Effect of Mass-Transfer Limitations on the Selectivity of Immobilized α-Chymotrypsin Biocatalysts Prepared for Use in Organic Medium

Raúl J. Barros,[1,2] Ernst Wehtje,[1] Patrick Adlercreutz[1]

[1]Department of Biotechnology, Center for Chemistry and Chemical Engineering, Lund University, P.O. Box 124, S-221 00 Lund, Sweden
[2]Unit for Agrarian Science and Technology, University of Algarve, Campus de Gambelas, P-8000 Faro, Portugal; telephone: +351 89 800900, ext. 7391; fax: +351 89 815927; e-mail: rbarros@ualg.pt

Received 26 April 1999; accepted 31 August 1999

Abstract: The selectivity of preparations of α-chymotrypsin immobilized on Celite or polyamide and carrying out syntheses of di- and tripeptides in acetonitrile medium were studied. The study concerns the effect of mass-transfer limitations on three different kinds of selectivity: acyl donor, stereo- and nucleophile selectivities, defined respectively as the ratio of initial rates with different acyl donors; the enantioselectivity factor (*E*); and the ratio of initial rates of peptide synthesis and hydrolysis of the acyl donor. Strong mass-transfer limitations caused by increased enzyme loading had a very strong effect on acyl donor selectivity, with reductions of up to 79%, and on stereoselectivity, with reductions of up to 77% in relation to optimum values, both on Celite. Nucleophile selectivity was not affected as strongly by mass-transfer limitations. Using a small molecule ($AlaNH_2$) as nucleophile, the onset of these limitations caused only minor reductions in selectivity, while when using a larger nucleophilic species ($AlaPheNH_2$) it was reduced by up to 60% when increasing enzyme loading on Celite from 2 to 100 mg/g. The different way these kinds of selectivity are affected by the onset of mass-transfer limitations can be explained by a combination of different aspects: the kinetic behavior of the enzyme toward nucleophile and acyl donor concentrations, the relative concentrations of reagents used in the reaction media, and their relative diffusion coefficients. In short, higher concentrations of nucleophile than acyl donor are generally used, and the nucleophile most often used in the experiments hereby described ($AlaNH_2$) diffuses faster than the acyl donors employed. These factors combined are expected to give rise to concentration gradients inside porous biocatalyst particles higher for acyl donor than for nucleophile under conditions of mass-transfer limitations. This explains why acyl donor selectivity and stereoselectivity are much more influenced by mass transfer limitations than nucleophile selectivity. *Biotechnol Bioeng* **67:** 319–326, 2000.

Correspondence to: R. J. Barros

Contract grant sponsors: Portuguese Foundation for Science and Technology (FCT, MCT); Swedish Research Council for Engineering Sciences (TFR)

Contract grant number: Grant BD/2839/94 of the program PRAXIS XXI (PFST)

Keywords: immobilized α-chymotrypsin; kinetically controlled peptide synthesis; acyl donor selectivity; stereoselectivity; nucleophile selectivity; internal diffusion

INTRODUCTION

Even though immobilization of enzymes on porous supports is recognized as a good strategy in order to facilitate the use of biocatalysts in organic media (Adlercreutz, 1996; Dordick, 1989), being a heterogeneous catalyst system, it gives rise to problems that do not exist with dissolved enzymes. It has been shown that mass-transfer limitations have a very strong effect on the activity of immobilized enzymes in organic media (Barros et al., 1998a; Bernard and Barth, 1995; Indlekofer et al., 1992; Ison et al., 1994; Luck et al., 1988). In the particular case of protease-catalyzed dipeptide synthesis, we have recently shown that internal diffusional limitations strongly influence the observed rates of product synthesis, reducing observed specific activity strongly (Barros et al., 1998a). This kind of mass-transfer limitation depends on the effective diffusion coefficients of the reacting compounds inside the porous biocatalyst particles, being stronger with slower diffusing species. It also depends on the rate of the reaction, the limitations being generally stronger with faster reactions because these give rise to higher concentration gradients inside the porous biocatalyst particles. It is thus expected that internal diffusional limitations differently affect distinct reactions, depending on their intrinsic rates and on the diffusion coefficients of the reacting compounds. In this way, this kind of mass-transfer limitation is expected to influence the observed enzyme selectivity. In protease-catalyzed peptide synthesis reactions, one can think of different kinds of enzyme selectivity, such as selectivity toward the employed acyl donor; stereoselectivity, if racemates or different enantiomers are used as reagents; and selectivity for the employed nucleophile. Water has to be present in the reaction medium, and it can also play a role in kinetically controlled peptide synthesis using activated acyl donors (esters) by

 CCC 0006-3592/00/030319-08

acting as nucleophile and hydrolyzing them. Hence the ratio of the rates of synthetic and hydrolytic reactions is a good measure of nucleophile selectivity, and is the parameter we have studied.

In this work, the effects of internal diffusional limitations on these kinds of selectivity have been studied by employing different immobilization conditions for the enzyme—different loadings and support materials—and observing the effect on a range of kinetically controlled dipeptide and tripeptide synthesis reactions.

We had already observed before that the nature of the acyl donor strongly influences reaction rates (Barros et al., 1998a,b). Using a common nucleophile ($AlaNH_2$) the α-chymotrypsin-catalyzed dipeptide synthesis rate is faster in the following order of acyl donors:

rate (BzAlaOMe) < rate (AcPheOEt) < rate (BzTyrOEt).

