than methyltrimethoxysilane and tetraethoxysilane. Although the measured specific activity was lower for the sol–gel encapsulated than for the soluble Alcalase, the important advantages of immobilized biocatalysts, like reusability, can overcome this activity loss. The measured activity values are influenced by a combination of factors, including protein accessibility, the fraction of active protein, mass transfer of the substrate to the silica matrix, diffusion of substrate and product within the matrix. The activity decrease can be explained by lower diffusion rate of materials in and out of the silica matrix, resulting in activity values that do not directly reflect the protein behaviour.

3.3. Effect of organic silane precursor nature on amidation activity of hybrid sol–gel entrapped protease

The amidation of Z-Ala-Phe-OMe has been carried out in a mixture of hydrophilic organic solvents, ^{t}BuOH/DMF (82.5:17.5, v/v) containing 0.11% water (determined by Karl–Fischer titration). As it was shown in Fig. 1, this substrate can be converted by protease into an amide or in a hydrolysis product (Z-Ala-Phe-OH). To reduce the amount of hydrolysis, the water content of the system must be kept at the lowest possible value. Obviously, the water contained in the solvent system and the immobilized preparations led to some extent of the hydrolysis side reaction. The amidation/hydrolysis (A/H) ratio was considered the best determinant of peptide amide synthesis efficiency.

Fig. 3 shows the immobilization yields and initial amidation rates (calculated at 2 h reaction time) of the hybrid sol–gel entrapped protease preparations obtained from various organic silanes and TMOS, at 1:1 molar ratio. The hybrid gel-entrapped protease derived from APrTMOS, that showed the highest percentage of immobilized protein, was without activity in the amidation of Z-Ala-Phe-OMe, probably due to the closed gel structure of the inorganic silica. Increasing the chain length of the alkyl group, the activity increased and reached a maximum with *i*-BuTMOS. In general, propyl or butyl groups were found to exert the most favorable hydrophobic interactions with the active site of enzymes such as lipase and protease, in organic solvents [33]. Alkyltrimethoxysilanes with large alkyl groups (i.e. OcTMOS) proved to be detrimental for the immobilization process, resulting in lower entrapment yield, probable by release of protein from the hydrogel. Better performance was achieved by increasing the number of methyl groups. Maximum activity (1.76 μmol/h/mg gel) was obtained with derivatives resulting from the encapsulation of protease in gels prepared with DMDMOS as precursor. However, the specific activity of this best performing immobilized enzyme, 10.2 μmol/h/mg protein, represented only 30.2% of the native enzyme activity (33.8 μmol/h/mg protein, not shown in Fig. 3). This activity decrease can be explained by the mass transfer limitation of substrate and/or product through the sol–gel matrix.

For possible industrial application, the reaction yield is usually more important than the initial activity. In this reason, the reactions were monitored over time and the yields of the two competing reactions, amidation and hydrolysis, as well as the amidation/hydrolysis ratio (A/H), are listed in Table 2. Among the silane precursors used, DMDMOS and TMOS were the most suitable for Z-Ala-Phe-NH_2 synthesis, giving the highest A/H ratio (13.3), and amide product yield (93%). The catalytic efficiency of the immobilized Alcalase was higher in sol–gel matrices with higher density of hydrophobic methyl groups, but was lowered by increasing chain length of the alkyl group. Some hydrophobic amino acid residues exist in the vicinity of an active site of protease, and interact with the hydrophobic/hydrophilic environment around the enzyme. Such residues are closely associated with the conformation of active protease sites, leading to an important influence of hydrophobic interactions on the active conformation of protease [34]. Using soluble Alcalase, the achieved substrate conversion was total, but the higher water content of the commercial aqueous enzyme preparation led to a lower A/H ratio, 8.6.

It must be noticed that the nature of the sol–gel matrix had a different influence in the hydrolysis of Z-Ala-Phe-OBu^t and amidation of Z-Ala-Phe-OMe. Excepting the preparation derived from APrTMOS and TMOS, all sol–gel immobilized preparations were efficient biocatalysts for the hydrolysis of Z-Ala-Phe-OBu^t, unlike the amidation reaction, where the nature of silane precursors had an important influence on enzyme activity and amide yields.

