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Effect of Diffusional Resistances on the Action Pattern of Immobilized Alpha-amylase

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ABSTRACT

Alpha-amylase from Aspergillus oryzae *has been immobilized onto corn grits and porous silica (specific areas 180 and 440 $m^2\ g^{-1}$). Kinetic parameters of immobilized enzyme have been determined.*

Immobilization of alpha-amylase results in the formation of less polymerized products resulting in an apparent decrease in the number of transglycosylation reactions, for both maltotetraose and starch as substrates, when compared with free enzyme.

Diffusional limitations for substrate and products have been quantified in the case of the three supports used. External diffusional resistances were important in all cases for the reaction products, whilst they became negligible for the substrate in the case of silica supports. Moreover, internal transfer limitations were identified with silica 180 $m^2\ g^{-1}$ support. It was demonstrated that diffusional resistances were in direct relation to the apparent modification of the enzyme action pattern after immobilization.

Key words: alpha-amylase, immobilization, reaction products distribution, diffusional resistances.

NOTATION

C_B Concentration in the bulk phase (g dm^{-3})
C_S Concentration in the external surface of the support (g dm^{-3})

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d_p Average support particle diameter (m)
D_S Diffusivity in bulk solution ($m^2\ s^{-1}$)
D_S^{eff} Effective diffusivity in porous support ($m^2\ s^{-1}$)
G1 Glucose
G2 Maltose
G3 Maltotriose
G4 Maltotetraose
G5 Maltopentaose
G6 Maltohexaose
G7 Maltoheptaose
G>4 Maltotetraose + maltohexaose + maltoheptaose
k External mass transfer coefficient ($m\ s^{-1}$)
N Density of flux per external surface area of support ($g\ s^{-1}\ m^{-2}$)
r Observed reaction rate ($g\ dm^{-3}\ s^{-1}$)
R Support particle radius (m)
S External catalyst surface (m^2)
T Temperature (K)
V Reactive volume (dm^3)
V_c Catalyst volume (m^3)
ΔC $C_B - C_S$ ($g\ dm^{-3}$)
ε Void fraction of porous catalyst
η_w Viscosity of water (cP)
ρ Apparent density of supports ($kg\ m^{-3}$)
τ Tortuosity factor
ϕ Thiele modulus
ϕ_m Modified Thiele modulus

1 INTRODUCTION

Immobilization of enzymes onto water-insoluble supports has become a subject of interest for many industries. The advantages of this technique include the possibility of enzyme re-utilization, enhanced stability,[1] easy separation of the catalyst from the reaction mixture and ready application to automated continuous processes.

It has been demonstrated that immobilization of alpha-amylase changes the enzyme action pattern on starch-like substrates from its natural endoenzymic[2] to an exoenzymic mode.[3] This change in behavior has been attributed to steric inhibition of interactions between active sites of the immobilized enzyme and amylaceous substrates.[4] The influence of transfer limitations for the substrate on enzyme activity pattern has been discarded, since the stirring speed had no effect on the rate of starch conversion.[5] Nevertheless, in the case of alpha-amylase, which involves a reaction with intermediates and formation of multiple products, diffusional resistances can affect not only the overall rate of hydrolysis, but also the products distribution resulting from enzyme activity, by modifying the concentration of these different species in the microenvironment of the enzyme. It is thus of interest to

quantify the importance of diffusional resistances for the different products and substrate. By using a short-chain-length substrate (maltotetraose) the action patterns of free and immobilized alpha-amylase have been studied in detail and compared. The effect of products and substrate diffusional limitations, in relation to the observed apparent changes in action pattern, has been investigated.

Since alpha-amylase has many applications in the food and fermentation industries, it is not surprising that a number of methods for immobilizing the enzyme have been developed.[6] Covalent linking onto two different supports (corn grits and silica) was chosen for the present work. Corn grits constitute an inexpensive and widely available support with good mechanical properties. Their high (30%) lignocellulosic content allows their utilization in packed-bed reactors at pilot scale.[7] Moreover, their macroporous structure is a useful factor in diminishing diffusional problems. Porous silica is available with a great variety of specific areas and pore diameters, allowing the study of the influence of these parameters on immobilized enzyme activity and specifity, and on diffusional resistances.