This agrees with the P_1 site selectivity (as defined by Schechter and Berger, 1967) of this enzyme, which is higher for amino acid residues with aromatic side chains (Schellenberger and Jakubke, 1991). This enzyme also shows a very high stereoselectivity for amino acid residues of the L-configuration at the P_1 site (Nagashima et al., 1992; Silver and Matta, 1972; Ståhl et al., 1991). With respect to the P_1' site, though, α-chymotrypsin does not show very marked selectivity with respect for the nature of the amino acid side chain or its configuration (Clapés et al., 1990; Jönsson et al., 1996; Nagashima et al., 1992; West and Wong, 1986). In contrast, the selectivity on the P_1' position is more influenced by the chemical nature of its C terminus: amides and peptides are preferred to esters or free amino acids as nucleophiles (Nagashima et al., 1992; Schellenberger and Jakubke, 1991). We have found no reports in the literature as to if the existence of mass-transfer limitations has an effect on these substrate preferences for α-chymotrypsin.

MATERIALS AND METHODS

Chemicals

Bovine pancreas α-chymotrypsin (CT, Specific activity 52 BTEE U · mg solid^{-1}), triethylamine (TEA), and 2-naphthol were purchased from Sigma Chemical Co., USA. *N*-Acetyl-L-phenylalanine ethyl ester (AcPheOEt), *N*-benzoyl-L-tyrosine ethyl ester (BzTyrOEt), *N*-benzoyl-L-alanine methyl ester (BzAlaOMe), *N*-benzoyl-D,L-phenylalanine-β-naphthyl ester (Bz-D,L-PheONaphthyl), L-alaninamide hydrochloride ($AlaNH_2 \cdot HCl$), L-alanyl-L-phenylalaninamide hydrochloride ($AlaPheNH_2 \cdot HCl$), and D-phenylalaninamide hydrochloride (D-$PheNH_2 \cdot HCl$) were purchased from Bachem Feinchemikalien AG, Switzerland. Acetophenone (99%+) was from Aldrich, Germany, and acetonitrile (HPLC grade), glacial acetic acid, and tris(hydroxymethyl)aminomethane (Tris) were from Merck, Germany.

Supports

Celite and polyamide supports of two particle size ranges, PAm 106–180, with mean particle diameter 178 μm, and PAm 300–500, with mean particle diameter 495 μm, were prepared as previously reported (Barros et al., 1998c). Determinations of specific surface area, area distribution with pore diameter, porosity, skeletal density, pore size distribution, and particle size distribution were performed on these granular materials (Barros et al., 1998c).

Immobilization Procedures

Enzyme preparations were made by wetting 1 g of the support material with 1 mL of an aqueous solution of adequate concentration of CT in buffer (50 m*M* Tris-HCl, pH 7.8), mixing thoroughly to ensure wetting of all the particles and subsequently drying overnight under vacuum (water pump) at room temperature. The enzyme loadings tested ranged between 1 and 100 mg CT per gram of support, adjusted through the concentration of enzyme solution used.

Reactions

In all cases the reaction solvent was acetonitrile containing 5 vol % aqueous buffer (50 m*M* Tris-HCl, pH 7.8). The reactions were carried out in 4-mL screw-cap vials (Chrompack) containing 1 or 2 mL of reaction mixture. The vials were kept at 25°C on a reciprocal shaker (185 rpm). The reactions were started by adding an adequate amount of enzyme preparation to the reaction mixture. Samples of 10 or 20 μL (depending on the protecting group of the acyl donor) were taken from the reaction vials at regular time intervals, diluted with the appropriate eluent, and analyzed by HPLC with a Merck-Hitachi LaChrom system composed of a Model L-7100 pump unit, a Model L-7250 autosampler, and a L-7400 UV detector, and using a reverse phase C18 column (Spherisorb ODS-2, 10 μm, 250 × 4 mm, Tracer Analitica). The samples were eluted with water/acetonitrile/acetic acid in different volumetric proportions depending on the reaction or reaction combination studied. Spectrophotometric detection at 254 nm was used in all cases.

Acyl Donor Selectivity

In order to study acyl donor selectivity, the reactions followed were the CT-catalyzed synthesis of the dipeptides *N*-acetyl-L-phenylalanyl-L-alanylamide ($AcPheAlaNH_2$), *N*-benzoyl-L-tyrosyl-L-alanylamide ($BzTyrAlaNH_2$), and *N*-benzoyl-L-alanyl-L-alanylamide ($BzAlaAlaNH_2$). In the separate reaction experiments the concentrations of reactants were 20 m*M* acyl donor (AcPheOEt, BzTyrOEt, or BzAlaOMe), 30 m*M* $AlaNH_2 \cdot HCl$, and 30 m*M* TEA, used to neutralize the hydrochloride of alaninamide and enhance its solubility and nucleophilicity. Competitive reaction experiments were carried out in similar conditions, except that

two acyl donors (AcPheOEt and BzTyrOEt or AcPheOEt and BzAlaOMe) were present simultaneously in the reaction medium at 20 m*M* concentration each. Initial rates were estimated from the slopes of the straight lines fitted by linear regression to the dipeptide conversion vs. time plots (usually 6 data points with conversions under 20%).

