Mass Transfer Studies on Immobilized α-Chymotrypsin Biocatalysts Prepared by Deposition for Use in Organic Medium

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Abstract: Mass transfer limitations were studied in enzyme preparations of α-chymotrypsin made by deposition on different porous support materials such as controlled pore glasses, Celite, and polyamides of different particle sizes. It is the onset of mass transfer limitations that determines the position of the activity optimum with respect to enzyme loading on each support. The evidence of various experiments indicates that internal diffusional limitations are the important mechanism for the observed mass transfer limitations. External diffusion was not found to play an important role under the conditions used, and it was also found that when immobilizing multilayers of enzyme the buried enzyme molecules are active to a large extent. An extreme situation is observed on Celite at very high loadings. Under these conditions, this support is expected to have its pores completely filled with packed enzyme molecules, and then it is the diffusion within the enzyme layer that determines the observed rate. As the enzyme loading increases, the area of contact between the deposited enzyme layers and the liquid solution inside the pores diminishes, causing a decrease on the observed rate of an intrinsically fast reaction which apparently is incongruous with the presence of more enzyme in the system. This work shows that mass transfer limitations can be an important factor when working with immobilized enzymes in organic media, and its study should be carried out in order to avoid undesired reduced enzyme activities and specificities. *Biotechnol Bioeng* **59**: 364–373, 1998.

Keywords: porous supports; internal and external diffusion; active site accessibility; enzyme loading; kinetically controlled dipeptide synthesis

INTRODUCTION

Immobilization of enzymes on porous supports for use in organic media is a broadly used strategy to eliminate some of the problems arising with the use of suspended enzyme powders. Suspended enzyme particles sometimes have a tendency to aggregate and attach to the walls of the reactor, specially at high hydration levels needed for the activity of some enzymes such as α-chymotrypsin (Clapés et al., 1992). Immobilization of the enzyme on a porous support reduces this problem, as the enzyme is spread on a large area and thus avoids the formation of aggregates (Dordick, 1989). In this way, a larger proportion of the active sites becomes available for catalytic function and mass transfer of substrates and products is facilitated (Adlercreutz, 1996). Other advantages with this strategy are that the enzyme recovery from the reaction medium becomes easier and it is also easier to pack immobilized enzyme into a column for continuous processing.

As enzymes are generally insoluble in the reaction media when organic solvents are employed, the immobilization procedures become extremely simple. In most cases there is no need for a strong binding force between the enzyme molecules and the support particles. A suitable immobilization procedure is deposition, where an enzyme solution in the appropriate buffer is allowed to wet the support material and enter its pores and is then evaporated under reduced pressure. In this way the only limits to the amount of enzyme to be immobilized are the solubility of enzyme in the solution used and the capacity of the support, in terms of pore volume, making it possible to perform multilayer immobilizations. This procedure has been widely used with good results to immobilize enzymes for use in organic media (Bovara et al., 1993; Day and Legge, 1995; Wehtje et al., 1993), especially for the synthesis of small peptides using proteases (Capellas et al., 1994, 1996; Clapés et al., 1992, 1995; Lozano et al., 1995; Jönsson et al., 1995).

Even though the procedure is so simple and used so often, not much is known about the enzyme preparations themselves, for example, what factors limit the activity or how the enzyme is distributed on the support. One specific problem that has to be investigated with immobilized preparations is that of mass transfer limitations. As with all forms of heterogeneous catalysis, this system is also potentially subject to the existence of this kind of limitations. Not much can be found in the literature about the specific problem of mass transfer limitations on immobilized enzymes operating in organic media. The problem with (nonimmobilized) sus-

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pended enzymes has been studied from a theoretical point of view by Kamat et al. (1992). Studies have been done on the operation of continuous reactors with immobilized lipases (Luck et al., 1988; Indlekofer et al., 1992; Ison et al., 1994), but these go seldom beyond the presentation of theoretical models, which are often not corroborated by experimental data. These models are then used to predict conditions under which the reactors can be operated in the absence of mass transfer limitations.

It has been reported before that the specific activity of enzymes immobilized by the deposition procedure depends strongly on the enzyme loading (Bovara et al., 1993; Capellas et al., 1996). In those works it was suggested that mass transfer limitations are responsible for the lowered enzyme specific activity at high loadings. Different mechanisms can be responsible for these limitations. Either some of the active sites are not accessible by the bulk liquid medium or there is diffusional limitation, which happens when substrate diffusion is the rate-limiting step of the process, giving rise to concentration gradients. In this case the enzyme molecules are no longer operating under the same concentration conditions existing in the bulk medium. Diffusional limitations can be further divided into external and internal, depending on whether the concentration gradients occur in the stagnant layer around the solid particles or in the pores inside the particles themselves.

Our goal with this work was to make a thorough study of what happens under conditions of mass transfer limitations and to distinguish which of the above mechanisms (inaccessibility or internal or external diffusion) is responsible for them. α-Chymotrypsin (CT, E.C. 3.4.21.1) was chosen as a model enzyme because it is available in a pure form and also because of the large amount of work done already with this enzyme using the deposition procedure (Capellas et al., 1996; Clapés et al., 1992, 1995; Lozano et al., 1995; Jönsson et al., 1995; Wehtje et al., 1993). Immobilizations were carried out using different support materials with varying physical characteristics that can influence mass transfer. The reactions chosen to measure enzyme activity were kinetically controlled dipeptide synthesis reactions, and the initial rates were determined. Three different dipeptide synthesis reactions were carried out in order to study the effect of different intrinsic reaction speeds. A differential reactor setup was used to study external mass transfer by varying the speed of the fluid around the particles in a packed bed (Bailey and Ollis, 1986).

We have previously correlated the observed enzymatic activity with the physical characteristics of the support materials (Barros et al., 1997), on the basis of the effects on the internal mass transfer rates, which we hereby demonstrate to be the rate-limiting process. In that work, the so-called ''support specific parameter,'' defined as the paricle diameter divided by the square root of the product of porosity and volumetric porosity, was introduced. The rate obtained on different supports at a constant enzyme loading was successfully correlated with this parameter.