2 MATERIALS AND METHODS

2.1 Enzyme

Fungamyl 800 L (Novo Industri, Bagsvaerd, Denmark), a liquid preparation of alpha-amylase from *Aspergillus oryzae*, was used. Protein concentration of the preparation was 225 g dm^{-3}.[8]

2.2 Immobilization of alpha-amylase onto insoluble supports

2.2.1 Corn grits

The enzyme was covalently linked onto corn grits (Eu-Grits 20/30, Eurama) by the method of Monsan,[9] avoiding the reduction step.[10] Average size of the support particle was 0·8 mm and its specific area was 1 m^2 g^{-1}.

Chemical activation of the support and immobilization of the enzyme were carried out by adding 200 cm^3 of the following reagents to 50 g of corn grits, in a 500-cm^3 screw-cap bottle, mixing by rotation. The conditions were: (a) for oxidation, 40 g dm^{-3} sodium metaperiodate solution in distilled water was used at 25°C for 24 h in the dark; (b) for amination, 1 mol dm^{-3} ethylene diamine solution in distilled water was used at 25°C for 72 h; (c) for activation, 2% glutaraldehyde solution in 0·05 mol dm^{-3} pyrophosphate buffer, pH 8·6, was used at 25°C for 6 h; and (d) for immobilization, 2 g dm^{-3} alpha-amylase solution in 0·2 mol dm^{-3} acetate buffer, pH 4·7, was used at 4°C for 24 h.

After each step, corn grits were washed with the solvent of the reagent used and with the solvent of the following reagent. Non-covalently-linked enzyme was eliminated with 1 mol dm^{-3} NaCl.

2.2.2 Porous silica

Spherosil (Rhone-Poulenc Ind.) with aromatic amino groups was also used as a support of differing porosities. Average particle diameter was 100–200 μm and volume of pores was 1 cm^3 g^{-1} in all cases. Specific areas were 6, 24, 180 and

440 $m^2 g^{-1}$ (silica 6, 24, 180 and 440, respectively). The respective average pore diameters were 550, 125, 15 and 7 nm.

The immobilization process was carried out by adding 20 cm^3 of each reagent to 100 mg of support in a 25-cm^3 screw-cap tube. Conditions were the same as for corn grits but, because silica was already aminated, the steps of oxidation and amination were not necessary. The duration of the activation step was 2 h.

2.3 Protein determination

The quantity of bound enzyme per gram of support was calculated by subtracting from the protein content in the initial solution, the residual protein in the same solution after the immobilization step and the protein discarded with 1 mol dm^{-3} NaCl. Protein was determined by the method of Lowry *et al.*[11] using alpha-amylase from *A. oryzae* type X-A (Sigma Chemical Co., St Louis, MO) as a standard.

2.4 Amylolytic activity measurement

2.4.1 Soluble enzyme

Amylolytic activity was measured using the DNS method as described previously[8] using 5% (w/v) soluble starch (Prolabo) as substrate, instead of maltodextrins.

2.4.2 Immobilized enzyme

Substrate solution (10 cm^3) was mixed with approximately 100 mg of support with covalently linked alpha-amylase, and the activity was measured as described above (subsection 2.4.1). The exact quantity of the support was determined gravimetrically, after overnight drying at 50°C.

2.4.3 Units

One unit of alpha-amylase activity (U) corresponds to the amount of enzyme that liberates 1 μmol of reducing sugars (maltose equivalent) per minute under the conditions of assay.

2.4.4 Immobilization yield

This is defined as the ratio of the specific activity of immobilized alpha-amylase to the specific activity of the free enzyme.

2.5 Reaction mechanism

2.5.1 Reaction conditions

The study of the alpha-amylase reaction mechanism has been conducted in a batch reactor, using a 2·8-cm diameter unbaffled vessel with a marine propeller agitator. A solution of 20 mg cm^{-3} maltotetraose (Boehringer Mannheim, North Ryde, NSW) in 0·02 mol dm^{-3}, pH 4·7, acetate buffer was used as substrate.

