Transesterification catalyzed by trypsin supported on MCM-41

J.M. Gómez[a], J. Deere[b], D. Goradia[b], J. Cooney[b], E. Magner[b], and B.K. Hodnett[b,*]

[a]Department of Chemical Engineering, Facultad de Químicas, Universidad Complutense de Madrid, 28040 Madrid, Spain
[b]Materials and Surface Science Institute and the Department of Chemical and Environmental Sciences, University of Limerick, Limerick, Ireland

Received 25th March 2003; accepted 28 March 2003

Trypsin supported on MCM-41 is demonstrated as a stable catalyst for the transesterfication of *N*-acetyl-L-tyrosine ethyl ester with propan-1-ol. An enzyme loading of 5 micromoles of trypsin per gram of silicate was readily achieved. The ultimate enzyme loading was shown to depend strongly on enzyme purity. The adsorbed enzyme exhibited the same turnover frequency for the transesterification reaction as native trypsin, the catalyst could be reused without loss of activity following separation by centrifuging and the enzyme did not leach during testing.

KEY WORDS: transesterification; trypsin; enzyme; MCM-41; mesoporous; stability.

1. Introduction

Ordered mesoporous silicates (MPS) were reported in chemical literature in 1992 [1,2], and by 1996 the first report of enzyme encapsulation or attachment inside an MPS was published by Balkus *et al.* [3]. Since then, there has been a small number of reports in the literature describing enzymes inside mesoporous materials, and a smaller number again describing the activity of the encapsulated enzymes. To date, this work is limited because the available mesoporous materials can only accommodate smaller proteins and enzymes. However, a consistent feature of these reports is that the encapsulated enzymes display similar activities to the free enzymes under similar reaction conditions [1–7].

Balkus *et al.* [3–5] studied protein immobilization (cytochrome c, papain and trypsin) in M41S (MCM-41 and MCM-48) and SBA-15 materials. They found that the efficiency of immobilization was dependent on the molecular size of the enzyme. He *et al.* [6] have shown that mesoporous MCM-41 material was an effective support for the immobilization of penicillin acylase (PA). Yiu *et al.* [7,8] studied trypsin adsorption in different types of MPS (MCM-41, MCM-48 and SBA-15) and in mesoporous materials with functionalized surfaces. All supports adsorbed more than 90% of trypsin from solution. However, 35–72% of the trypsin leached from the support. The amount of leaching decreased when the internal surface was functionalized with mercaptotriethoxysilane or 3-chloropropyltriethoxysilane [8]. Takahashi *et al.* [9] studied the influence of the surface characteristics of mesoporous silica (MCM-41, FSM-15 and SBA-15) in the adsorption of subtilisin (max. 198 mg g^{-1}) and horseradish peroxidase (HRP) (max. 183 mg g^{-1}). Optimal stability and catalytic activity was observed when the mesopore size just exceeded the enzyme molecular diameters. Han *et al.* [10] studied the immobilization of chloroperoxidase (CPO) into MPS materials. The loading capacity on mesocellular foam (MCF) was 122-mg protein per 1 g of silicate. These workers concluded that a weak interaction between enzyme and silicate was critical in maintaining the enzyme activity. Recently, Deere *et al.* [11] studied the adsorption of cytochrome c in different MPSs. For significant adsorption to occur, the surface charges of the protein and of the MPS must be complementary, in addition to the requirement that the pore diameter must be sufficiently large.

In all of these works, the catalytic activity of the enzyme adsorbed on the mesoporous material was measured with the typical assay for each enzyme. Only the work of Takahashi *et al.* [9] reported the catalytic activity of HRP adsorbed on mesoporous materials in a different reaction to the HRP assay. They employed the HRP enzyme in the oxidative reaction of 1,2-diaminobenzene in toluene with *tert*-butylhydroperoxide as oxidant, reaching a conversion of 75% after 7 h for FSM-16/51 immobilized HRP.