MATERIALS AND METHODS

Chemicals

Bovine pancreas α-chymotrypsin (specific activity 52 BTEE U (mg solid)$^{-1}$), bovine serum albumin (BSA, Fraction V, 96–99%), *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), and triethylamine (TEA) were purchased from Sigma Chemical Co. (St. Louis, MO). *N*-Acetyl-L-phenylalanine ethyl ester (AcPheOEt), *N*-benzoyl-L-tyrosine ethyl ester (BzTyrOEt), *N*-benzoyl-L-alanine methyl ester (BzAlaOMe), and L-alaninamide hydrochloride ($AlaNH_2 \cdot HCl$) were purchased from Bachem Feinchemikalien AG (Bubendorf, Switzerland). Acetonitrile (HPLC grade) was from Lab Scan (Dublin, Ireland), and glacial acetic acid and Tris(hydroxymethyl)aminomethane (Tris) were from Merck (Darmstadt, Germany).

Supports

Polyamide 6 granules (Accurel EG700) of about 3-mm diameter were a kind gift from AKZO (Obernburg, Germany). The polyamide granules were cryogenically ground using a stainless steel Waring commercial blender. The resulting powder was sieved on a Retsch vibrating device from Retsch (Haan, Germany) with water flow using sieves of 75-, 106-, 180-, 300-, and 500-μm nominal size (also from Retsch) to obtain fractions of different particle size (denoted PAm 75–106, PAm 106–180, PAm 180–300, and PAm 300–500). Celite (30–80 mesh) was from BDH (Poole, UK). It was washed with ethanol to remove organic matter, followed by several washings with deionized water. Between each washing the supernatant was carefully decanted to remove fines. It was then incubated overnight with 10% nitric acid at room temperature, further washed thoroughly with Millipore water, and finally dried overnight at 80°C. Glyceryl-controlled pore glasses of 500 (GG-500) and 3,000 Å(GG-3000) nominal pore diameter and mesh size 200–400 were obtained from Sigma (St. Louis, MO).

Support Characterization

Determinations of specific surface area, area distribution with pore diameter, porosity, skeletal density, pore size distribution, and particle size distribution were performed on all the supports studied as described elsewhere (Barros et al., 1997).

Enzyme Inactivation

α-Chymotrypsin (CT, 30 mg) was incubated for 2 days in 30 mL of 1 m*M* phosphate buffer, pH 7.8, containing 5% acetonitrile and 8.4 mg of TPCK (20-fold molar excess in relation to CT). TPCK was not completely soluble in the medium employed, so it was suspended with gentle agitation on a rocking shaker (Swelab Instrument, Stockholm, Sweden). After this time, the suspension was filtered

through glass wool three times and applied to gel filtration pre-packed disposable PD-10 columns (Pharmacia, Uppsala, Sweden). Aliquots of 2.5 mL were applied to each column and eluted with 3.5 mL of Millipore water. The columns were subsequently washed by passing 25 mL of Millipore water and reused. The fractions collected from each elution were pooled, frozen (−80°C), and freeze-dried for 24 h at 0.5 mbar and −50°C. A white powder, presumed to be inactivated enzyme, was recovered (25.3 mg). The activity of this preparation as determined spectrophotometrically by the hydrolysis of *N*-benzoyl-L-tyrosine ethyl ester in aqueous medium was negligible. Also the presence of inactivated enzyme did not influence the performance of fresh CT, which rules out contamination with TPCK. The specific activity obtained in the organic medium employed, when immobilizing the inactivated enzyme on Celite at 30 mg (g loading)$^{-1}$ and using the synthesis of the dipeptide AcPheAlaNH$_2$ (see procedure below) was of 2.5×10^{-3} μmol min^{-1} (mg of solid)$^{-1}$. This is about 2 orders of magnitude lower than that obtained with non-inactivated enzyme deposited on the same support. As we will see below, under these conditions the reaction with the fresh enzyme preparation is strongly mass transfer limited, so this comparison is actually a higher limit.

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 of CT per gram of support, adjusted through the concentration of enzyme solution used. The preparations containing inactivated enzyme or BSA were prepared exactly the same way, with the aqueous enzyme solutions containing the appropriate proportions of fresh and inactivated CT or BSA.

Reactions

The reactions followed were the CT-catalyzed synthesis of the dipeptides *N*-acetyl-L-phenylalanyl-L-alanylamide, *N*-benzoyl-L-tyrosyl-L-alanylamide, and *N*-benzoyl-L-alanyl-L-alanylamide. The reaction solvent was acetonitrile containing 5 vol % aqueous buffer (50 m*M* Tris-HCl, pH 7.8). The concentrations of reactants were 20 m*M* of the acyl donor (AcPheOEt, BzTyrOEt, or BzAlaOMe), 30 m*M* AlaNH$_2$ · HCl, and 30 m*M* TEA, used to neutralize the hydrochloride of alaninamide and enhance its solubility and nucleophilicity. The reactions were carried out in 4-mL screw-cap vials (Chrompack) containing 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 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 (Shimadzu SCL-6A) 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 volumetric proportions depending on the acyl donor used: 66:29:5 for AcPheOEt and BzTyrOEt and 75:20:5 for BzAlaOMe and detected spectrophotometrically at 254 nm. The conversions were calculated from the peak areas. For each reaction, the initial rate was estimated from the slope of the straight line fitted by linear regression to the dipeptide conversion vs time plot (usually six data points with conversions under 20%). Under the conditions used, hydrolysis of AcPheOEt, yielding *N*-acetyl-L-phenylalanine (AcPheOH) occurred to a minor extent. Usually the initial rate for this reaction was 15 to 20 times slower than the corresponding synthesis reaction. This means that the yield of hydrolysis product was always well under 2% during the measurements, so that any effects of the accumulation of this compound on initial synthetic rates can safely be ignored.