Stereoselectivity

In order to study stereoselectivity a racemic acyl donor (Bz-D,L-PheONaphthyl) was used, while D-PheNH$_2$ was employed as nucleophile to minimize further CT-catalyzed reactions on the C-terminus of the dipeptide products. The concentrations used were 10 m*M* for the racemate and 108 m*M* for the nucleophile. Acetophenone (0.42 m*M*) was used as internal standard. The reaction conversion was estimated from the amount of liberated 2-naphthol. Since the two possible dipeptide products (Bz-L-Phe-D-PheNH$_2$ and Bz-D-Phe-D-PheNH$_2$) are diastereomers, not enantiomers, it is possible to analyze them separately with the nonchiral HPLC column used, and thus it is possible to estimate the enantiomeric excess of the product (ee_P). The enantiomeric excess of the substrate (ee_S) was calculated by applying the equation

$$ee_S = \frac{ee_P \cdot C}{1 - C}. \quad (1)$$

The apparent enantioselectivity of the enzyme (E_{app}) for each experiment was estimated by plotting C vs ee_S for all the points of each reaction and fitting by nonlinear regression the curve of the kind

$$C = 1 - \left[\frac{(1 + ee_S)^{E_{app}}}{1 - ee_S}\right]^{1/(1-E_{app})}, \quad (2)$$

which gives the best E_{app} value. The fit was performed with help of Kaleidagraph software (version 3.0.5, © 1994 by Abelbeck Software). Eq. (2) is derived from the enantioselectivity (E) definition of Chen et al. (1982) for irreversible reactions:

$$E = \frac{\ln[(1 - C)(1 - ee_S)]}{\ln[(1 - C)(1 + ee_S)]}. \quad (3)$$

Nucleophile Selectivity

In order to study nucleophile selectivity all of the above reactions were studied, plus the synthesis of the tripeptide *N*-benzoyl-L-tyrosyl-L-alanyl-L-phenylalanylamide (BzTyrAlaPheNH$_2$) using BzTyrOEt as acyl donor and AlaPheNH$_2$ · HCl plus TEA as nucleophile. Nucleophile selectivity is defined here in competitive terms between the nucleophile amide used and water. It is defined as the ratio of the rates of the synthetic and hydrolytic reactions, yielding the peptide product and the hydrolyzed acyl donor, respectively.

RESULTS AND DISCUSSION

The extent of mass-transfer limitation can be controlled by using different supports and enzyme loadings. Three different granular materials were used in this study: Celite and polyamide of two particle size ranges. Celite is a poorly porous support wherein diffusion of small molecules is difficult compared to polyamide. The diameter of the particles influences internal diffusion because it determines how far, on average, substrate molecules have to travel inside the porous biocatalyst particles before running into an enzyme molecule that converts them: the larger the particle size, the stronger the diffusional limitation. Enzyme loading strongly influences mass-transfer limitations because a higher amount of enzyme present in the porous support particles gives rise to a faster consumption of reagents and in this way creates steeper concentration gradients. The higher the enzyme loading, the stronger the expected internal diffusional limitations.

Because the onset of mass transfer limitations depends on the intrinsic rate of the reactions that take place, we have summarized the information on the specific activity of α-chymotrypsin on each reaction we have studied, and presented it in Table I. One can see that the fastest reaction is the synthesis of the dipeptide BzTyrAlaNH$_2$, the slowest is the synthesis of the dipeptide BzAlaAlaNH$_2$, and the others have rates of the same order of magnitude.

Acyl Donor Selectivity

We had observed before that the variation of enzyme activity with enzyme loading was different on different supports and with different acyl donors. This translates into a changed apparent selectivity of the enzyme for the acyl donor employed with increased enzyme loading, as we have called the attention to previously (Barros et al., 1998a). Figure 1A and B shows this for the supports tested. The data in these figures are calculated from the initial rates of separate experiments running the different reactions involved: the syntheses of the dipeptides BzTyrAlaNH$_2$, AcPheAlaNH$_2$, and BzAlaAlaNH$_2$. In both comparisons shown the trend is quite clear: a strong decrease in apparent selectivity of the enzyme for the acyl donor is observed with increased enzyme loading. Also, when comparing the used supports, Celite, the one with which stronger diffusional limitations are expected, is the one where lower apparent selectivities are observed. Higher selectivity is observed with PAm 106–180, which is explained by the same reasons: this support, having the smaller particle size and the largest porosity, is the one where internal diffusional limitations are expected to play a lesser role.

Further experiments were performed to confirm this trend. Running reactions separately is not considered as a good practice in assessing enzyme selectivity. If substrates have very different K_M values in the case of classical Michaelis–Menten kinetics, the relative rate at which they are converted can be different if the reactions are run separately

Table I. Maximum specific activities obtained for each reaction on different supports.

Reaction	Support	Max specific activity [μmol ester consumed /(min · mg enzyme)]	Enzyme loading (mg enzyme/g support)
BzTyrOEt	PAm 106–180	6.2	10
+ $AlaNH_2$	PAm 300–500	4.1	5
	Celite	8.0	2
AcPheOEt	PAm 106–180	0.59	10
+ $AlaNH_2$	PAm 300–500	0.63	5
	Celite	0.98	2
BzAlaOMe	PAm 106–180	0.0082	20
+ $AlaNH_2$	PAm 300–500	0.0081	30
	Celite	0.0152	10
Bz-D,L-PheONaphthyl	PAm 106–180	0.58	10
+ D-$PheNH_2$	PAm 300–500	0.20	10
	Celite	0.58	2
BzTyrOEt	PAm 106–180	1.74	10
+$PheAlaNH_2$	PAm 300–500	0.54	10
	Celite	0.36	10

or competitively. Competitive reactions with two acyl donors simultaneously present were then run to confirm the acyl donor selectivity trend. The results are represented in Fig. 1C and D. The same trend of decreased apparent selectivity with increased enzyme loading can be observed. Even though it is harder to distinguish, the support with smaller particle size also shows higher apparent selectivities. Also a similar range of apparent selectivity is observed in both combinations of reactions studied, independently of them happening simultaneously in the same reaction vessel or not, which is observed by comparing Fig. 1A with C and Fig. 1B with D, respectively.