The reaction was started by addition of 20 mm^3 of Fungamyl 800 L, diluted 200 times with distilled water, to 2 cm^3 of substrate, or by addition of 20 cm^3 of substrate to adequate amount of immobilized alpha-amylase to obtain the same number of units as with the free enzyme. Incubations were made at 37°C (substrate and enzyme were previously heated to this temperature) during 24 h, with mechanical agitation. At regular time intervals, 25 mm^3 of the reaction mixture was

removed; diluted with equal volume of milliQ (Millipore) quality water, and frozen by immersion in liquid nitrogen to stop the reaction. Samples were thawed immediately before HPLC analysis.

In another series of experiments, a solution of 5% (w/v) soluble starch (Prolabo) in 0·02 mol dm^{-3}, pH 4·7, acetate buffer was used as substrate. Conditions for reaction were the same. Samples were purified before HPLC analysis.[12]

2.5.2 HPLC analysis

If necessary, samples were desalted by using a mixture of two ion-exchange resins (Amberlite IRA-67 and IR-120 from Rohm & Haas) prior to HPLC analysis.

The chromatographic system consisted of a Waters Associates (Mildford, MA) model M-6000 A pump, Rheodyne 7125 injector with a 20-mm^3 loop, Gilson model 131 differential refractometer, and a Waters 740 Data Module integrator. A Nucleosil C_{18} 10 μ column (4·6 × 250 mm) was used, with a guard column (30 mm) of the same packing. The eluent was water of milliQ quality, at a flow rate of 0·8 cm^3 min^{-1}, at 35°C. Standards of maltotriose (*G3*), maltotetraose (*G4*), maltopentaose (*G5*), maltohexaose (*G6*) and maltoheptaose (*G7*) were purchased from Boehringer Mannheim. Glucose (*G1*) and maltose (*G2*) were obtained from Sigma.

3 RESULTS AND DISCUSSION

3.1 Immobilization and kinetic constants of alpha-amylase

Alpha-amylase was immobilized onto two different kinds of porous supports: corn grits and aminated silica. As can be seen in Table 1, the amount of enzyme bound and the activity per unit weight of support increased with specific area. An exception was with silica 440 which showed an average pore diameter of 7 nm, too small to allow alpha-amylase penetration of the pores, the molecular dimension of the enzyme being 8 nm.[13] Consequently, binding appears to have occurred mainly on external surfaces of this matrix.

The observed decrease in immobilization yield (and hence in specific activity) is directly related to increase of enzyme concentration on the support, and can be

TABLE 1
Important Parameters for Alpha-amylase Immobilization onto Different Support Matrices

	Bound enzyme (%)	*mg enzyme* g^{-1} *support*	*U* g^{-1} *support*	*Immobilization yield* (%)
Corn grits[a]	91	7·3	502	24·4
Silica 6 m^2/g[b]	2	10·6	613	19·1
Silica 24 m^2/g[b]	4	22·8	686	10·0
Silica 180 m^2/g[b]	25	100·0	950	3·6
Silica 440 m^2/g[b]	5	24·2	519	7·4

[a] During immobilization step, 8 mg alpha-amylase are in contact with 1 g support.
[b] During immobilization step, 400 mg alpha-amylase are in contact with 1 g support.

TABLE 2
Kinetic Parameters of Immobilized Alpha-amylase[a]

	K'_M (g dm^{-3})	V_M (U g^{-1} support)	V_M (U mg^{-1} enzyme)
Enzyme linked onto corn grits	16·7	677·3	92·8
Enzyme linked onto silica 180 m^2 g^{-1}	52·6	1543·2	15·4
Enzyme linked onto silica 440 m^2 g^{-1}	40·0	793·7	32·8

[a] Experimental conditions: 100 mg support with covalently linked alpha-amylase are added to 10 cm^3 starch solution in acetate buffer pH 4·7, 0·1 mol dm^{-3}; the temperature is 37° C.

attributed to steric hindrances in the immediate vicinity of the enzyme molecules. These hindrances are probably caused by the shielding effect of the matrix and by the excessive packing of enzymes, which would render their active sites less accessible to the substrate. It is difficult to distinguish between these effects and the reduced diffusivity of the substrate in the porous medium. Thus, when enzymatic molecules are too tightly packed on the support, the rate of substrate degradation can exceed its diffusivity into the matrix. This is particularly so with macromolecular substrates like starch, and such reactions cannot be kinetically controlled.