Study of External Mass Transfer

The differential reactor setup is represented on Fig. 1 and consisted of a slight modification of that described by Bailey and Ollis (1986). The reactor itself was a glass cylinder of precision glass (Viridian) of 4.95-mm diameter. The enzyme preparation (20 mg of CT per g of PAm 300–500) was packed between two microporous polypropylene sieves of 1.50-mm thickness (PIAB AB, Stockholm, Sweden). A large amount of support particles without enzyme were also used so that the reactor would have a convenient length-to-diameter ratio, with the active preparation in the middle, to avoid inlet and outlet mixture effects. An HPLC pump (Shimadzu LC 6A) was used to create a constant flow which was changed in different experiments from 0.1 to 2.0 mL min^{-1}. Samples were taken at regular time intervals from the tank, which consisted of a 20-mL glass bottle with a rubber cap perforated to fit the inlet and outlet tubes (flow

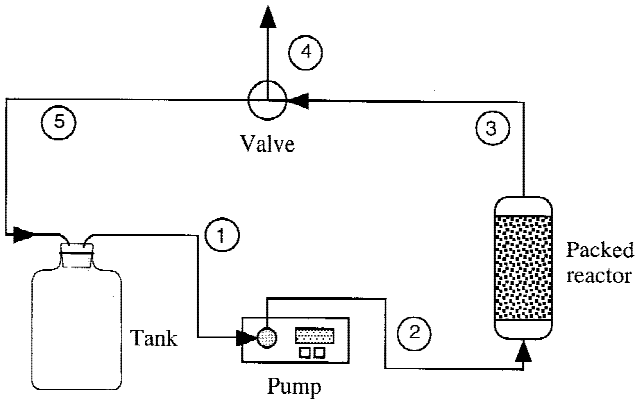

Figure 1. Setup of the differential reactor used to study external mass transfer.

lines 5 and 1 in Fig. 1), and from flow line 4 via a three-way valve, and analyzed by HPLC as described above. AcPheOEt or BzTyrOEt were used as acyl donors. For the AcPheOEt reaction, 12 mg of enzyme preparation were used with a total weight of 154 mg of solid particles, and for the BzTyrOEt reaction 6 mg of enzyme preparation were used with a total weight of 139 mg of solid particles. Before starting the sampling, lines 1, 2, 3, 5, and the reactor were continuously flushed first with 3 mL of solvent and then with reaction mixture until the composition in line 5 was constant. After this, the loop was closed by placing lines 1 and 5 in the tank. A 10-mL amount of reaction medium was placed in the tank. A simple mass balance indicates that the rate of accumulation of products inside the tank corresponds to the rate of the enzymatic reaction in the packed bed reactor. The initial rate for each reaction was estimated as described above using the samples taken directly from the tank using a syringe to perforate the rubber cap. Samples taken from line 4 were used just to check that the conversions per pass were not too high, which would mean that there would be large concentration gradients inside the reactor making it nondifferential. The plots of conversion vs time of the samples taken from line 4 and from the tank were perfectly parallel straight lines, which means that this problem is negligible. The maximum distances between these lines were 4.2% for the AcPheOEt reaction and 13.0% for the BzTyrOEt reaction with the lowest flow rate.

RESULTS AND DISCUSSION

Support Characteristics

The observed specific activity of α-chymotrypsin immobilized by the deposition procedure catalyzing the synthesis of $AcPheAlaNH_2$ strongly depends on the enzyme loading. However, the specific activity profiles show a characteristic shape with all the porous supports used. As shown in Fig. 2, each support has an optimum loading at which the specific activity is maximized. Similar profiles have been observed previously with Lipoprotein Lipase deposited on Celite (Bovara et al., 1993). The observed activity is lower at lower enzyme loading due to direct inactivation of the enzyme by the support. This is probably because there is enzyme conformational change/unfolding at the support surface. This effect has been observed before, and that decreases with the increasing coverage of the support by the enzyme at increased loading (Bosley, 1991; Wehtje et al., 1993; Day and Legge, 1995). At higher enzyme loading the onset of mass transfer limitations poses another difficulty that prevents the enzyme from exerting its activity at a higher rate. Similar profiles were observed even with non-immobilized Chymotrypsin suspended in ethanol when the amount of suspended enzyme was varied (Yamamoto and Kise, 1993). In the case of these observations, it is the size of the enzyme aggregates that changes with increased amount of enzyme, and thus mass transfer limitations become more important. On the other hand, there is always a very small amount of enzyme that is inactivated by the medium employed, which explains why the observed specific activity also decreases with decreasing amount of enzyme.

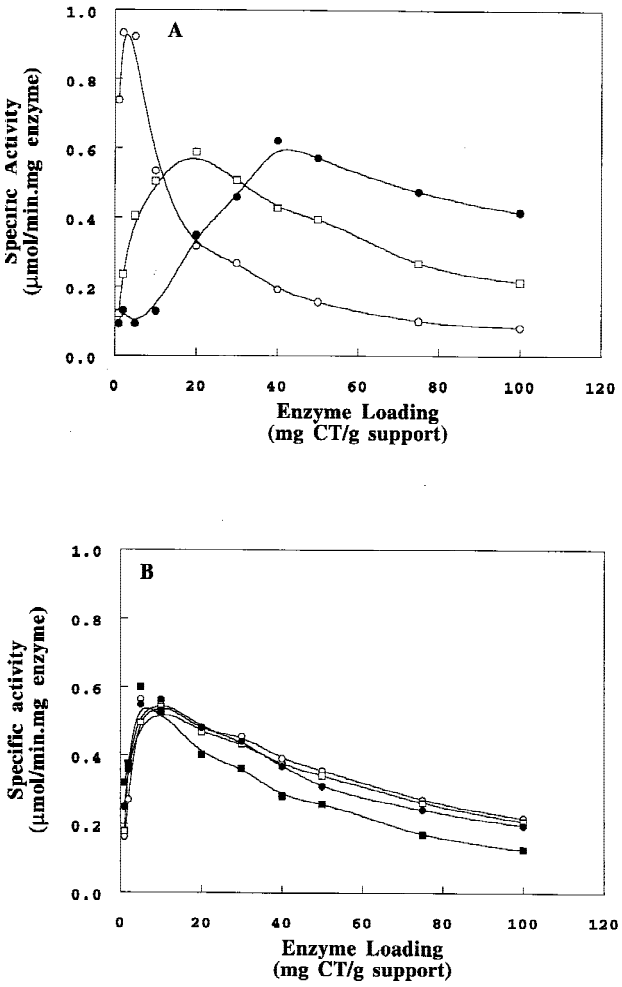

Figure 2. Specific activity variations with enzyme loading obtained in different supports for the α-chymotrypsin-catalyzed synthesis of $AcPheAlaNH_2$. (A) Celite (○); GG-3000 (□); and GG-500 (●). (B) Polyamides of different nominal particle sizes: 75–106 μm (○); 106–180 μm (□); 180–300 μm (●); and 300–500 μm (■).

