Utilization of Immobilized Trypsin on Commercial Silica Gel as Catalyst

G. Hodaifa[a,*] and M. D. Romero[b]

[a]Molecular Biology and Biochemical Engineering Department, Pablo de Olavide University, 41013 Seville, Spain

[b]Chemical Engineering Department, Complutense University of Madrid, 28040 Madrid, Spain

Original scientific paper
Received: September 25, 2011
Accepted: February 27, 2012

In this study, trypsin was immobilized onto commercial silica gel and the transesterification of N-acetyl-L-tyrosine ethyl ester (ATEE) and 1-propanol in an organic solvent was used as a model reaction. The amount of trypsin adsorbed onto the support affected the time required to achieve full conversion. The concentration of immobilized trypsin that provided a maximum yield of product in a minimal amount of time was 43 μmol of trypsin/g silica gel. At the end of transesterification reactions, the turnover frequency ($9.3 \cdot 10^{-5}$ to $6.8 \cdot 10^{-3}$ s^{-1}) and turnover number (8 to 520) of transesterification markedly increased when the initial concentration of the substrate had increased from 0.8 to 100 mmol L^{-1}. The kinetic parameters K_m (64.8 mmol L^{-1} $ATEE_{initial}$) and V_{max} (0.364 mmol L^{-1} ethanol min^{-1}) were determined. The immobilized trypsin maintained enzymatic activity and reusability after one month of storage at 4 °C.

Key words:
Transesterification, trypsin, enzyme immobilization, silica-gel

Introduction

The most valuable property of enzymes is their selectivity in reaction catalysis. Enzymatic reactions in organic solvents provide numerous industrially attractive advantages, such as increased solubility of non-polar substrates, reversal of the thermodynamic equilibrium of hydrolysis reactions, suppression of water-dependent side reactions, alternation of substrate specificity and enantioselectivity, and elimination of microbial contamination.[1–3]

In general, hydrophilic solvents are preferred because the enzyme activity is retained in these solvents; however, polar substrates are often used in many esterification reactions. The solvent influences the activity and/or stability of the enzymes, as well as the equilibrium of the reaction.[4]

Proteases, such as trypsin, are widely used in industrial and biomedical applications;[5] however, the application of native enzymes is limited in industrial processes due to enzyme instability and the rapid loss of biocatalytic activity during operation and storage (autolysis effect). Furthermore, it is difficult to remove the enzyme from solution, which may lead to a contaminated product.[6–8]

Immobilization should allow the enzyme to maintain its catalytic activity and diminish other processes that are detrimental to the enzyme, such as autolysis.[9] Many researchers have shown that immobilized enzymes possess many advantages over native enzymes, including improved stability and ease of removal.[10,11] Among the various immobilization methods, physical adsorption is the most widely used.

The enzymatic reactions of proteases have been employed in various industrial processes.[12,13] Trypsin is commonly used in the hydrolysis of casinomacropeptide,[14] wherein the hydrolytic product (peptides) is of immense importance in the food industry and is recognised as a functional food. The design and development of processes for the continuous production of peptides on a large scale at a competitive cost are highly desirable. Immobilized hydrolytic enzymes, such as trypsin, are also important in the manufacturing of hypoallergenic baby food,[13] the semi-synthesis of human insulin,[15] the resolution of enantiomeric *O*- or *N, O*-derivatised amino acids,[16] and in esterification and transesterification reactions.[4,17]

Peptide synthesis that are catalyzed by proteases have been extensively investigated as an alternative to the chemical synthesis of polypeptides. In general, protease-based peptide synthesis does not require special activation of the carboxyl group, is highly specific, and does not require a protecting group.[18] In comparison with peptide synthesis, ester formation and transesterification with proteases have been less extensively investigated.

The objective of the present study was to determine the efficiency of immobilized trypsin on com-

*Corresponding author. Tel.: +34 978 206 546; fax: +34 954 349 813; E-mail address: ghodaifa@upo.es (G. Hodaifa).

mercial silica gel as a catalyst. To that end, the transesterification reaction of N-acetyl-L-tyrosine ethyl ester monohydrate was used as a model reaction. This reaction was conducted using different concentrations of substrate and trypsin, and the production of ethanol and N-acetyl-L-tyrosine propyl ester was monitored over time.

Materials and methods

Reagent and materials

Porcine pancreatic trypsin (T4799, lyophilised powder, 1000–2000 Na-benzoyl-L-arginine ethyl ester solution (BAEE) units per mg of solid, herein after referred to as impure trypsin), N-acetyl-L-tyrosine ethyl ester monohydrate, buffer phosphate solution ($NaH_2PO_4 \cdot 2H_2O$ and $Na_2HPO_4 \cdot 12H_2O$), commercial silica gel (pore size diameter = 15 nm and 75- to 175-μm particle size), and 1-propanol (HPLC grade) were obtained from Sigma-Aldrich, Logistik GmbH, and Kappelweg, respectively (1, D-91625 Schnelldorf, Germany).