The next logical step in the development of these systems is testing their suitability as catalysts for a range of chemical reactions. In this work, we describe the attachment of trypsin to MCM-41 and its subsequent use as a catalyst for the transesterification of *N*-acetyl-L-tyrosine ethyl ester (scheme 1). Features of the reaction investigated include the influence of enzyme purity on the amount adsorbed, the reproducibility of the reaction and the influence of water content.

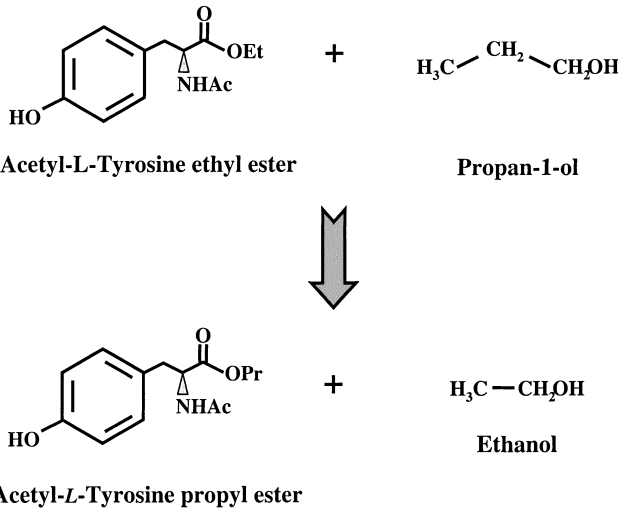
2. Experimental

2.1. Reagents

Cetyltrimethylammonium bromide (CTAB, 99%), trypsin from porcine pancreas (1000–2000 BAEE units

* To whom correspondence should be addressed.

1011-372X/03/0600–0183/0



Scheme 1. Transesterification reaction of *N*-acetyl-L-tyrosine ethyl ester and 1-propanol.

per mg solid hereafter referred to as *impure trypsin*) and from bovine pancreas (minimum 10 000 BAEE units per mg protein hereafter referred to as *pure trypsin*), *N*-acetyl-L-tyrosine ethyl ester monohydrate (ATEE, Sigma grade), 1-propanol (HPLC grade) and ethanol (HPLC grade) were obtained from Sigma-Aldrich. Tetraethoxysilane (TEOS, 98%) was purchased from Lancaster.

2.2. Synthesis of MCM-41

MCM-41 was prepared following a modified method of Mokaya *et al.* [12]. In this method the synthesis was carried out by mixing 10 g of CTAB, 1 g of NaOH and 90 g of H_2O at 35 °C. On dissolution of the surfactant, 9 ml of TEOS was added and stirred at 35 °C for 30 min. The mixture was then heated to 150 °C for 24 h. The solid product was filtered and added to 200 ml of H_2O, followed by stirring and heating to 70 °C for 10 min. The surfactant was removed by calcination at 650 °C for 6 h (ramp rate of 1 °C min^{-1}). MCM-41 presented a pore size of 45 Å (BJH method) with a BET surface area of 970 $m^2 g^{-1}$. In the following section, this material will be identified as MCM-41/45.

2.3. Protein adsorption

Adsorption isotherms for trypsin on MCM41/45 were determined at equilibrium concentrations in the range 0 to 1.5 μM. Protein adsorption was carried out by mixing 0.5 ml of trypsin solution with 0.5 ml of MCM-41 suspension (2 mg of MCM-41/45 per ml) in Eppendorf tubes at 25 °C. The trypsin solution and the silicate suspension were prepared in phosphate buffer (25 mM) at pH 6.5. The amount of trypsin adsorbed was measured by a difference method with trypsin concentrations determined before and after adsorption by UV absorption at 280 nm (trypsin, $\varepsilon_{molar} = 14\ 300\ M^{-1}\ cm^{-1}$ [13]). The batch of material used for the catalytic tests was prepared by mixing 0.5 ml of 10 mg ml^{-1} trypsin solution with 0.5 ml of MCM-41/45 suspension resulting in trypsin loading of *ca.* 5 μmol g^{-1} silicate.