It is the combination of these two effects, inactivation and onset of mass transfer limitations, that determines the position of the activity optimum in the immobilized preparations. Lower capacities for inactivation and stronger problems with mass transfer will give a maximum at low enzyme loading, which is the case of Celite. The opposite will happen with supports where mass transfer is easy, but a high capacity for enzyme inactivation exists, as is the case of the controlled pore glasses. Roughly, one can say that a support with a higher accessible surface area has also a higher capacity to inactivate proteins, because the degree of contact between them can be much higher. Also roughly, the mass transfer is much easier on a very porous support than on a less porous one, because then the substrates and products have much more free space to move and less obstacles to their diffusion. This influences the effective diffusion coef-

ficient inside the particles and thus internal mass transfer. Also the particle size influences internal mass transfer in the way that the distances that have to be traveled by substrates and products depend on the radius of the biocatalyst particle, and thus internal diffusion will become more limiting with larger particles.

Table 1 shows some of the physical characteristics of the support materials used. One can see that the positions of the maxima in Fig. 2 have a good qualitative correlation with these characteristics. The support with highest surface area and highest porosity, GG-500, exhibits the optimum at the highest enzyme loading, 40 mg g^{-1}, while Celite, which has the lowest porosity and the lowest surface area, shows the maximum at only 2 mg g^{-1}, the lowest value of all the supports studied. The other supports follow the expected tendency. For the polyamides of different particle sizes, the differences are not clear and all the maxima lie around 5–10 mg g^{-1}.

It can be seen more easily that the decrease in specific activity is caused by mass transfer limitations if the results are plotted in a different way, as in Fig. 3. There the reaction rate per amount of preparation is represented. It is clear that for each support studied the rate has the tendency to reach a constant plateau at a given enzyme loading. The value of this plateau represents the maximum rate at which the substrates can be transported from the bulk solution to the enzyme molecules placed inside the pores of the support particles. The rate obtained at the plateau also correlates well qualitatively with those physical characteristics of the support that influence mass transfer. More porous supports have this plateau at a higher rate (Fig. 3A), and supports with larger particle sizes have it at a lower value (Fig. 3B). We have studied this point more thoroughly (Barros et al., 1997) and found out that with basis on the definition of Thiele modulus, the different physical characteristics of the support can be associated in a so-called ''support characteristic parameter''. This correlates well with the activity observed on different supports under conditions of internal mass transfer limitation.

Table I. Physical characteristics of the supports studied.

Support	D_{mean}[a] (μm)	P[b] (cm^3 g^{-1})	ρ (g cm^{-3})	ε[c]	a[d] (m^2 g^{-1})
PAm 300–500	495	0.56	1.25[e]	0.41	1.98
PAm 180–300	278	0.56	1.25[e]	0.41	1.98
PAm 106–180	178	0.56	1.25[e]	0.41	1.98
PAm 75–106	112	0.56	1.25[e]	0.41	1.98
GG-500	88	1.07	2.41[f]	0.72	44
GG-3000	91	0.86	2.30[f]	0.62	5.9
Celite	146	0.06	1.59[e]	0.09	0.18

[a]Mean particle diameter as determined from particle size distributions.
[b]Porosity as determined by mercury porosimetry.
[c]Volumetric porosity, calculated from porosity and skeletal density.
[d]Accessible surface area, calculated as the surface area on pores with diameter larger than 200 Å.
[e]Skeletal density determined by mercury porosimetry.
[f]Skeletal density determined by helium picnometry.

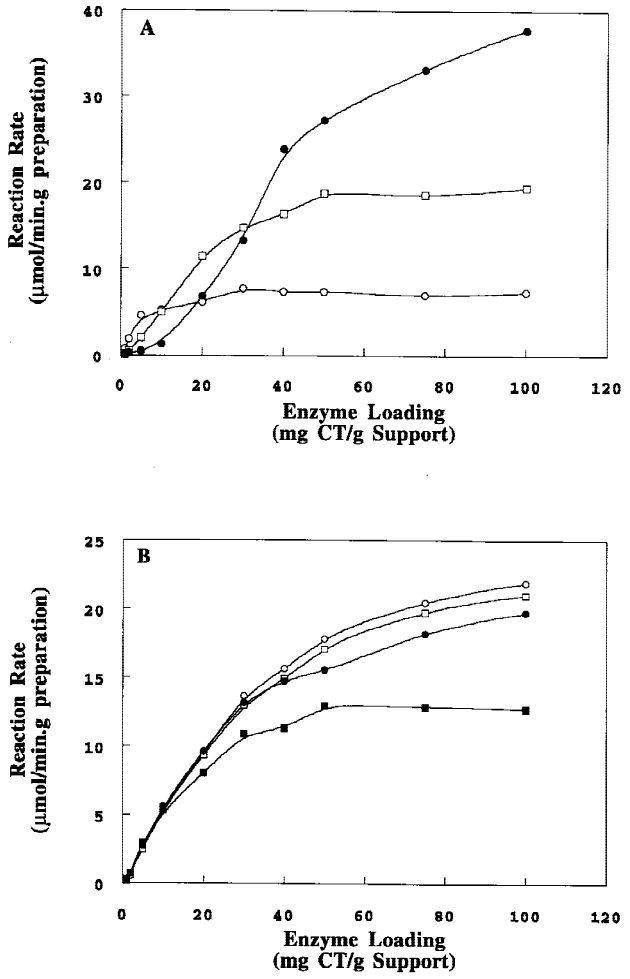

Figure 3. Different representation of the data in Fig. 2. Activity per amount of preparation is plotted instead of specific activity. (A) Celite (○); GG-3000 (□); and GG-500 (●). (B) Polyamides of different nominal particle sizes: 75–106 μm (○); 106–180 μm (□); 180–300 μm (●); and 300–500 μm (■).