The results are what one should expect, taking into account simultaneous reaction and diffusion inside the porous biocatalyst particles: to ensure mass transport for the faster reactions, steeper substrate concentration gradients will exist inside the particles, and thus larger limitations to enzymatic catalysis. Diffusional limitations will affect more the faster reactions, which explains why the observed selectivity of the enzyme for the faster reactions is reduced under conditions when strong mass-transfer limitations are expected to occur: large enzyme loadings and supports of poor porosity or large particle size.

Stereoselectivity

It is known that α-chymotrypsin is a very stereospecific enzyme with respect to the acyl donor employed, strongly preferring the L- conformation of amino acid derivatives that act as good acyl donors (Silver and Matta, 1972; Ståhl et al., 1991). It is thus interesting to investigate to what extent mass-transfer limitations can affect the stereoselectivity of immobilized preparations of this enzyme. The theoretical effect of mass transfer limitations on the resolution of stereoisomers by spheric solid enzyme preparations has been studied before (Lopez et al., 1990), but no experimental observations have been reported on the literature. Figure 2 shows the apparent enantioselectivity of the resolution of Bz-D,L-Phe-ONaphthyl using D-$PheNH_2$ as nucleophile as a function of enzyme loading and support material. With all supports studied the apparent enantioselectivity shows optimum values at intermediate enzyme loadings: 20 mg enzyme · $(g\ support)^{-1}$ with polyamides and 30 mg enzyme · $(g\ support)^{-1}$ with Celite. The existence of a spontaneous non-stereoselective reaction under the experimental conditions used accounts for low apparent enantioselectivities at low enzyme loadings. This spontaneous reaction was confirmed in the absence of biocatalyst. Under the same experimental conditions as the rest of the experiments were run, about 10% of the ester was converted non-stereoselectively to dipeptide in 96 h. This compares with about 30 h for each experiment depicted in Fig. 2, with conversions reaching 50% quite fast, and going up to 60%. While the experiments represented in Fig. 2 were run using 25 mg of enzyme preparation, increasing the amount of preparation led to higher apparent enantioselectivities at low loadings but not at high loadings, as shown in Table II with PAm 106–180 as support material. At high enzyme loading the rate of the nonselective spontaneous process is negligible when compared with the highly stereoselective enzyme catalysis, which explains the invariance of apparent enantioselectivity with amount of preparation. With all the supports employed, however, apparent enantioselectivity decreased at enzyme loadings above the observed optima. This can be explained by the onset of mass-transfer limitations: while at high enzyme loading internal diffusion limits the rate at which the fast enantiomer (the L-ester) is transported inside the biocatalyst particles and transformed, the slow enantiomer is not subjected to that limitation, because it reacts much more slowly, and negligible concentration gradients will be created inside the particles. Increased loading will then increase the rate at which the slow enantiomer reacts, without changing the rate of reaction of the fast