Mercury porosimetry

Mercury porosimetry (Thermo Electron Corporation, Pascal 440 and 140 Series) was conducted to determine the porosity of the silica gels.[19]

Experimental device

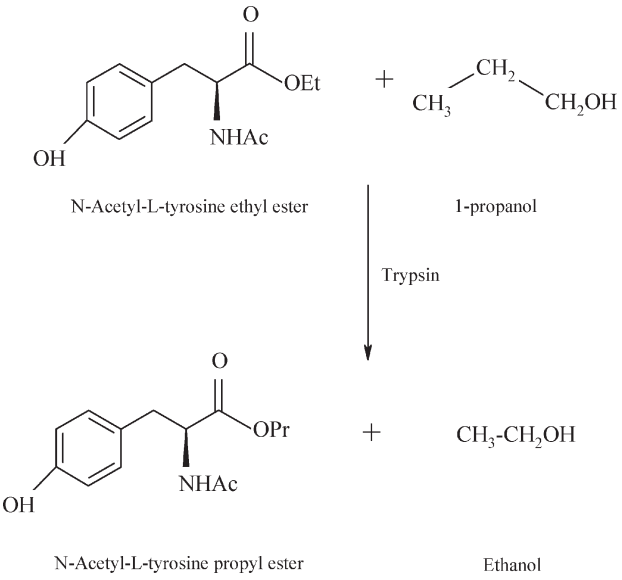
All the experiments were made in a batch reactor that was agitated with Heidolph type RZR-1 motor. The working volume and the overall volume of the reactor were 0.125 dm^3 and 0.210 dm^3, respectively.[19]

Trypsin adsorption and transesterification reactions

Enzyme adsorption was achieved by stirring the enzyme (0–30 mg cm^{-3}) and a suspension of silica gel (final concentration of 10 mg cm^{-3}) in a 0.125 dm^3 reactor at 25 °C and at a pH of 6.5 (maintained with a 25 mmol L^{-1} phosphate buffer solution). The amount of adsorbed trypsin was obtained by calculating the difference in trypsin concentration before and after adsorption, wherein the trypsin concentration was spectrophotometrically evaluated at 280 nm. The concentration of trypsin after enzyme immobilization was determined by centrifugation and evaluation of the supernatant. After centrifugation, the solid phase was washed twice with a phosphate buffer solution (pH = 6.5) for 5 minutes to eliminate free trypsin. The enzyme desorbed during these washing steps was determined, and the enzyme adsorbed was corrected considering the enzyme desorption.

Desorption experiments were realized by washing immobilized trypsin with a 25 mmol L^{-1} phosphate buffer solution (pH = 6.5) for 120 min.

The trypsin-catalyzed transesterification of N-acetyl-L-tyrosine ethyl ester monohydrate (ATEE) and 1-propanol to N-acetyl-L-tyrosine propyl ester and ethanol was monitored, as in previous work, to determine the catalytic activity of the enzyme (Scheme 1).[17]



Scheme 1 – *Transesterification of N-acetyl-L-tyrosine ethyl ester monohydrate and 1-propanol catalyzed by immobilized trypsin on commercial silica gel*

The reaction was conducted in a 0.125 dm^3 reactor at 25 °C, wherein a suspension of immobilized enzyme (solid phase) was combined with 1-propanol, ATEE (10 mmol L^{-1}) and φ = 5 % H_2O (or buffer solution (25 mmol L^{-1}) phosphate solution, pH = 6.5). The ethanol concentration was monitored by gas chromatography to determine the conversion of transesterification.

Transesterification was conducted using various amounts of immobilized trypsin (4.7–61.4 μmol trypsin adsorbed/g silica gel) at a constant ATEE concentration of 10 mmol L^{-1}. Furthermore, different concentrations of ATEE (0.8–100 mmol L^{-1}) were evaluated at a constant trypsin concentration (4.7 μmol trypsin adsorbed/g silica gel).

Trypsin activity assay

Using gas chromatography, the catalytic activities of free and immobilised trypsin were evaluated by measuring the amount of ethanol liberated during the transesterification (Varian 3400 chromatograph).

Specific activities were expressed as the turnover frequency (TOF = moles of ethanol/moles of trypsin per second), whereas the catalytic efficiency was expressed as the turnover number (TON = moles of ethanol/moles of trypsin).

Reusability and storage stability

Transesterification was performed with two samples containing different concentrations of adsorbed trypsin (4.7 and 61.4 μmol of trypsin/g of silica gel) and different volumes of reaction media (10 and 125 cm^3). Both samples were reused twice after several washes with 1-propanol. The storage stability of the second sample (61.4 μmol of trypsin/g of silica gel) after transesterification was evaluated at 4 °C. The enzyme-adsorbed support was separated by centrifugation and stored for four weeks. The reaction was conducted with 61.4 μmol of adsorbed trypsin/g of silica gel and 10 mmol L^{-1} of ATEE in a φ = 5 % of 25 mmol L^{-1} phosphate solution (pH = 6.5), 1-propanol at 25 °C for 120 min.

Gas chromatography method

The ethanol concentration was determined by gas chromatography (Varian 3400 chromatograph) with a sample of 0.4 μL injected, using a flame-ionisation detector and Megabore column DB-WAX (30 m × 0.55 mm). The operating conditions for the determination of ethanol were:

– Column temperature: initially 55 °C for 1 min, 55 °C min^{-1} up to 150 °C (5 °C min^{-1}).

– Detector (FID): temperature: 250 °C.

– Injector temperature: 200 °C.

– Helium carrier flow was 2 cm^3 min^{-1}.

The ethanol yields were determined using a calibration line between peak areas versus ethanol yields for known standards.

Calculation methods and reproducibility

All immobilization experiments and analytical methods were made at least in duplicate. Calculations and statistical analyses were conducted with OriginPro software, version 8.0.