3.4. Reproducibility of the immobilization method

The reproducibility of the immobilization technique was investigated by evaluating the catalytic efficiency and the enzyme loading of three enzymatic preparations obtained with different silane precursors (DMDMOS, MPDMOS, VTMOS), which were repeated five times under the same conditions. These silanes showed previously the highest activity for the amidation reaction with a good percentage of protein immobilization. The results are presented in Table 3. Maximum catalytic efficiency was obtained with preparations obtained by the encapsulation of Alcalase in gels from DMDMOS and TMOS precursors. Using these preparations, total conversion of the substrate peptide ester was achieved after 21 h, and the product contained about 91.5% of the desired amide, Z-Ala-Phe-NH_2. Using DMDMOS as precursor, the percentage of peptide amide in the final product, as well as the specific activity in the initial stage of the reaction, showed excellent reproducibility for the five batches, with standard deviations of 0.38% and 1.7%, respec-

Table 1
Influence of silane precursors on the catalytic efficiency of Z-Ala-Phe-OBu^t hydrolysis catalyzed by sol–gel immobilized Alcalase after 2 h of incubation. Reaction conditions: 10 mM Z-Ala-Phe-OBu^t, 0.1 M phosphate buffer pH 7.0, 50% DMF (%, vol), 40 °C.

Silane precursors (1:1 molar ratio)	Hydrolysis yield (%)	Hydrolytic activity (μmol/h/g gel)	Entrapped protein (mg/g gel)	Specific activity (μmol/h/mg protein)	Activity retention[a] (%)
Free Alcalase	49.7	1096[b]	–	27.4	–
PrTMOS:TMOS	36.9	987	131	7.49	27.3
OcTMOS:TMOS	39.2	1133	123	9.29	33.9
PhTMOS:TMOS	38.3	1072	111	9.56	34.9
VTMOS:TMOS	38.3	1090	110	9.91	36.2
i-BuTMOS:TMOS	38.2	1098	163	6.78	24.7
APrTMOS:TMOS	9.8	287	83	3.49	12.7
MPDMOS:TMOS	39.2	1124	132	8.53	31.1
DMDMOS:TMOS	41.3	1147	142	8.11	29.6

[a] The activity retention (%) of immobilized enzyme was determined as the ratio of specific activity of the immobilized enzyme to the specific activity of the free enzyme in the same reaction.
[b] Activity expressed in μmol/h/ml.

Table 2
Influence of silane precursors on the yield and efficiency of Z-Ala-Phe-OMe amidation catalyzed by sol–gel immobilized Alcalase after 21 h of incubation. Reaction conditions: 10 mM Z-Ala-Phe-OMe, ^{t}BuOH/DMF (82.5:17.5, v/v), 30 °C.

Silane precursors (1:1 molar ratio)	Yield (%)		A/H ratio
	Amidation	Hydrolysis	
Free Alcalase	89.6	10.4	8.6
MeTMOS:TMOS	31.2	3.6	8.7
PrTMOS:TMOS	85.1	9.6	8.9
OcTMOS:TMOS	58.1	7.5	7.8
PhTMOS:TMOS	75.4	9.4	8.0
VTMOS:TMOS	81.2	9.3	8.7
i-BuTMOS:TMOS	83.2	9.0	9.2
APrTMOS:TMOS	3.3	0.0	–
MPDMOS:TMOS	88.4	8.2	10.8
DMDMOS:TMOS	93.0	7.0	13.3

Table 3
Influence of silane precursors on the reproducibility of entrapment yield, catalytic efficiency and specific activity in the ammonolysis of Z-Ala-Phe-OMe. Reaction conditions were: 10 mM Z-Ala-Phe-OMe, 100 mM ammonium carbamate, solvent mixture tBuOH/DMF (82.5:17.5, v/v), reaction time 21 h.