Support Coverage

Given the differences in accessible surface areas, the same amount of enzyme on different supports represents a different coverage of the support. One could think that only the outermost layer of enzyme is active or that there is a limit to the number of layers that are active. Therefore we have plotted the data of reaction rates vs support coverage for some of the supports. This is represented on Fig. 4. The accessible surface areas present in Table I were used. The calculated value for monolayer coverage is represented, and was obtained assuming that each enzyme molecule occupies 51 × 40 Å and has a molecular weight of 25,000, the dimensions of a CT molecule (Stryer, 1988). This means that 1 mg of CT will occupy 2.0 m^2. It can be seen that the rate of the reaction increases even with higher loadings than monolayer coverage for all supports. Even for GG-500, where there is only data up to monolayer coverage, the tendency is for the rate to increase. This rules out the idea that only the outermost layer of enzyme is active, because

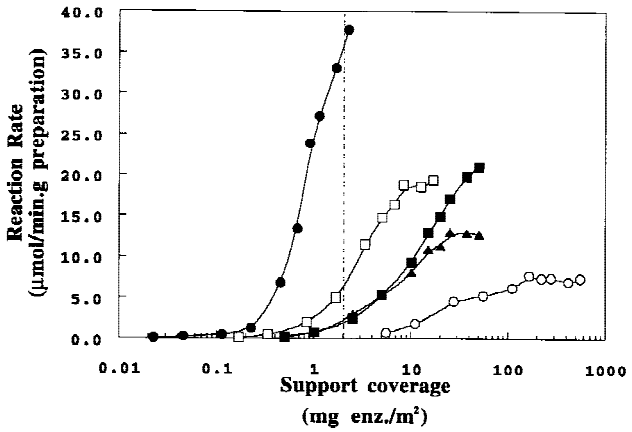

Figure 4. Effect of the support coverage on the rate obtained with different supports. To calculate the coverages, the accessible surface areas (Table I) were used. Monolayer coverage (dashed line) was calculated assuming that a CT molecule as a molecular weight of 25,000 and occupies an area of 51 Å× 40 Å(Stryer, 1988). Celite (○); GG-3000 (□); GG-500 (●); PAm 106–180 (■); and PAm 300–500 (▲).

adding enzyme to the support well beyond this value still increases the observed rate. This is not surprising, because there are always empty spaces between packed enzyme molecules where solvent and small substrate molecules can circulate, and reach the enzyme active sites buried under other layers of enzyme (Faber, 1991). On the other hand, one cannot say that there is a constant upper limit to the thickness of the enzyme layer that shows activity on all the supports. The plateaus are obtained at quite different coverages for different supports. While with Celite the plateau is obtained with 200 mg m^{-2} (about 100 enzyme layers), it appears with only 7 mg m^{-2} for GG-3000 and 30 mg m^{-2} with PAm 300–500 (3 and 15 layers, respectively). Comparing the curves for the polyamides of different particle size, it can be seen that even though the materials have the same surface area the plateaus are reached at different coverages. In the extreme case of Celite, where the calculated enzyme layer thicknesses at high loadings start to be comparable with particle sizes (1.4 vs 146 μm at 100 mg/g), layer diffusion might be the limiting mechanism. In all other cases, however, the evidence indicates that this is not the mechanism that determines the position of the rate plateaus with each support, but rather that there is some other factor responsible for their onset.

External Mass Transfer

The experimental setup represented in Fig. 1 allows a perfect control of the speed of fluid motion around the particles packed inside the reactor by changing the fluid flow rate. The higher the fluid speed, the thinner the stagnant layer will be around the particles, and thus the lower the external mass transfer limitations will be. The results obtained are represented in Fig. 5. Two reactions were studied at different flow rates. It can be seen that for the synthesis of $AcPheAlaNH_2$, the flow rate does not have any influence on the observed activity, which rules out any external mass transfer limitations within the range studied (0.1–2.0 mL min^{-1}). For the faster synthesis of $BzTyrAlaNH_2$, however, there is a trend for the reaction rate to increase with increasing flow rates, indicative of the presence of some external mass transfer limitation at the lower flow rates. In order to compare the fluid motion around the particles in this system and inside the capped vials used for all the other reactions here reported, the Reynolds number was estimated in each situation. The density of the support material plays a very important role in the case of the shaking vials. If it is too close to that of the liquid, the particles will have no movement relative to the surrounding fluid. If it is too low or too high, the particles will float or sink to the bottom of the vial, and thus their movement relative to the liquid will also be hampered. The skeletal density data of the particles is also shown on Table I. The Reynolds number was estimated to vary between 0.15 and 3.0 in the packed bed reactor for flow rates of 0.1 and 2.0 mL min^{-1}, respectively. If it is admitted that the shaking of the vials is good enough that the speed of the particles in relation to the liquid is the same as on particles falling free in the liquid, a generally used engineering condition, then the estimated Reynolds number is 2.7, very near the upper limit of the range studied. For both reactions this is already on a rather flat region of the plots on Fig. 5. On the basis of these results and calculations, we believe that in this case external mass transfer is not the predominating mechanism for the onset of mass transfer limitations at high enzyme loadings.