Results and discussion

Immobilization of trypsin

Trypsin was immobilized onto commercial silica gel that had been obtained from Sigma-Aldrich. This specific silica gel with the aforementioned characteristics (SG-15) was selected based on the results of our previous study.[19]

Fig. 1 depicts the amount of trypsin that became immobilized on the silica gel as a function of time. Equilibrium was achieved after 60 min in all the experiments. Furthermore, over 80 % of the maximum amount of immobilized trypsin was incorporated into the pores of the silica gel within the first two minutes, indicating that this process is facile. Initially, the pores on the surface of the silica gel were vacant, which resulted in rapid immobilization and a large concentration gradient between the support and the solution, allowing the enzyme greater access to the pores. Over time, the amount of accessible surface had decreased, which limited the diffusion of trypsin into the silica gel. Thus, after 2 minutes of adsorption, the ability of the silica gel to immobilize the enzyme had decreased (Fig. 1).

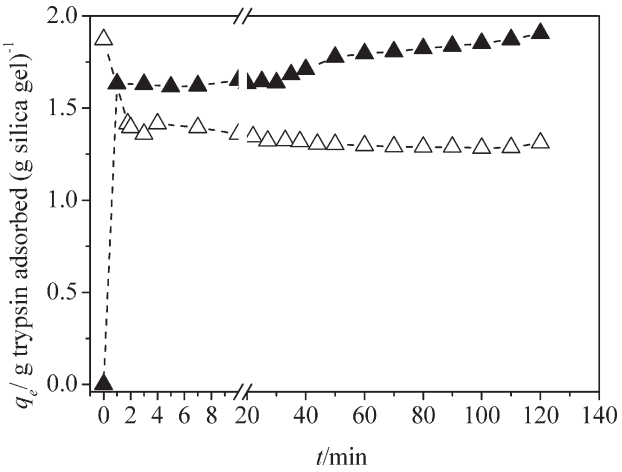


Fig. 1 – *Amount of trypsin adsorbed onto commercial silica gel as a function of time (▲). The concentration of immobilized trypsin after one washing step (Δ). Experimental conditions: phosphate buffer solution (pH = 6.5), 25 °C; initial concentration of silica gel (15-nm pore diameter and 75 to 150-μm particle size) = 10 mg* cm^{-3}*; initial concentration of trypsin = 34 mg* cm^{-3}.

In the desorption experiment, the amount of trypsin removed from the support was monitored over time (Fig. 1). In the first two minutes, the amount of adsorbed trypsin had decreased by 0.4 g g^{-1}. Over time, the amount of trypsin removed from the support remained almost constant, reaching a final concentration of 0.43 g g^{-1}. Therefore, a 5 min. wash removed the majority of the nonimmobilized trypsin. Moreover, the trypsin that was not firmly immobilized in the pores of the support was also removed; hence, any trypsin that had been deposited onto the outer surface of the support was removed by the washing step.

Moreover, in subsequent washes, the presence of desorbed enzyme was not detected in the supernatant, which confirmed that the trypsin was

trapped on the silica gel and it was not likely that an equilibrium existed between attached enzyme and free enzyme.

The amount of trypsin that had become immobilized onto the support prior to the washing step represents the apparent immobilised trypsin concentration. The apparent immobilized trypsin concentration was 79.3 μmol/g silica gel, which decreased to 61.4 μmol/g silica gel after 120 minutes of washing. These results indicate that the addition of a washing step reduced the concentration of trypsin by approximately 30 %. Thus, to achieve a proper assessment of enzymatic activity, it was necessary to wash away the non-adsorbed trypsin before the transesterification reaction. In general, approximately a five-minute wash removed 93 % of the free enzyme.

Characterisation of commercial silica gel before and after the adsorption of trypsin

Table 1 summarises the physical properties of the commercial silica gel before and after the adsorption of trypsin (determined by mercury porosimetry). The pore size distribution of the silica gel prior to trypsin adsorption could be divided into two ranges. More specifically, the pore sizes that ranged from 4 to 200 nm with a specific volume of 1.0 $cm^3\ g^{-1}$ corresponded to 62 % of the total specific volume, whereas pores larger than 200 nm with a specific volume of 1.7 $cm^3\ g^{-1}$ corresponded to 38 % of the total specific volume.

As can be observed in Table 1, the immobilization of trypsin altered the pore size distribution of the silica gel and resulted in a lower total specific surface area and total volume. The specific cumulative volume of the pores before and after immobilization ($[Trypsin]_{initial}$ = 34 mg cm^{-3}) is depicted in Fig. 2. Therein, the results indicate that the majority of the pores within the silica gel possessed an average diameter of 8.5 nm (Fig. 2) prior to trypsin immobilization, which decreased to 6.5 nm after trypsin adsorption. Thus, changes in the specific