Silane II/TMOS s	Specific activity ± SD (μmol/h/mg protein)	Immobilization yield ± SD (%)	Composition of the reaction product		
			DP-NH_2 ± SD (%)	DP-OH ± SD (%)	DP-OMe ± SD (%)
VTMOS	2.5 ± 1.2	84 ± 1.9	21.4 ± 12.4	6.7 ± 1.12	71.9 ± 13.4
MPDMOS	13.7 ± 8.9	75 ± 3.9	88.5 ± 3.2	8.6 ± 0.98	2.9 ± 2.7
DMDMOS	10.5 ± 1.7	80 ± 2.7	91.5 ± 0.38	8.5 ± 0.38	0.0 ± 0.0

SD – standard deviation

tively. Although the maximum entrapment efficiency of 84% was obtained with vinyltrimethoxysilane (VTMOS), the activity and the product yield values were lower compared to the previous study, which indicate a bad reproducibility of this silane monomer, also emphasized by the high values of standard deviation. This can be caused by unsaturated bonds available for additional cross-linking.

3.5. *Effect of enzyme loading on catalytic efficiency of sol–gel immobilized Alcalase*

We investigated the efficiency of immobilization for a range of initial protein concentrations, to determine the immobilization capacity of the biosilica matrix. Experiments were performed to determine the lowest enzyme concentration that can be used for immobilization to obtain high catalytic activity of the biocatalysts. Initial protein mass ratio between 2.8 and 9.7 mg per mmol silanes was used in the immobilization experiments. Determination of the protein content in the water washing solutions revealed that between 82 and 92% of the enzyme had been immobilized and the degree of immobilization was almost independent of the amount of Alcalase used. Subsequently, the enzyme loading in the sol–gel was calculated from the actual amount of immobilized enzyme and the weight of dried sol–gel preparation. Fig. 4 shows that the optimum capacity for enzyme loading in the silica matrix was 115 mg of protein per g of dry xerogel. Protein overloading above this value resulted in activity decrease. Therefore, in subsequent studies, a protein loading of 115 mg protein per g xerogel was used.

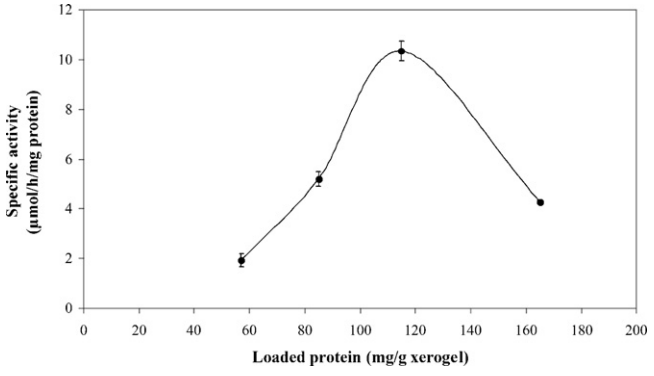

Fig. 4. Effect of loaded protein on the sol–gel immobilized Alcalase activity.

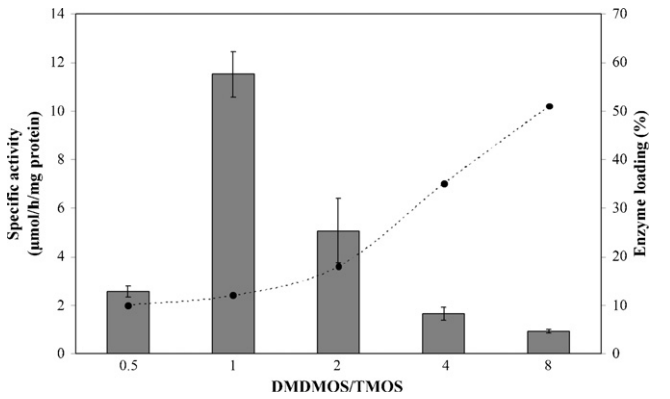

Fig. 5. Effect of the molar ratio of DMDMOS to TMOS on the enzyme loading (●) and specific activity (gray bars) for the amidation of Z-Ala-Phe-OMe.