Intrinsic Reaction Rate

Having the acyl donor specificity of CT in mind, reactions were run using acyl donors that react at considerably different intrinsic speeds, but that should have diffusion coefficients of the same order of magnitude. Figure 6 shows the

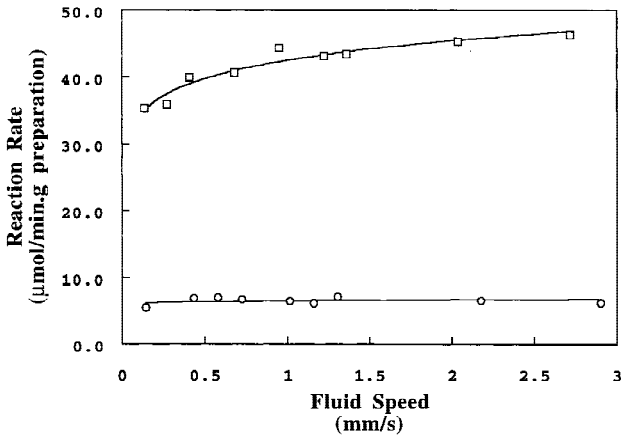

Figure 5. Effect of the variation of the linear velocity of liquid around the packed particles on the reaction rate. CT was immobilized in PAm 300–500 at a loading of 20 mg of CT (g of PAm 300–500)$^{-1}$. Two different reactions were studied: The synthesis of $AcPheAlaNH_2$ (○) and the intrinsically faster synthesis of $BzTyrAlaNH_2$ (□).

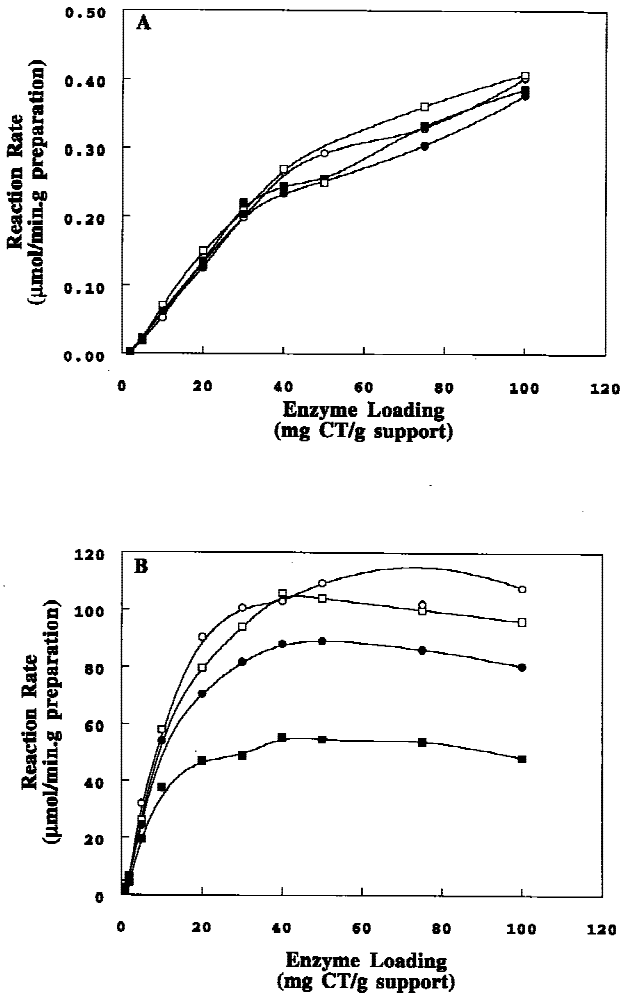

Figure 6. Use of different reactions. (A) The intrinsically slower synthesis of $BzAlaAlaNH_2$. (B) The intrinsically faster synthesis of $BzTyrAlaNH_2$. The supports used were polyamides of different nominal particle sizes: 75–106 μm (○); 106–180 μm (□); 180–300 μm (●); and 300–500 μm (■). These plots should be compared with Fig. 3B.

results obtained when running reactions using BzAlaOMe (Fig. 6A), a slower acyl donor than AcPheOEt (Fig. 3B), and BzTyrOEt (Fig. 6B), a faster one, with the polyamides of different particle sizes. Comparison of the data for these three reactions shows that the faster the reaction, the more the differences are visible between the different polyamides. While with BzAlaOMe the enzyme shows approximately the same performance on all polyamides (Fig. 6A), the differences start to be noticeable with AcPheOEt (Fig. 3B) and are rather clear with BzTyrOEt (Fig. 6B), the observed activities being higher with the supports that have smaller particle size. This is a signal that internal diffusion is an important mechanism of mass transfer limitation.

The profiles also reach a plateau at lower enzyme loading as the intrinsic reaction speed increases. The slowest reaction has the profile that approximates better the linear situation expected in the absence of mass transfer limitations (Fig. 6A), while the fastest shows the biggest deviations from that situation, even with a slight decrease in rate for enzyme loading above 50 mg of CT (g of support)$^{-1}$. This means the apparent specificity of the immobilized enzyme toward the fastest reagents will be lowered with increased enzyme loading. Relaxed specificity is a signal that diffusional limitations are affecting the observed rates. As can be seen in Fig. 7, where ratios of the rates obtained with different acyl donors are plotted vs enzyme loading, the apparent specificity decreases considerably with increasing enzyme loading, when harsher mass transfer limitations are expected to occur. With PAm 180–300, when going from 2 to 100 mg of CT/(g of support), the specificity BzTyrOEt/AcPheOEt diminished from 9 to 4, more than 2-fold decrease, and the specificity AcPheOEt/BzAlaOMe diminished from 250 to 50, a 5-fold decrease.

Enzymatic Activity Content at Constant Enzyme Loading

Another way to determine if the mass transfer problem is the presence of diffusional limitations is to compare with what happens if the activity content of the immobilized enzyme preparation is diminished without changing total enzyme loading. In order to do that, immobilization of active enzyme was performed in the presence of enzyme previously inactivated by the irreversible inhibitor TPCK. Keeping in each series of experiments the sum of active and inactivated enzyme constant, there were no major morphologic differences between the preparations, except for the total activity of the enzyme preparation. The synthesis of $AcPheAlaNH_2$ was studied using CT immobilized on two polyamides, PAm 106–180 and PAm 300–500, and in each at three different enzyme loadings: a low loading where no mass transfer limitations are supposed to occur, an intermediate situation and a high loading where the activities are already on the plateaus shown in Fig. 3. The fraction of active enzyme in the preparation was varied between 5 and 100%. The results, expressed as specific activity based on active enzyme weight, are represented in Fig. 8. It can be