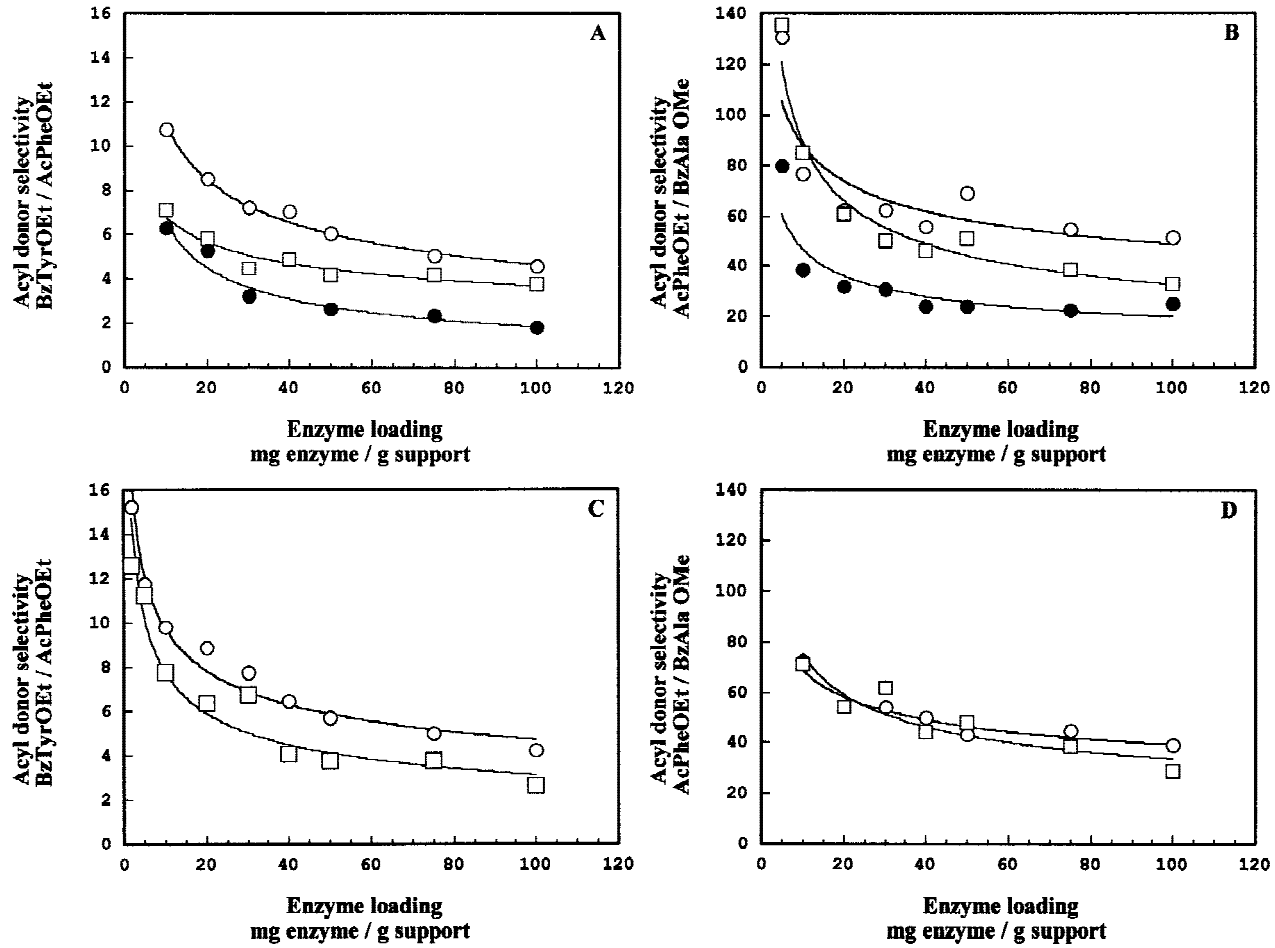

Figure 1. Acyl donor selectivity as a function of enzyme loading and support material for dipeptide synthesis reactions using $AlaNH_2$ as nucleophile; 20 m*M* acyl donor and 30 m*M* nucleophile were used. Reactions were run separately for each acyl donor (A and B) or simultaneously with two acyl donors (C and D). Acyl donors used: BzTyrOEt and AcPheOEt (A and C) or AcPheOEt and BzAlaOMe (B and D). Supports: PAm 106–180 (○); PAm 300–500 (□); and Celite (●).

enantiomer, which explains why the apparent enantioselectivity of the preparation decreases.

Nucleophile Selectivity

Figure 3 shows the effects of support and enzyme loading on the nucleophile selectivity of the immobilized enzyme preparations for the dipeptide synthesis reactions used to study acyl donor selectivity. Only a slight reduction in selectivity can be distinguished in all cases. The nucleophile selectivity ranges between 19 ± 1 and 22 ± 1 using BzTyrOEt as acyl donor (Fig. 3A) and does not seem to show any significant differences with support material used. It is quite difficult to interpret the results in Fig. 3B, given their scattering. This is due to the lower sensitivity of the HPLC analyses when the acyl donor is AcPheOEt, which has a much lower UV response factor than the *N*-benzoyl-protected amino acid esters used in all other cases. Because of this, the slight trend for decrease of selectivity with increased enzyme loading is probably less important than the scattering of the data. With BzAlaOMe as acyl donor (Fig.

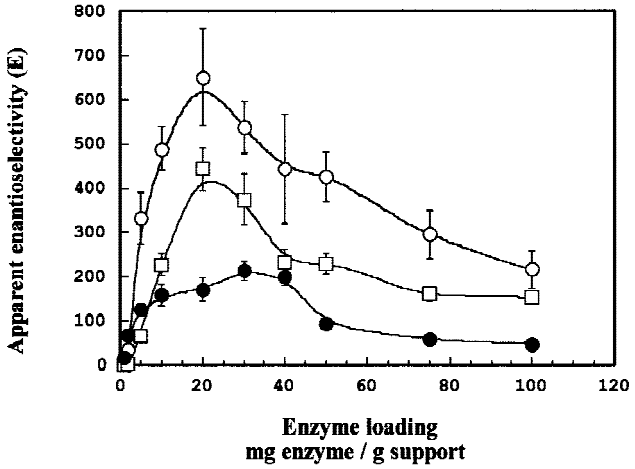

Figure 2. Variation of apparent enantioselectivity ratio (*E*) with enzyme loading and support material; 10 m*M* Bz-D,L-PheONaphthyl and 108 m*M* D-$PheNH_2$, 1 mL of reaction medium and 25 mg of enzyme preparation were used. Supports: PAm 106–180 (○); PAm 300–500 (□); and Celite (●).

Table II. Effect of amount of biocatalyst preparation on apparent enantioselectivity.

Enzyme loading (mg enzyme/g support)	Amount of preparation (mg)	Apparent E
2	25	5.8 ± 0.3
	100	26 ± 2
5	25	76 ± 7
	100	175 ± 18
50	25	373 ± 47
	80	326 ± 48

Reaction conditions: 10 m*M* Bz-D,L-PheONaphthyl, 108 m*M* D-PheNH$_2$, 2 mL of reaction medium. The immobilization support used was PAm 106–180.

3C), there seems to be a higher nucleophile selectivity when using Celite as support material. The nucleophile selectivity ranges between 14 ± 3.5 and 16 ± 1 with Celite, while with the polyamide supports it ranges between 8.4 ± 0.7 and 12.7 ± 0.9 with consistently lower values than with Celite. Still, enzyme loading influences this parameter only to a small extent. The conclusion is that mass transfer limitations show only a small effect on the nucleophile selectivity of these three dipeptide synthesis reactions.

Nucleophile selectivity was also determined in the experiments where stereoselectivity was studied. With the polyamides there was no major variation of nucleophile selectivity with enzyme loading, with values around 14, while with Celite the nucleophile selectivity strongly decreased with enzyme loading: from 15 ± 1.8 at 5 mg/g to 2 ± 1 at 100 mg/g. The reasons for this difference in behavior with different supports are not at all clear. The decrease in nucleophile selectivity at high loadings is due to lowered synthetic rates, not to increased hydrolytic rates. Somehow it becomes more difficult for the nucleophile to participate in the reaction, but the reasons for this are obscure because this phenomenon is not observed with other reactions and is specific for Celite.