2.4. Enzymatic activity

The transesterification of *N*-acetyl-L-tyrosine ethyl ester and 1-propanol (scheme 1) was selected to measure the catalytic activity of the enzyme. Typical reaction conditions for the sodium phase trypsin were on a 10-ml scale and for the adsorbed trypsin on MCM-41 on a 1-ml scale. For the former, the native trypsin concentration was 0.3 mg of lyophilized trypsin per ml of 1-propanol. Lyophilized trypsin was prepared by freeze-drying the protein from buffer using an Edwards Super Modulus Lyophilizer. A suspension of enzyme in propanol was placed on an agitator. Initial ester concentration was 10 mM and the water or buffer (25 mM phosphate, pH 6.5) content was varied between 0 and 5% v/v. The reaction was followed by gas chromatography analysis (GC-17A Shimadzu) fitted with a flame ionization detector (FID) and capillary column (30QC2.5/BPX5-0.25). Leaching of trypsin was checked after a typical reaction by centrifuging the solid and removing the supernatant liquor. A second or third batch of substrate was thus added, whereupon the reaction restarted. In some cases, a fresh batch of substrate was added to the supernatant liquor to test the reactivity of any leached protein.

3. Results and discussion

Figure 1 shows adsorption isotherms for pure and impure trypsin on MCM-41 in buffer solution (25 mM phosphate) at pH 6.5. Adsorption using an impure

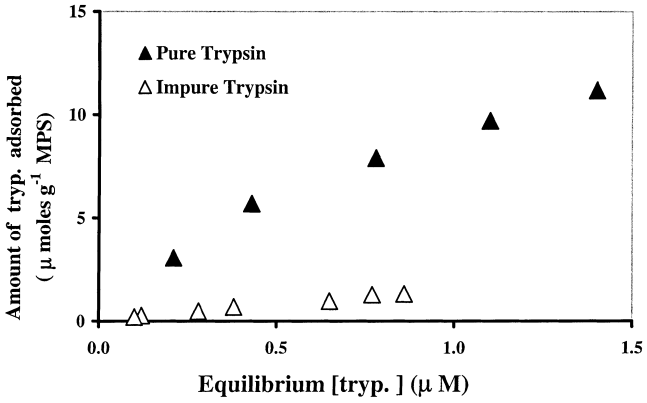


Figure 1. Adsorption isotherms for pure (▲) and impure (△) trypsin on MCM-41/45 at 25 °C, buffer solution (25 mM phosphate, Ph 6.5).

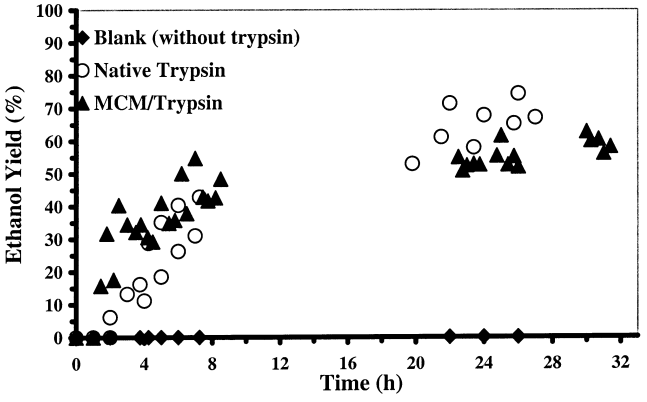


Figure 2. Ethanol yield from: (◆) blank (without trypsin), (▲) native trypsin (ester : trypsin = 780 molar) and (○) MCM-41/trypsin (ester : trypsin = 1170 molar). Reaction conditions: [ester]: 10 mM, 1-propanol: 95% v/v and buffer solution (25 mM phosphate, pH 6.5): 5% v/v.