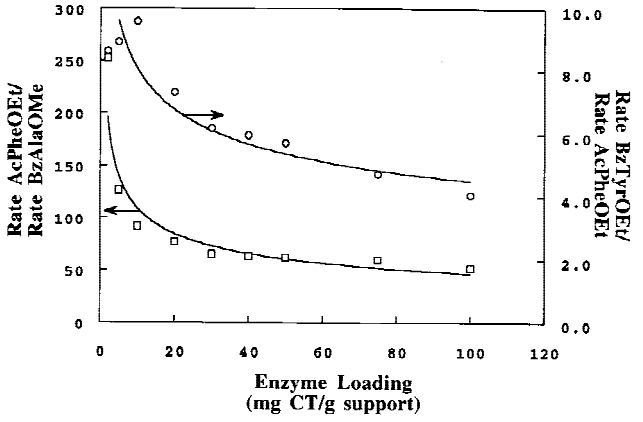

Figure 7. Effect of enzyme loading on the acyl donor specificity. (○) Specificity for BzTyrOEt compared to AcPheOEt; (□) specificity for AcPheOEt compared to BzAlaOMe. The support used was polyamide with nominal particle size 180–300 μm.

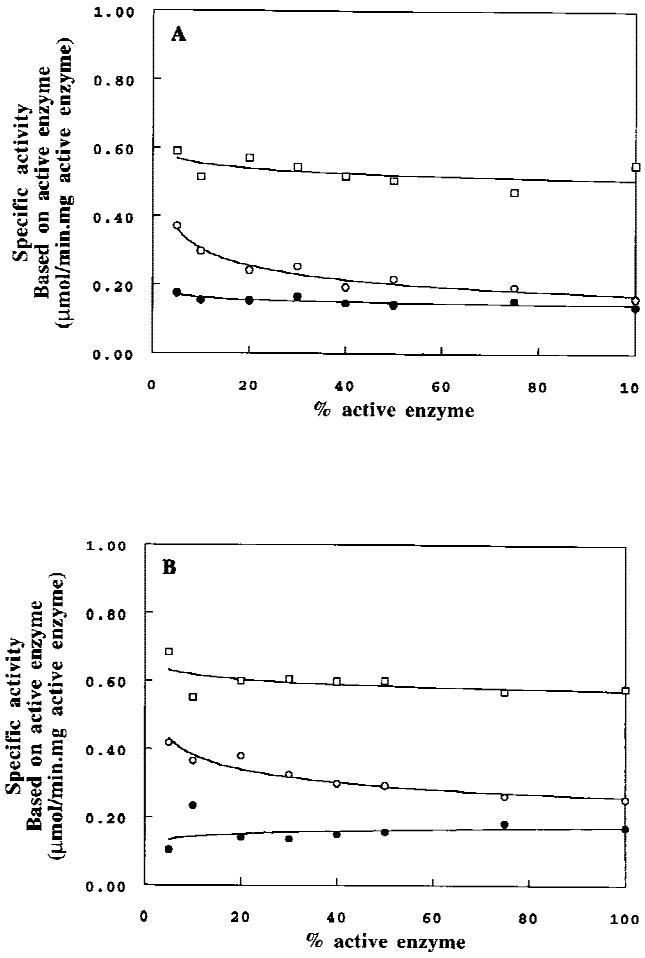

Figure 8. Effect of immobilizing different proportions of active and inactive enzyme on the specific activity of the active fraction. Two different polyamides were used with three different total protein loadings each: (A) PAm 300–500 and (B) PAm 106–180 with loadings of 100 (○), 10 (□), and 1 mg g^{-1} (●).

seen that all the plots are approximately flat within the whole range of ratios studied, except for those with 100 mg (g of total protein loading)$^{-1}$. With these preparations the specific activities diminished with increasing content of active enzyme. With PAm 300–500 the decrease was from 0.37 to 0.16 μmol min^{-1} (mg of active enzyme)$^{-1}$ when going from 5 to 100% active enzyme, a decrease of almost 60%. With PAm 106–180 the decrease was from 0.42 to 0.25 μmol min^{-1} (mg of active enzyme)$^{-1}$, which corresponds to 40% decrease. This decrease suggests that diffusional limitations are an important mechanism to explain the mass transfer difficulties experienced, a conclusion that is corroborated by the higher magnitude of the decrease on the larger particles. For 10 mg of total protein (g of support)$^{-1}$ a very slight decrease is observed, suggesting that at this enzyme loading this mechanism starts to play a role but is far from dominant. At 1 mg of total protein (g of support)$^{-1}$ the results suggest that no diffusional limitations are present. This is because the reaction rate is slow, and substrate diffusion is fast enough to meet the demands for optimal activity of the enzyme molecules on the support.

Analog experiments were carried out with Celite as support but using bovine serum albumin as the inert protein fraction instead of inactivated CT. The results obtained confirm those obtained with the polyamides. With a total protein loading of 5 mg g^{-1}, the specific activity decreases from 1.26 to 0.98 μmol min^{-1} (mg active enzyme)$^{-1}$ when increasing the active fraction form 20 to 100%. This indicates that diffusional limitations are already important at this loading. In fact, careful observation of Fig. 2A shows that the specific activity is at the optimum value at this loading, and as we have discussed before, this optimum exists due to the balancing of the effects of enzyme inactivation by the support and mass transfer limitations. With 30 mg g^{-1} total protein loading, well within the rate plateau represented in Fig. 3A, the decrease is much more dramatic when going from 3 to 100% active enzyme, from 0.56 to 0.19 μmol min^{-1} (mg of active enzyme)$^{-1}$, a whole 67% reduction in specific activity.

Correction of Porosity of the Enzyme Preparation

Careful observation of Fig. 3B shows that, even though the profiles are quite flat at loadings above 50 mg g^{-1}, an indication of mass transfer limitations, there are no large differences on the results observed using the three polyamides of smaller particle sizes. This suggests that for these preparations internal diffusion is being affected in such a way that the differences caused by the particle size are attenuated. The internal diffusion rate depends on the effective diffusion coefficient of the substrates inside the particle pores. This in turn depends on the proportion of free space available for the substrates to diffuse inside the porous matrix of the support, the volumetric porosity. It can be thought that in preparations where large amounts of enzyme has been immobilized, the volume occupied by the enzyme becomes an important proportion of the original volumetric porosity of the support, leaving less space available for substrate diffusion. Corrections for the volume occupied by the enzyme molecules in the polyamides and in Celite are presented in Table II. It can be seen that with the polyamides,

Table II. Volumetric porosities of the enzyme preparations depending on enzyme loading.