It was a bit surprising to notice that the onset of mass-transfer limitations has an influence on nucleophile selectivity so small, when compared with other kinds of selectivity, independently of the reaction rate. An explanation for this kind of observation lies in the kinetics of these reactions. At a constant water content in acetonitrile medium, which is the case dealt with here, the experimental intrinsic kinetics of the dipeptide synthesis and ester hydrolysis reactions have been observed to obey Eqs. (4) and (5):

$$\frac{d[\mathrm{Dip}]}{dt} = \frac{k_{\mathrm{Synth}} \cdot [\mathrm{AcD}] \cdot [\mathrm{Nuc}] \cdot [E_0]}{k_{\mathrm{N}} + [\mathrm{Nuc}]}, \quad (4)$$

$$\frac{d[\mathrm{Hyp}]}{dt} = \frac{k_{\mathrm{Hydr}} \cdot [\mathrm{AcD}] \cdot [E_0]}{k_{\mathrm{N}} + [\mathrm{Nuc}]}, \quad (5)$$

respectively , where [AcD] = concentration of acyl donor, m*M;* [Nuc] = concentration of nucleophile, m*M;* [E_0] = amount of enzyme, mg/mL. [Dip] = concentration of dipeptide product, m*M;* [HyP] = concentration of hydrolysis product, m*M*, and k_{Synth}, k_{N}, and k_{Hydr} are empirical kinetic constants (Barros et al., 1998b). The ratio between the rates of synthesis and hydrolysis can then be expected to be given by

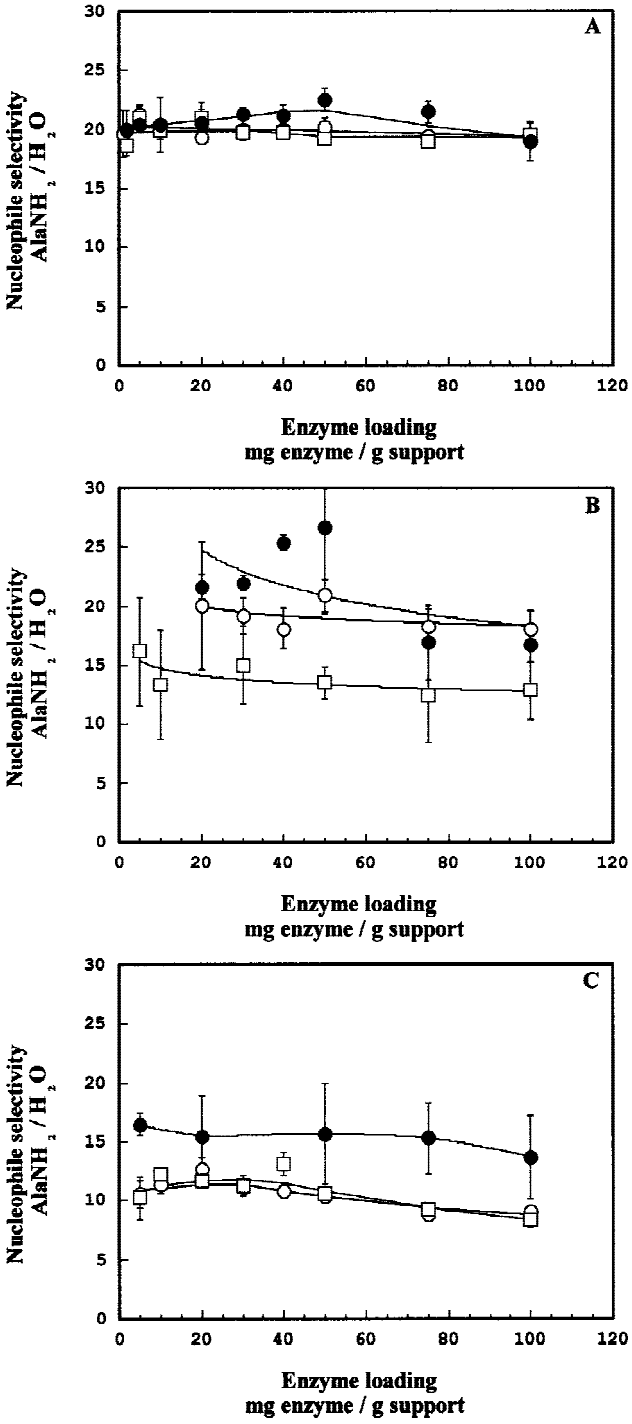

Figure 3. Nucleophile selectivity (rate of dipeptide synthesis/rate of acyl donor hydrolysis) as a function of enzyme loading and support material for dipeptide synthesis reactions using AlaNH$_2$ as nucleophile; 20 m*M* acyl donor and 30 m*M* nucleophile were used. Acyl donors used: BzTyrOEt (A), AcPheOEt (B), and BzAlaOMe (C). Supports: PAm 106–180 (○); PAm 300–500 (□); and Celite (●).