3.6. Influence of DMDMOS/TMOS molar ratio on the encapsulated protease activity

To obtain biocatalysts with high performances, it is important to determine the optimum molar ratio within the best silane precursor's mixture. The relative molar ratio of DMDMOS was varied between 0.5 and 8. Increasing the ratio of DMDMOS to TMOS above an optimal value resulted in more difficult gel formation and lower activity of the immobilized enzyme (Fig. 5). The amount of the dry xerogel was lower, as well as the percentage of immobilized protein of the preparations. This might be explained by the increased difficulty in hydrolyzing and condensing silanes at higher concentration of hydrophobic groups. The texture of the forming silica gel is influenced by the relative rates of hydrolysis and condensation reactions, as it was stated by Pierre [35]. Maximum activity of 11.5 μmol/h/mg protein for the amidation of Z-Ala-Phe-OMe was obtained at 1:1 molar ratio of silane precursors. This optimal ratio was used in the subsequent experiments.

3.7. Reaction kinetics of immobilized and native enzyme

To ensure a proper comparison of native and immobilized Alcalase, the kinetic and the subsequent stability studies were performed with biocatalyst amounts providing the same enzyme activity units. The kinetic parameters for the ammonolysis activity of the native and immobilized protease were assayed at substrate concentrations from 5 to 350 mM. The entrapment of protease within silica matrices increased the K_M value from 55 to 117 mM (Table 4), indicating a decreased affinity of enzyme for its substrate, probably caused by an increase in mass transfer limitation for substrate through silica matrix, as it was also observed by Madadlou et al. [36].

At the same time, the maximum velocity increased 4.4-fold following immobilization. The overall catalytic efficiency of the enzyme-catalyzed reaction, expressed by the k_{cat}/K_M ratio, was two times higher for the immobilized enzyme, validating the effectiveness of the immobilization process.

Table 4
Kinetic parameters for soluble and immobilized Alcalase.

Enzyme	Kinetic parameters		
	K_M[a] (mM)	V_{max}[b] (μmol min^{-1})	k_{cat}/K_M[c] (mM^{-1} min^{-1})
Native Alcalase	55	0.28	2.0×10^{-3}
Immobilized Alcalase	117	1.24	4.1×10^{-3}

[a] K_M: Michaelis–Menten constant.
[b] V_{max}: maximum velocity of enzymatically catalyzed reaction.
[c] k_{cat}/K_M: catalytic efficiency of enzyme.

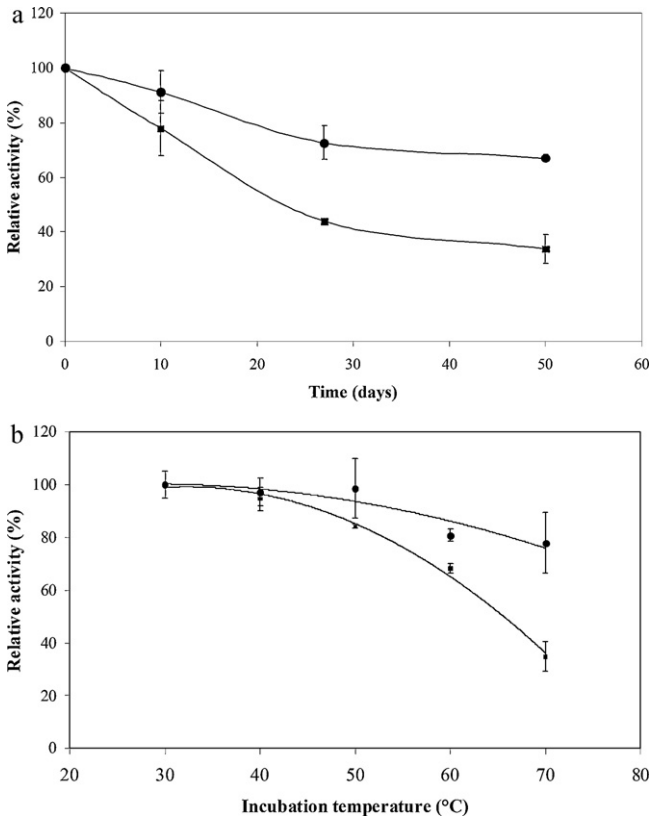

Fig. 6. Stability of encapsulated (-●-) and free (-■-) protease from *Bacillus licheniformis* at ambient temperature (a) and incubated at different temperatures for 10 min (b).