Enzyme loading (mg of CT (g of support)$^{-1}$.	ε^a	
	Celite	Polyamide
No enzyme	0.09	0.41
20	0.06	0.40
30	0.05	0.39
40	0.03	0.38
50	0.02	0.37
75	–	0.35
100	–	0.33

[a]The volumetric porosity for the preparations containing enzyme was calculated taking into account that 1 g of α-chymotrypsin occupies 1.03 cm^3. This last value is based on MW = 25,000 and an ellipsoid shape with radii of 51, 40, and 40 Å (Stryer, 1988).

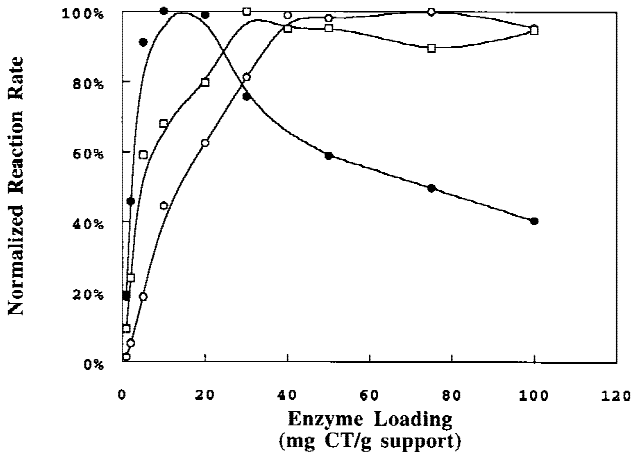

Figure 9. Normalized rate profiles obtained with the three different dipeptide synthesis reactions with Celite as the support. For the synthesis of $BzAlaAlaNH_2$ (○), 100% corresponds to 0.31 μmol min^{-1} (g of preparation)$^{-1}$; with $AcPheAlaNH_2$ (□), it is 7.8 μmol min^{-1} (g of preparation)$^{-1}$, while with $BzTyrAlaNH_2$ (●), it corresponds to 33 μmol min^{-1} (g of preparation)$^{-1}$.

at a loading of 100 mg g^{-1} the space occupied by the enzyme corresponds to 20% of the original pore volume of the support. With Celite the situation is even more complex. The porosity of this support is so low that the volume of 75 mg of enzyme is already higher than the pore volume available in 1 g of the support. Even with only 20 mg g^{-1}, already one-third of the porosity is occupied. The situation is probably even worse, because it is reasonable to think that the enzyme will not be able to penetrate the narrower pores of the support, and also because there might be a nonuniform enzyme distribution along the radius of the particles, with more enzyme near the surface than in the center. The results obtained with Celite confirm the problems predicted. Figure 9 shows the normalized rate profiles for the three reactions studied. As it had been observed with the polyamides, the faster the reaction, the lower the enzyme loading at which the profile reaches a maximum. It can be seen from this figure that even the slow synthesis of $BzAlaAlaNH_2$ reaches a very flat plateau above 40 mg g^{-1}, while with the quick synthesis of $BzTyrAlaNH_2$ there is even a decrease in the total rate with increasing enzyme loading above 10 mg g^{-1}. A similar situation has been reported before for this enzyme on a polyamide support (Capellas et al., 1996). This situation, which seems contradictory with the presence of a higher amount of enzyme, suggests that there is extremely severe clogging of the pores of the support with increasing amounts of enzyme. One can think that as the pores are about to be completely filled with enzyme, which is deposited on their walls, the area of the interface between the liquid medium inside the pores and the enzyme layer decreases. Due to diffusional limitation inside this quite thick layer most of the buried enzyme molecules experience a very low substrate concentration. In practice, even though more enzyme is present on the preparation, less of it experiences a substrate concentration comparable to that of the bulk medium, which translates into a lower reaction rate. At the highest loadings, probably all the support particles are completely covered with enzyme, and these preparations will in some way resemble non-immobilized enzyme.

CONCLUSIONS

We have shown that the reactions catalyzed by the enzyme preparations described can be subject to strong mass transfer limitations. The onset and extent of these limitations depends strongly on the characteristics of the support material used and on the intrinsic speed of the reaction studied. Apparently in the cases studied external mass transfer does not seem to be the predominating mechanism. However, this cannot be generalized in the case of other enzymatic reactions, especially if they are intrinsically faster. From the plots of observed rates vs enzyme coverage it seems that it is not the thickness of the enzyme layer deposited on the walls of the pores of the support that determines the onset of the observed limitations. The calculated thicknesses of the deposited layers of enzyme are several orders of magnitude lower than the particle sizes, in all cases except with Celite. This might be the only support where layer diffusion is limiting, but this has to be confirmed by further experiments. For layer diffusion to be rate-limiting it would take effective diffusion coefficients several orders of magnitude lower in the enzyme layer than in the pores, which is not very probable. Internal diffusion is then the dominating mechanism for the observed mass transfer limitations. More porous supports with lower particle sizes are less subject to these limitations, which affect more the intrinsically faster reactions. With some preparations that have high enzyme loading, reduction of the porosity of the final preparation and increased packing of the pores with enzyme molecules has proved to give rise to important unexpected effects.

This kind of study is important in order to optimize the operating conditions of an enzyme-catalyzed process. An immobilized enzyme operating under conditions of mass transfer limitation will show decreased apparent activity and selectivity. These undesired characteristics should be avoided in laboratory studies if meaningful conclusions are to be drawn and also in practical synthetic applications in order to optimize the performance of the enzyme. In the latter case, however, it is many times necessary to compromise with other factors. As an example, it is not practical to reduce the size of the biocatalyst particles too much, because then they become too small to be efficiently separated from the liquid medium, or give too large pressure drops in packed reactors. An ''economically optimal'' particle size should then be found that compromises the factors considered.

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