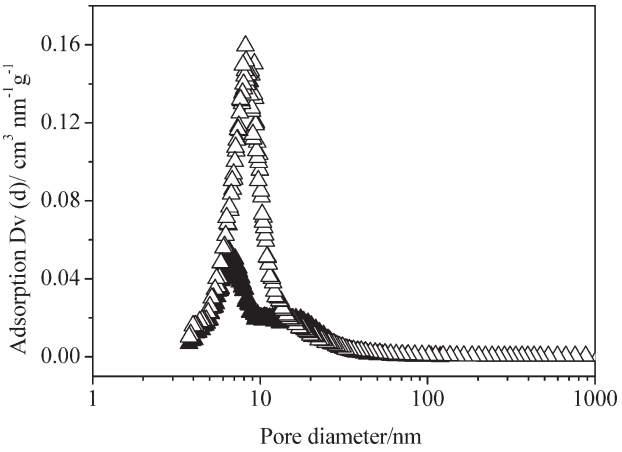


Fig. 2 – *The pore size distribution obtained by mercury porosimetry before and after trypsin immobilization: commercial silica gel before (Δ) and after trypsin immobilization (▲). Immobilization conditions: 10 mg cm^{-3} of silica gel, 34 mg cm^{-3} of trypsin (42 μmol of trypsin g^{-1}) in a φ = 5 % phosphate buffer solution (pH = 6.5) for 120 min at 25 °C.*

volume of the pores before and after immobilization were a result of enzyme adsorption.

In general, all types of pore participated in the adsorption process. Pore distribution curves indicate that immobilization predominantly occurred in pores of less than 30 nm in diameter. Furthermore, the results reveal that the pore size decreased after the adsorption of trypsin.[20] For pore sizes of less than 200 nm in diameter, the cumulative specific volume of these pores decreased from 1.0 to 0.54 $cm^3\ g^{-1}$ after immobilization. Moreover, a decrease in the specific volume of pores with diameters of less than 30 nm (Fig. 2) confirmed that immobilization primarily occurred in pores with diameters of less than 30 nm.

Transesterification of N-acetyl-L-tyrosine ethyl ester monohydrate (ATEE)

In order to study the catalytic capacity of immobilized trypsin onto commercial silica gel, the transesterification of N-acetyl-L-tyrosine ethyl ester

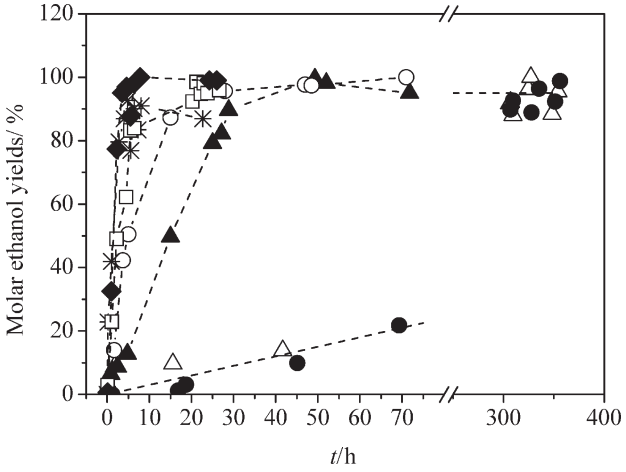
Table 1 – *Physical properties of the commercial silica gel used in the transesterification reactions. Commercial silica gel (pore diameter = 15 nm and particle size = 75–150 μm) before and after trypsin immobilization (q_e = 42.9 μmol trypsin g^{-1}).*

Silica gel	Total specific surface area ($m^2\ g^{-1}$)	Total specific cumulative volume ($cm^3\ g^{-1}$)	Pore diameter ranges (nm)	Specific volume ($cm^3\ g^{-1}$)	Specific volume (%)
Before immobilization*	383	2.7	4–200	1.0	62
			>200	1.7	38
After immobilization	185	1.3	4–200	0.54	43
			>200	0.72	57

*The silica gel was characterised after 25 h of agitation in 1-propanol and φ = 5 % phosphate buffer solution (pH = 6.5) at 25°C.

monohydrate (ATEE) and 1-propanol was conducted in the presence of trypsin to obtain N-acetyl-L-tyrosine propyl ester and ethanol.

In order to compare the catalytic capacity of free and immobilized trypsin, transesterification reactions were catalyzed by 4.7 μmol of free and immobilized trypsin. After a reaction time of approximately 244 h (Fig. 3), the results of both reactions were similar, and conversion values in excess of 92 % were obtained using both forms of trypsin. Thus, immobilized trypsin was confirmed to retain its catalytic activity after being immobilized onto silica gel. On the other hand, ethanol was not detected in the reactions performed with no enzyme.



F i g . 3 – *Molar yields of ethanol at different concentrations of immobilized trypsin as a function of time. (●) 4.7, (▲) 7.3, (○) 14.6, (*) 26.8, (□) 42.9, (◆) 61.4 μmol trypsin immobilized/g silica gel, and (Δ) 4.7 μmol free trypsin. Experimental conditions: 10 mmol L^{-1} ATEE, φ = 5 % phosphate buffer solution (pH = 6.5), 1-propanol, 25 °C.*

Variations in the catalytic activity and catalytic efficiency of immobilized trypsin were monitored over the course of the reaction by determining the turnover frequency (TOF) and the turnover number (TON), eqs. (1) and (2) respectively:

$$\text{TOF} = \frac{\mu\text{mol Product}}{\mu\text{mol Enzyme} \cdot \text{s}} \quad (1)$$

$$\text{TON} = \frac{\mu\text{mol Product}}{\mu\text{mol Enzyme}} \quad (2)$$

TON and TOF results they will be discussed later.

The effect of immobilized trypsin concentration

Fig. 3 depicts the yield of ethanol over time (ATEE = 10 mmol L^{-1}) for different concentrations of immobilized trypsin (4.7–61.4 μmol trypsin/g silica gel). In all of the experiments, an ethanol yield of approximately 100 % was obtained. The rate of ethanol production was directly related to the concentration of immobilized enzyme, wherein a higher concentration of immobilized enzyme resulted in shorter reaction times.