$$\frac{r_{\text{Synth}}}{r_{\text{Hydr}}} = \frac{k_{\text{Synth}}[\text{Nuc}]}{k_{\text{Hydr}}}, \quad (6)$$

meaning that for a given dipeptide synthesis reaction, the nucleophile selectivity will depend only on the concentration of nucleophile in the vicinity of the enzyme molecules, a conclusion that agrees with theoretical analyses of kinetic schemes for kinetically controlled peptide synthesis (Gololobov et al., 1988; Schellenberger and Jakubke, 1991). Then, a small variation of nucleophile selectivity with the onset of mass-transfer limitations can only mean that these limitations do not create a steep nucleophile concentration gradient inside the porous biocatalyst particles, and all the enzyme molecules operate at a similar nucleophile concentration, even at very high loadings and in poorly porous supports. Under conditions of high mass-transfer limitations, then, is the existence of a high acyl donor concentration gradient (demonstrated by the experiments on acyl donor selectivity) compatible with the existence of a low nucleophile concentration gradient (suggested by the experiments on nucleophile selectivity)? Yes, if the diffusion coefficients of these two species are different enough, higher for the nucleophile. This seems to be the case in the experiments depicted in Fig. 3: the acyl donors have molecular mass ranging from 207.2 to 313.4, while the unprotonated nucleophile has a molecular mass of 88.1. The bulkier acyl donors will then have lower diffusion coefficients than the nucleophile (according to the estimation method of Wilke and Chang, as cited by Reid et al. (1987), the diffusion coefficient of a solute at infinite dilution varies inversely with the 0.6th power of the molar volume, thus having an indirect relation with the molecular mass of the diffusing species), which means that in order to have diffusive transport of these species at a similar rate, a higher concentration gradient is needed for the acyl donors. Furthermore, the concentration of nucleophilic reagent used in these experiments (30 m*M*) was higher than the acyl donor concentration (20 m*M*). That makes the relative importance of a difference in concentrations slightly higher for the case of the acyl donor reagents. The importance of substrate concentration and diffusion coefficient ratios on internal diffusion effects has been theoretically realized before for two-substrate reactions (Indlekofer et al., 1992). Notice also that the curve described by Eq. (4)—linear with acyl donor concentration and Michaelis–Menten-like with nucleophile concentration—means that the predominant synthetic reaction is more sensitive to change in concentration of acyl donor than to change in concentration of nucleophile.

In order to test the hypothesis of the relative magnitude of diffusion coefficients, we measured nucleophile selectivity with a bulkier nucleophile reagent. The choice fell upon the dipeptide derivative $AlaPheNH_2$ (molecular mass 235.3), and the acyl donor used was BzTyrOEt (molecular mass 313.4). The diffusion coefficients of these species are not expected to differ a lot. The results are depicted in Fig. 4. With all supports, an appreciable decrease in nucleophile selectivity can be observed. When loading is changed from

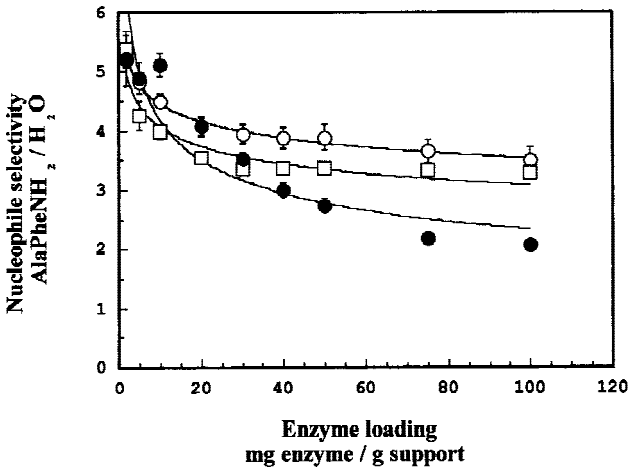

Figure 4. Nucleophile selectivity (rate of dipeptide synthesis/rate of acyl donor hydrolysis) as a function of enzyme loading and support material for tripeptide synthesis reactions; 20 m*M* BzTyrOEt and 30 m*M* $AlaPheNH_2$ were used. Supports: PAm 106–180 (○); PAm 300–500 (□); and Celite (●).

2 to 100 mg/g, the nucleophile selectivity decreases are as follows: from 5.2 ± 0.13 to 3.5 ± 0.22 (with PAm 106–180); from 5.4 ± 0.25 to 3.28 ± 0.09 (with PAm 300–500) and from 5.2 ± 0.46 to 2.08 ± 0.07 (with Celite). This comes in contrast with what was observed using $AlaNH_2$ as nucleophile (Fig. 3A), even though the reaction rates are lower in the tripeptide synthesis reaction (cf. Table I). As expected, with the bulkier nucleophile a strong decrease in selectivity with loading was observed, and this was more marked with the support where stronger mass-transfer limitations are expected to occur.

It should be noticed, however, that enzyme loading seems to have a stronger effect on acyl donor selectivity and stereoselectivity than on nucleophile selectivity, even when using this bulky nucleophilic molecule.

CONCLUSIONS

Given the importance of good selectivity to achieve an efficient use of biocatalysts, our goals with this work were to sort out what effect the existence of internal diffusional limitations has on selectivity and to evaluate to what degree they are disadvantageous to the performance of an immobilized enzyme preparation. We have shown that the selectivity of immobilized α-chymotrypsin-catalyzing peptide synthesis in organic medium can be dramatically influenced by mass-transfer limitations. The stronger the mass-transfer limitations, the lower the selectivity of the preparations toward acyl donors or different enantiomers (see Figs. 1 and 2). This agrees with the order of magnitude expected for reagent concentration gradients inside the porous biocatalyst particles: under mass-transfer limiting conditions steeper gradients are established to ensure the diffusive transport of fast reacting molecules when compared to others with lower intrinsic reactivity, leading to the apparent