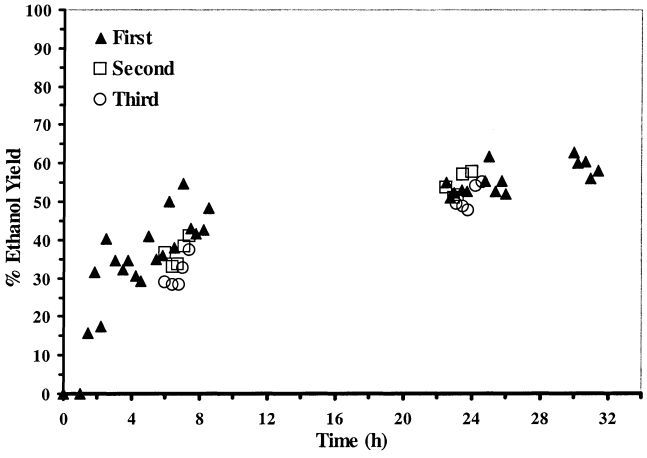


Figure 3. Catalytic activity of reused MCM-41/trypsin: (◆) First reaction, (...) Second reaction, (...) Third reaction. Reaction conditions: [ester]: 10 mM, 1-propanol: 95% v/v, buffer solution (25 mM phosphate, pH 6.5): 5% v/v and ester : trypsin molar ratio of 1170 : 1.

source of trypsin reached saturation in less than 1 h and was stable thereafter for at least 20 h. Equilibrium took *ca.* 48 h with pure trypsin. Typically, five times more trypsin was adsorbed when pure trypsin was used.

Figure 2 shows the percentage yield of ethanol for the transesterification of *N*-acetyl-L-tyrosine ethyl ester in 95% 1-propanol and 5% buffer (25 mM phosphate, pH = 6.5). In the absence of trypsin, no reaction was observed. When solution phase trypsin at an initial ester : trypsin molar ratio of 780 : 1 was used as catalyst, the ethanol yield reached 65–70 mol% in 30 h at 20 °C. For trypsin adsorbed onto MCM-41 (loading *ca.* 5 μmol g^{-1}) at an ester : trypsin molar ratio of 1170 : 1, the maximum ethanol yield was in the range 50 to 60%. The data in figure 2 in fact came from five separate experiments, indicating excellent reproducibility of this system.

The results of reusing the MCM-41/45 loaded trypsin, isolated by centrifuging the reaction mixture removing the supernatant liquor and reusing the solid by introducing a fresh batch of ester, are shown in figure 3. Within experimental error, the full activity of the MCM-41/45-supported trypsin could be recovered for the second and the third reuses of these materials. In our experimental condition, each use of the MCM-41/45-supported trypsin corresponds to a turnover number (TON) of around 600 molecules of ethanol formed per molecule of trypsin. In addition, when the supernatant liquid was tested by adding a further change of ester following removal of the solid, no further reaction was observed, indicating that leached trypsin was not responsible for the observed reactivity.

Figure 4 demonstrates the influence of water content on the reactivity of the enzyme. No reaction was observed in the absence of water for the native trypsin or trypsin attached onto MCM-41/45. Addition of 5% water or buffer activated the free and attached enzyme.

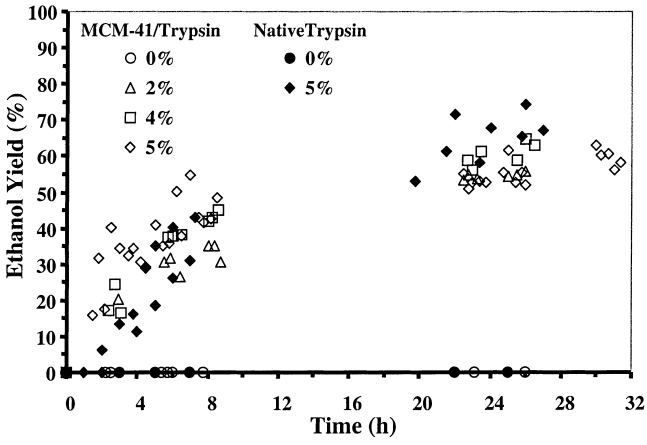


Figure 4. Influence of water content in the catalytic activity: native trypsin: (●) 0% v/v, (◆) 5% v/v of water; MCM-41/trypsin: (...) 0% v/v, (...) 2% v/v (...) 5% v/v. Reaction conditions: [ester]: 10 mM, 1-propanol: 95 to 100% v/v, native trypsin: ester : trypsin molar ratio of 780 : 1, MC-41/trypsin: ester : trypsin molar ratio of 1170 : 1.