Table 2 presents a ratio of trypsin/silica gel (φ/%) at different concentrations of immobilized trypsin (ATEE = 10 mmol L^{-1}). At trypsin concentrations less than 15 μmol g^{-1}, the trypsin/silica gel ratio was less than 1, indicating that the silica gel pores were partially occupied by trypsin; however, at concentrations greater than 26 μmol g^{-1} of trypsin, the trypsin/silica gel ratio was greater than 1, indicating that the pores were fully occupied by trypsin. At higher concentrations, trypsin may begin to deposit on the outer surface of the silica gel (in the macropores). Thus, at immobilized enzyme concentrations in excess of 26 μmol g^{-1}, the saturation of silica gel resulted in a constant reaction time.

T a b l e 2 – *The reaction time and occupied volume of pores at different concentrations of adsorbed trypsin*

Concentration of adsorbed trypsin (μmol trypsin/g silica gel)	Trypsin/silica gel volume ratio* (φ)
4.7	0.27
7.3	0.42
14.6	0.84
26.8	1.54
42.9	1.42
61.4	3.54

*In order to calculate the volume of trypsin, the molecule diameter was assumed to be equal to 3.8 nm. Occupied pores in the support corresponded to the specific volume of pores with diameters of less than 200 nm. Transesterification was considered to be complete when the molar yield of ethanol was approximately 100 % ([μmol L^{-1} ethanol] · 100/[μmol L^{-1} ATEE]). Transesterification conditions: [ATEE] = 10 mmol L^{-1}, φ = 5 % phosphate buffer solution (pH = 6.5), 25 °C.

A comparison of the pore size distribution before and after immobilization revealed that the cumulative specific volume of the pores was modified by the adsorption of trypsin (Fig. 2). Considering that trypsin is shaped like a sphere with a diameter of 3.8 nm,[21] the ratio between the volume of immobilized trypsin to the cumulative specific volume of pores with diameters of less than 200 nm at a concentration of 42.9 μmol of trypsin g^{-1} of support was equal to 1.42 (Table 2). Thus, the enzyme occupied all of the interior pores and a portion of the outer surface, which indicates that transesterification reactions could be catalysed in part by enzymes located at the surface of the support. The localisation of trypsin within silica gel is important for enzyme accessibility and stability.[20]

The initial reaction rates, v_0, were measured during the initial-rate phase of that period of reaction over which there is a constant change in product concentration per unit time by linear regression according to eq. (3),[22]

$$\frac{dP}{dt} = v_0 = cte \qquad (3)$$

where 'P' is the product formation and 't' is the time of reaction.

The variation of the initial reaction rate with enzyme concentration is shown in Fig. 4. The biocatalysts show increased initial reaction rate with increased enzyme loading (in the range studied). Similar observation has been obtained by Jurado *et al.*[23] when they studied the hydrolysis of emulsified tributyrin by *Thermomyces lanuginosus* lipase (TLL).[23]

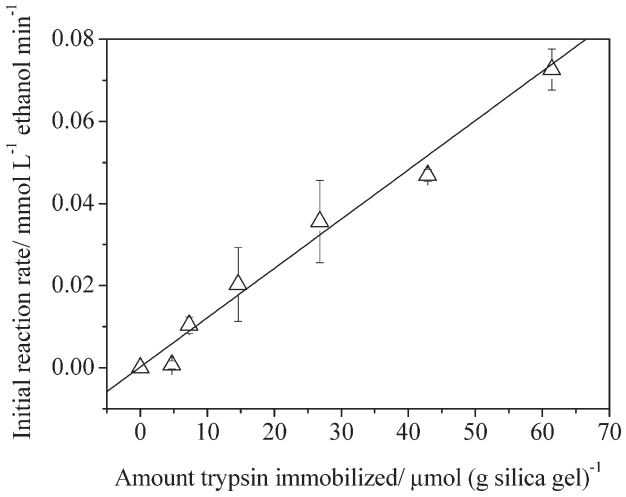


Fig. 4 – *Initial reaction rates of ATEE transesterification at different concentrations of adsorbed trypsin on commercial silica gel. Experimental conditions: 10 mmol L^{-1} of ATEE, φ = 5 % phosphate buffer solution (pH = 6.5), 25 °C.*

Catalytic activity (TOF) was plotted against the immobilized trypsin concentration after 4 h of transesterification as shown in Fig. 5. In general, the TOF at low concentrations of immobilized trypsin was high, indicating that access to immobilized trypsin by ATEE was limited at high concentrations. As can be observed in Fig. 5, the TOF remained constant at high concentrations of immobilised trypsin (> 4.6 μmol trypsin/g silica gel), implying that the reaction was limited by enzyme catalyzed step. Therefore, the catalysis did depend on the concentration of immobilized enzyme. Similar results were observed by Kang *et al.*[24] in the immobilization of trypsin on soap-free poly[(methyl methacrylate)-co-(ethyl acrylate)-co-(acrylic acid)] 'P(MMA-EA-AA)', latex particles. On the other