3.8. Enzyme stability studies

Stability is one of the most important issues regarding a broad implementation of enzymatic catalysis in industry. In our study, both storage stability at ambient temperature and thermal stability at temperatures up to 70 °C have been investigated. As shown in Fig. 6a, the sol–gel immobilized Alcalase retained about 75% of its original activity over a period of 50 days. Under the same storage conditions, the native enzyme retained only about 35% of its initial activity.

The thermostability of free and encapsulated enzymes was investigated to find out whether the silica could protect the encapsulated enzyme molecules from thermal denaturation. The activity of free protease decreased much faster than of the immobilized one, with increasing incubation temperature. The native enzyme lost about 65% of its initial activity when incubated at 70 °C, indicating a profound sensitivity to heat. Under the same conditions, the encapsulated enzyme retained 78% of its initial activity (Fig. 6b). The improved thermostability of encapsulated protease is most probably due to multipoint attachments of enzyme molecules to the support that prevent them from conformational changes upon heating. Bruins et al. showed a number of advantages provided by the enhanced thermostability of immobilized proteases, such as improved substrate solubility, higher mass transfer rates and reduced microbial risk [37]. In the opinion of other authors [38,39], the increased stability of immobilized proteases over their soluble counterparts may be due to the prevention of autolysis and thermal denaturation. These results demonstrate that sol–gel immobilized Alcalase possesses improved stability compared to the

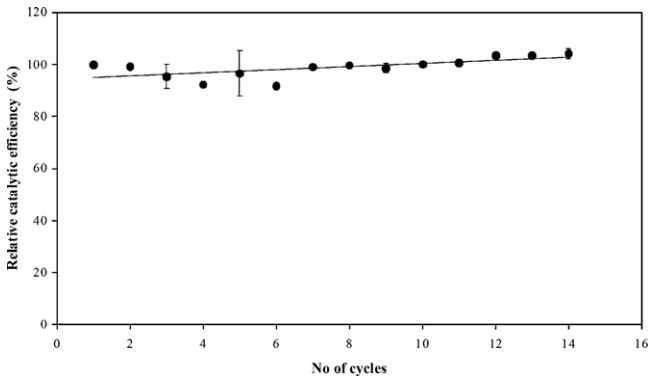

Fig. 7. Reuse of sol–gel entrapped Alcalase as catalyst in the amidation of Z-Ala-Phe-OMe. Biotransformation batch runs (22 h) were performed at 30 °C in ^{t}BuOH/DMF (82.5:17.5, v/v).

native enzyme and may be an attractive biocatalyst for industrial applications.

Reusability is another crucial feature in practical applications of biocatalysts. Inactivation and enzyme leaching are the most prominent drawbacks for large-scale use of immobilized enzymes. We investigated the repeated use of sol–gel immobilized Alcalase preparations in several batch ammonolysis runs at 30 °C (Fig. 7). No decrease in catalytic efficiency of protease after 22 h biotransformation was observed, even after 14 cycles of repeated use. The experimental data gathered also highlight mechanical stability of the immobilization matrix with prolonged exposure to organic solvent. The reusability of immobilized protease is essential for cost-effective use of the enzyme either in repeated batch or in continuous processes [10].

4. Conclusions

The obtained results highlight the suitability of sol–gel immobilized Alcalase to serve as a biocatalyst in the modification of peptides. Commercial Alcalase was successfully entrapped in dimethyldimethoxysilane (DMDMOS) and tetramethoxysilane (TMOS) yielding sol–gel preparations with high degree of immobilization, high reproducibility and high efficiency in the synthesis of Z-Ala-Phe-NH_2. The encapsulated protease had good storage stability at ambient temperature and against heat treatment up to 70 °C, making it an ideal choice for peptide synthesis. The immobilized biocatalyst was successfully reused in repeated batch biotransformation runs with almost no decrease in the final product yield, showing a good operational and mechanical stability. Therefore, the sol–gel immobilized Alcalase could be a promising candidate for the development of a highly effective set-up for the production of biologically active peptides.

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