reductions in selectivity observed. An exception to these observations is nucleophile selectivity when using a small substrate as nucleophile (Fig. 3). Since that molecule is predicted to have a diffusion coefficient significantly higher than the bulkier acyl donors used, it is diffusively transported at similar rates with less steep concentration gradients. The effect of mass-transfer limitations on nucleophile selectivity becomes important as soon as a bulkier nucleophile is used (see Fig. 4), because then similar concentration gradients exist for both substrates. Thus lowered apparent nucleophile selectivity is observed with stronger limitations (high enzyme loadings and Celite as support). Since the conclusions achieved are general concerning the nature of the enzyme immobilized and of the reactions carried out, these reductions in selectivity also call the attention to the importance of immobilization conditions on the efficiency of enzymatic conversions. The logical choice of support material will fall upon that which minimizes mass-transfer limitations, as long as it has other desirable characteristics, such as suitable mechanical properties and chemical compatibility with enzyme, substrate, and solvent used. Concerning enzyme loading, however, optimization of operation conditions will be needed, and a compromise between fast and selective conversions will have to be found to optimize the economy of the enzymatic transformation.

This work was financially supported by the Portuguese Foundation for Science and Technology (FCT, MCT) through the grant BD/2839/94 of the program PRAXIS XXI and the Swedish Research Council for Engineering Sciences (TFR).

References

Adlercreutz P. 1996. Modes of using enzymes in organic media. In: Koskinen AMP, Klibanov AM, editors. Enzymatic reactions in organic media. Glasgow: Chapman & Hall. p 9–42.

Barros RJ, Wehtje E, Adlercreutz P. 1998a. Mass transfer studies on immobilized α-chymotrypsin biocatalysts prepared by deposition for use in organic medium. Biotechnol Bioeng 59:364–373.

Barros RJ, Paar A, Wehtje E, Adlercreutz P. 1998b. Intrinsic α-chymotrypsin activity determination in acetonitrile containing small amounts of aqueous buffer. Ann NY Acad Sci 864:188–191.

Barros RJ, Wehtje E, Garcia FAP, Adlercreutz P. 1998c. Physical characterization of porous materials and correlation with the activity of immobilized enzyme in organic medium. Biocatal Biotransf 16:67–85.

Bernard P, Barth D. 1995. Internal mass transfer limitation during enzymatic esterification in supercritical carbon dioxide and hexane. Biocatal Biotransf 12:299–308.

Chen C-S, Fujimoto Y, Girdaukas G, Sih CJ. 1982. Quantitative analyses of biochemical kinetic resolutions of enantiomers. J Am Chem Soc 104:7294–7299.

Dordick JS. 1989. Enzymatic catalysis in monophasic organic solvents. Enzyme Microb Technol 11:194–211.

Clapés P, Adlercreutz P, Mattiasson B. 1990. Enzymatic peptide synthesis in organic media: Nucleophile specificity and medium engineering in α-chymotrypsin-catalyzed reactions. Biotechnol Appl Biochem 12: 376–386.

Gololobov MY, Borisov IL, Belikov VM, Svedas VK. 1988. Acyl group transfer by proteases forming acyl-enzyme intermediate: Kinetic model analysis. Biotechnol Bioeng 32:866–872.

Indlekofer M, Hoeschele M, Rizzi M, Reuss M. 1992. Kinetics and engineering studies of lipase-catalyzed transesterification in organic solvent. In: Tramper J, Vermüe MH, Beeftink HH, von Stockar U editors. Progress in biotechnology, Vol 8, Biocatalysis in non-conventional media. Amsterdam, The Netherlands: Elsevier. p 645–652.

Ison AP, Macrae AR, Smith CG, Bosley J. 1994. Mass transfer effects in solvent-free fat interesterification reactions: Influences on catalyst design. Biotechnol Bioeng 43:122–130.

Jönsson Å, Wehtje E, Adlercreutz P, Mattiasson B. 1996. Temperature effects on protease catalyzed acyl transfer reactions in organic media. J Mol Catal B: Enzymatic 2:43–51.

Lopez JL, Wald SA, Matson SL, Quinn JA. 1990. Multiphase membrane reactors for separating stereoisomers. Ann NY Acad Sci 613:155–166.

Luck T, Kiesser T, Bauer W. 1988. Engineering parameters for the application of immobilized lipases in a solvent-free system. In: Applewhite TH, editor. Biotechnology for the fats and oils industry. World Conference. Champaign, IL: American Oil Chemists' Society. p 343–345.

Nagashima T, Watanabe A, Kise H. 1992. Peptide synthesis by proteases in organic solvents: Medium effect on substrate specificity. Enzyme Microb Technol 14:842–847.

Reid RC, Prausnitz JM, Poling BE. 1987. The properties of gases & liquids. 4th edition. New York: McGraw-Hill.

Schechter I, Berger A. 1967. On the size of the active site in proteases. I. Papain. Biochem Biophys Res Commun 27:157–162

Schellenberger V, Jakubke H-D. 1991. Protease-catalyzed kinetically controlled peptide synthesis. Angew Chem, Int Ed Engl 30:1437–1449.

Silver MS, Matta MS. 1972. A comparison of the utilization of methyl and p-nitrophenyl esters in determining the specificity of α-chymotrypsin. Arch Biochem Biophys 151:62–67.

Ståhl M, Jeppsson-Wistrand U, Månsson M-O, Mosbach K. 1991. Induced stereoselectivity and substrate selectivity of bio-imprinted α-chymotrypsin in anhydrous organic media. J Am Chem Soc 113:9366–9368.

West JB, Wong C-H. 1986. Enzyme-catalyzed irreversible formation of peptides containing D-amino acids. J Org Chem 51:2728–2735.