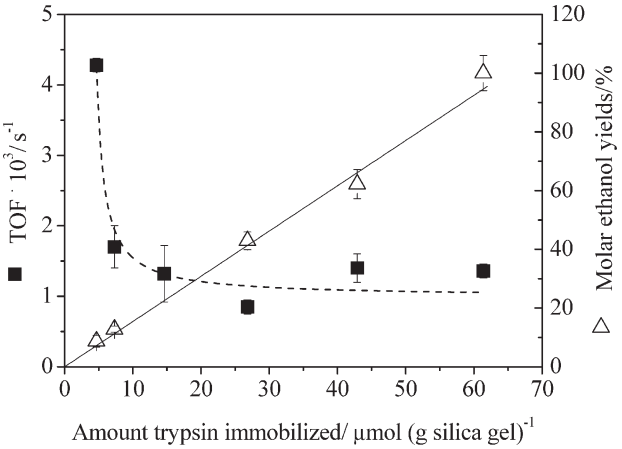


Fig. 5 – *ATEE transesterification at different concentrations of adsorbed trypsin on commercial silica gel. (■) Turnover frequency = μmol product/(μmol enzyme · s) and (Δ) Molar ethanol yields after 4 h of transesterification. Experimental conditions: 10 mmol L^{-1} of ATEE, φ = 5 % phosphate buffer solution (pH = 6.5), 25 °C.*

hand, after 4 h of transesterification, the yield of ethanol linearly increased as a function of increasing immobilized trypsin concentration (Fig. 5).

Effect of initial ATEE concentrations

Transesterification was evaluated at ATEE concentrations between 0.8 and 100 mmol L^{-1} and at a constant immobilized trypsin concentration [43 μmol trypsin/g silica gel, φ = 5 % phosphate buffer, pH = 6.5, 1-propanol at 25 °C], wherein the TOF and the TON were determined throughout each reaction.

Fig. 6 depicts the effect of substrate concentration on the TON. The results indicate that the TON

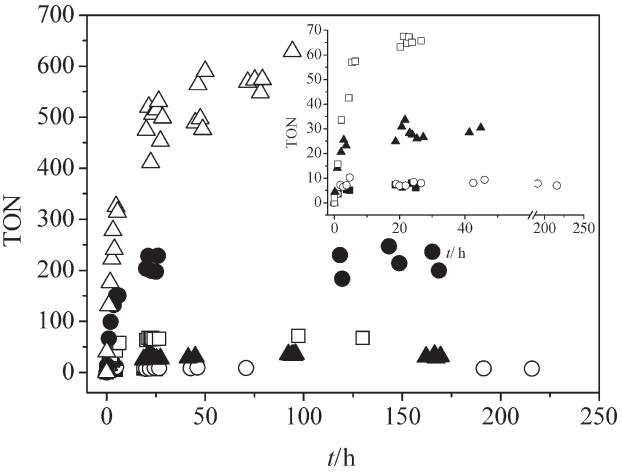


Fig. 6 – *Enzymatic efficiency (ATEE transesterification) profiles of trypsin immobilized onto commercial silica gel. Experimental conditions: [ATEE]$_{initial}$ = (■) 0.8, (○) 1, (▲) 5, (□) 10, (●) 40, (Δ) 100 mmol L^{-1}, φ = 5 % phosphate buffer solution (pH = 6.5), 1-propanol, concentration of immobilized trypsin = 42.9 μmol trypsin/g silica gel, 25 °C.*

increased from 6 to 520 as the initial concentration of the ATEE had increased from 0.8 to 100 mmol L^{-1}, respectively. In all the experiments, the time required to achieve the highest TON value was less than 50 h, which confirmed that the concentration of the trypsin that was adsorbed onto the silica gel provided maximum yields and a full conversion within 50 h.

A linear relationship was observed between the ATEE concentration and the values of the TOF and the TON after one hour of transesterification using initial substrate concentrations that ranged from 0.8 to 100 mmol L^{-1}.

The TOF varied from $8.1 \cdot 10^{-4}$ to 0.04 s^{-1}, which is similar to the results obtained by Suzuki *et al.*[25] at 30 min of N-α-benzoyl-DL-arginine-4-nitroanilide (BAPNA) hydrolysis using trypsin-immobilised carboxy-functionalised monodispersed mesoporous silica spheres (MMSS) as a catalyst (20 °C, pH = 7.0). Immobilized trypsin was able to produce 131 molecules of ethanol per immobilized trypsin at an ATEE concentration of 100 mmol L^{-1} in non-saturated conditions. The TOF decreased to four molecules of ethanol at an ATEE concentration of 0.8 mmol L^{-1}.

At the end of the experiments (after 50 h approximately), the TOF and the TON dramatically increased when the initial ATEE concentration had increased from 0.8 to 100 mmol L^{-1}. The increases in the TOF (from $9.3 \cdot 10^{-5}$ to $6.8 \cdot 10^{-3}$ s^{-1}) and the TON (from 8 to 520) as a function of increasing ATEE concentration were due to the increased contact between the immobilized enzyme and the substrate, which increased the reaction rate. Moreover, at the end of the experiments (as compared to one hour of transesterification), linear relationships between the initial ATEE concentration and the TOF and TON were observed.