Figure 4 shows the change in ethanol yield as the water content was varied in the range 0 to 5%.

4. Conclusions

These results confirm the very strong interaction between trypsin and MCM-41. A notable feature of the results is the influence of enzyme purity on the amounts adsorbed. This feature of our results is the subject of further investigation. However, the abrupt arrest of adsorption, when the less pure trypsin sample was used, is consistent with pore blockage by impurities. Indeed, when pure trypsin was added to the MPS material that had previously been saturated with the impure trypsin solution, no further adsorption was observed that was consistent with an irreversible poisoning, possibly blocking off the silicate porosity by impurities. Indeed, protein purity may also explain the conflicting reports in the literature concerning the suitability of other MPSs as hosts for the encapsulation of enzymes [10–14].

The main feature of this work is the excellent activity of trypsin encapsulated in MCM-41 compared to the native enzyme. Other gratifying features include the good reproducibility (five separate reactions gave almost identical results), the lack of leaching (the supernatant liquor did not exhibit any reactivity when recharged with ester following the removal of the catalyst) and the stability of the catalyst (no deactivation was observed during three uses of the encapsulated enzymes).

Acknowledgments

This work was carried out with a postdoctoral grant of the University Complutense/Flores Valles Company International Grants Program and was supported by the Programme for Research in Third-level Institutions and Basic Research Grants Programmes of the Irish Government.

References

[1] J.S. Beck, J.C. Vartuli, W.J. Roth, M.E. Leonowicz, C.T. Kresge, K.D. Schmitt, C.T.-W. Chu, D.H. Olson, E.W. Sheppard, S.B. McCullen, J.B. Higgins and J.L. Schlenker, J. Am. Chem. Soc. 114 (1992) 10834.
[2] C.T. Kresge, M.E. Leonowicz, W.J. Roth, J.C. Vartuli and J.S. Beck, Nature 359 (1992) 710.
[3] J.F. Diaz and K.J. Balkus Jr., J. Mol. Catal., B 2 (1996) 115.
[4] M.E. Gimon-Kinsel, V.L. Jimenez, L. Washmon and K.J. Balkus Jr., Stud. Surf. Sci. Catal. 117 (1998) 373.
[5] L. Washmon-Kriel, V.L. Jimenez and K.J. Balkus Jr., J. Mol. Catal., B: Enzym. 10 (2000) 453.
[6] J. He, X. Li, D.G. Evans, X. Duan and C. Li, J. Mol. Catal. B: Enzym. 11 (2000) 45.
[7] H.H.P. Yiu, P.A. Wright and N.P. Botting, Micropor. Mesopor. Mater. 44–45 (2001) 763.
[8] H.H.P. Yiu, P.A. Wright and N.P. Botting, J. Mol. Catal. B: Enzym. 15 (2001) 81.
[9] H. Takahashi, B. Li, T. Sassaki, C. Miyazaki, T. Kajino and S. Inagaki, Micropor. Mesopor. Mater. 4445 (2001) 755.
[10] Y. Han, J.T. Watson, G.D. Stucky and A. Butler, J. Mol. Catal. B: Enzym. 17 (2002) 1.
[11] J. Deere, E. Magner, J.G. Wall and B.K. Hodnett, J. Phys. Chem. B 106, (2002) 7340.
[12] R.J. Mokaya, W. Zhou and W. Jones, Chem. Commun. (2000) 51.
[13] K. Walsh and H. Neurath, Proc. Natl. Acad. Sci. U.S.A. 52 (1964) 884.
[14] H. Takahasi, E. Li, T. Sasaki, C. Miyazaki, T. Kajino and S. Inagaki, Chem. Mater. 12 (2000) 13301.