For the kinetic parameters K_m and V_{max} determination, the initial reaction rates were plotted against the initial ATEE concentration (Fig. 7). Experimental data were fitted to the Michaelis-Menten equation by nonlinear regression, as follows in eq. (4):

$$v_0 = \frac{V_{max}[\mathrm{ATEE}_{initial}]}{K_m + [\mathrm{ATEE}_{initial}]} \quad (4)$$

which gave the maximum initial reaction rate, V_{max} = 0.364 mmol L^{-1} ethanol min^{-1}, and the Michaelis-Menten contant, K_m = 64.8 mmol L^{-1} $ATEE_{initial}$. These values are of the same order of magnitude as that reported by Purich,[26] and Ding *et al.*[27] for trypsin using different substrates. The parameters of the goodness of fit were r^2 = 0.999 and SSE = $2.542 \cdot 10^{-5}$.

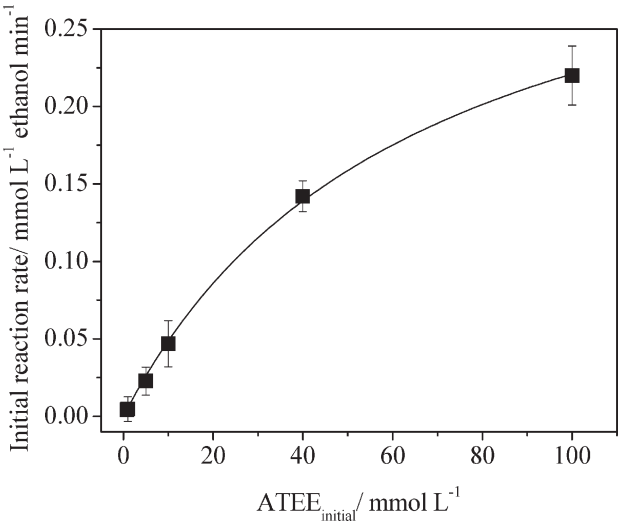


Fig. 7 – *Initial reaction rates determined versus different concentrations of ATEE, (■) experimental data, – Michaelis-Menten equation. Experimental conditions: $[ATEE]_{initial}$ = 0.8, 1, 5, 10, 40, 100 mmol L^{-1}, φ = 5 % phosphate buffer solution (pH = 6.5); 1-propanol, concentration of immobilized trypsin = 42.9 μmol trypsin/g silica gel; 25 °C.*

Reusability and storage stability

After transesterification, immobilized trypsin on commercial silica gel was separated by natural settling for 30 minutes and washed twice with 1-propanol. As can be observed in Fig. 8, different concentrations of purified trypsin were reused in the transesterification of fresh ATEE. Specifically, 4.7 μmol trypsin/g support and 61.4 μmol trypsin/g support were reacted with 10 mmol L^{-1} ATEE. The results indicate that a trypsin concentration of 4.7 μmol trypsin/g provided (at 260 h of reaction time) a mean value of 75 % yield of ethanol and the standard deviation 2.47 in the first use, and a mean

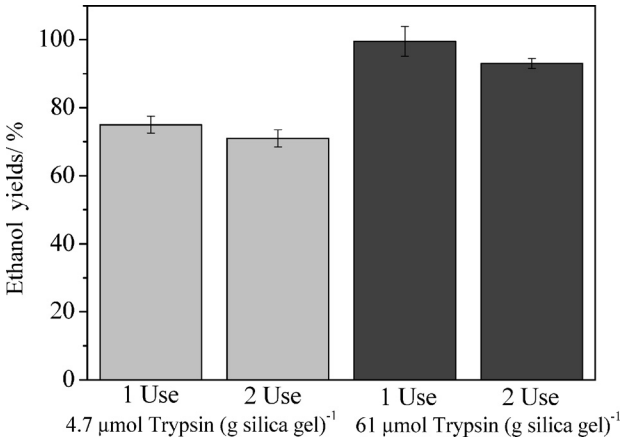


Fig. 8 – *Reusability of adsorbed trypsin (4.7 and 61.4 μmol trypsin/g silica gel) in the transesterification of ATEE. Experimental conditions: $[ATEE]_{initial}$ = 10 mmol L^{-1}, φ = 5 % phosphate buffer solution (pH 6.5), 1-propanol, 25 °C, reaction time = 260–300 h and 21–22 h for 4.7 and 61.4 μmol trypsin/g silica gel, respectively.*

value of 71 % yield of ethanol and the standard deviation 2.50 in the second use (at 300 h of reaction time). However, in the third using (at 305 h of reaction time) the mean value of ethanol yield was equal to 61 % and the standard deviation 1.50. Alternatively, at a trypsin concentration of 61.4 μmol g^{-1}, the mean yields of the first (at 21 h of reaction time) and second uses (at 22 h of reaction time) were 99.5 % (standard deviation 4.40) and 93 % (standard deviation 1.50), respectively. Variations in reaction performance at different concentrations of immobilized trypsin were due to sample manipulation and the loss of immobilized trypsin. A loss in activity after repeated use is a common phenomenon in enzyme catalyzed reactions.[28] Moreover, immobilized trypsin separated by centrifugation remained active with a loss of 5 % of its activity after storage for one month at 4 °C.

Conclusions

The results of this study confirm that trypsin immobilized onto commercial silica gel is an effective catalyst in the transesterification of ATEE. A comparison of free and immobilized trypsin confirmed that the adsorption of trypsin onto silica gel did not alter the catalytic activity of the enzyme. Furthermore, the results indicate that the optimal concentration of immobilized trypsin is 43 μmol trypsin/g silica gel (reaction time 21.2 h). Linear relationships between the substrate concentration (ATEE) and the TOF and TON were observed. The kinetic parameters K_m (64.8 mmol L^{-1} $ATEE_{initial}$) and V_{max} (0.364 mmol L^{-1} ethanol min^{-1}) were determined. The catalyst obtained by the immobilization of trypsin onto commercial silica gel is reusable twice and active after one month of storage at 4 °C.

ACKNOWLEDGEMENTS

Financial support was provided by the Santander Group and the Complutense University of Madrid under project Nº. PR34/07-15825.

Symbols and abbreviations

ATEE – N-acetyl-L-tyrosine ethyl ester
BAEE – Na-benzoyl-L-arginine ethyl ester solution
BAPNA– N-α-benzoyl-DL-arginine-4-nitroanilide
cte – constant value
Dv (d) – derivative of the pore volume with respect to the pore diameter, cm^3 nm^{-1} g^{-1}
K_m – Michaelis-Menten constant, mmol L^{-1} $ATEE_{initial}$
MMSS – monodispersed mesoporous silica spheres
P – product formation, mmol
P(MMA-EA-AA) – poly[(methyl methacrylate)-co-(ethyl acrylate)-co-(acrylic acid)]
t – time reaction, h
TOF – turnover frequency, s^{-1}
TON – turnover number
TLL – *Thermomyces lanuginosus* lipase
v_0 – initial reaction rate, mmol L^{-1} ethanol min^{-1}
V_{max} – maximum initial reaction rate, mmol L^{-1} ethanol min^{-1}
φ – volume fraction, %

References

1. *Doukyu, N., Ogino, H.*, Biochem. Eng. J. **48** (3) (2010) 270.
2. *Irazoqui, G., Giacomini, C., Batista-Viera, F., Brena, B. M.,* J. Molecular Catal. B: Enzymatic **46** (2007) 43.
3. *Kim, J., Jia, H., Wang, P.*, Biotechnol. Adv. **24** (2006) 296.
4. *Simon, L. M., Kotorman, M., Marászi, K., László, K.*, J. Mol. Catal. B: Enzym. **10** (2000) 565.
5. *Xi, F., Wu, J., Jia, Z., Lin, X.*, Process Biochem. **40** (2005) 2833.
6. *Villalonga, R., Villalong, M. L., Gomez, V.,* J. Mol. Catal. B: Enzym. **10** (2000) 483.
7. *Zhang, Z., He, Z., He, M.,* J. Mol. Catal. B: Enzym. **14** (2001) 85.
8. *Ruckenstein, E., Guo, W.,* J. Membrane Sci. **187** (2001) 277.
9. *Jarzebski, A. B., Szymanska, K., Bryjak. J., Mrowiec-Białon*, J. Catal. Today **124** (2007) 2.
10. *Sakai-Kato, K., Kato, M., Toyo'oka, T.* Anal. Chem. **74** (2002) 2943.
11. *Nouaimi, M., Moschel, K., Bisswanger, H.*, Enzym. Microbial. Technol. **29** (2001) 567.
12. *Seabra, I. J., Gil, M. H.,* Braz. J. Pharm. Sci. **43** (4) (2007) 535.
13. *Prata, V. M., Bouhallab, S., Henry, G., Aimar, P.,* Biochem. Eng. **8** (3) (2001) 195.
14. *Margot, E., Flaschel, E., Renken, A.,* Process Biochem. **33** (2) (1998) 125.
15. *Morihara, K., Ueno, K.,* Biotechnol. Bioeng. **37** (7) (1991) 693.
16. *Kasai, K.,* J. Chromatogr. **597** (1–2) (1992) 3.
17. *Gomez, J. M., Deere, J., Goradia, D., Cooney, J., Magner, E., Hodnett, B. K.,* Catal. Lett. **88** (3–4) (2003) 183.
18. *Wong, C. H., Wang, K. T.,* Experientia **47** (11–12) (1991) 1123**.**
19. *Gomez, J. M., Romero, M. D., Hodaifa, G., de la Parras, E.,* Eng. Life Sci. **9** (4) (2009) 336.
20. *Takimoto, A., Shiomi, T., Ino, K., Tsunoda, T., Kawai, A., Mizukami, F., Sakaguchi, K.,* Micropor. Mesopor. Mat. **116** (2008) 601.
21. *Diaz, J. F. Jr., Balkus, K. J.,* J. Mol. Catal. B: Enzym. **2** (1996) 115.
22. *Purich, D. L.,* Enzyme Kinetics: Catalysis and Control, Elsevier Inc. (2010) 215.
23. *Jurado, E., Camacho, F., Luzón, G., Fernández-Serrano, M. García-Ramón, M.,* Biochem. Eng. J. **40** (2008) 473.
24. *Kang, K., Kan, C., Yeung, A., Liu, D.,* Macromol. Biosci. **5** (2005) 344.
25. *Suzuki, T. M., Mizutani, M., Nakamura, T., Akimoto, Y., Yano, K.* Micropor. Mesopor. Mat. **116** (2008) 284.
26. *Purich, D. L.,* Enzyme Kinetics: Catalysis and Control, Elsevier Inc. (2010) 305.
27. *Ding, L., Jiang, Y., Huang, L., Li, Y., Huang, J.,* Appl. Biochem. Biotechnol. **95** (2001) 11.
28. *Aarasaratnam, V., Galaey, I. Y., Matiasson, B.,* Enzym. Microbial Technol. **27** (2000) 254.