Variations of kinetic constants (Table 2), calculated from Lineweaver–Burk[14] plots for alpha-amylase immobilized onto corn grits and porous silica (180 and 440), are consistent with these hypotheses. All K'_m values were greater than K_m (4 g dm^{-3} for the free enzyme), and they increased with decreasing specific activity. This could also be attributed to steric hindrances and diffusional limitations, rather than to real changes in enzyme substrate affinity.

A similar effect has been described[15] both for native and chemically modified beta-amylase immobilized onto Spherosil. Here, when the quantity of bound enzyme was increased, the relative activity was lowered. This was explained in terms of a partial lack of substrate in the vicinity of enzyme but the phenomenon was not quantified.

The kinetics of enzyme bound on a porous particle can be affected by external or internal diffusional resistances, which respectively correspond to the transport of the substrate and products from the bulk solution to the outer surface of the enzymic particle, and to the internal transport of these species inside the porous system of the particle.

Eadie–Hofstee[14] plots are useful for discerning between external (concave plots) and internal (sigmoïdal plots) diffusional limitations. Linear plots are found in the absence of these effects, i.e. for enzymes in solution.

In Fig. 1, Eadie–Hofstee plots for the studied immobilized preparations are presented, as evidence for the postulated hypothesis of the existence of diffusional resistances. Thus, external diffusional limitations are found for alpha-amylase bound onto corn grits and silica 440. Since enzyme molecules cannot penetrate the pores of the silica 440 during the immobilization phase, no internal diffusional

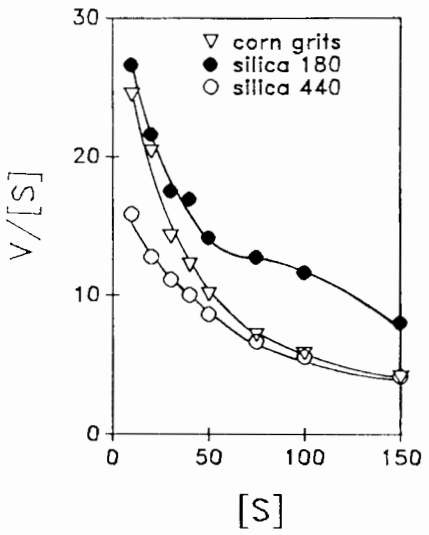

Fig. 1. Eadie–Hofstee plots for alpha-amylase immobilized onto corn grits and porous silica (specific areas 180 and 440 $m^2 g^{-1}$). V = reaction rate in U g^{-1} support. $[S]$ = starch concentration (mg cm^{-3}). (▽) Corn grits, (●) silica 180, (○) silica 440.

limitations are shown. However, when alpha-amylase is immobilized onto silica 180 (pores of 15-nm diameter), the reaction is affected by internal diffusional resistances.

3.2 Reactions with maltotetraose as substrate

It has been shown[16,17] that the reaction mechanism of alpha-amylase from *A. oryzae* involves transglycosylation reactions in addition to hydrolytic ones. This means that the enzyme transfers glucosyl residues to an acceptor containing a hydroxyl group. When this acceptor is water, hydrolytic reaction takes place. However, when the acceptor is a sugar, new oligosaccharides are formed.

Maltotetraose is a useful substrate for the study of alpha-amylase activity since, as all reaction products are recoverable, it enables not only hydrolysis but also transglycosylation reactions to be followed and quantified. Moreover, the small molecular weight and high solubility of this substrate, compared to starch, more commonly used as substrate, facilitate the use of HPLC analysis.

3.2.1 Hydrolysis products

Degradation products of *G4* by free and immobilized alpha-amylase are represented in Figs 2–4. These data show that, while the action pattern of free alpha-amylase on *G4* was characterized by the appearance of *G2* and *G3* as the most abundant products, and of *G1* to a much lesser extent (<4%), immobilized alpha-amylase exhibited an apparently more exoenzymic type of action. Thus, with the immobilized enzyme preparations, the relative amounts of *G1* and *G2* were higher, and of *G3* lower than with the free enzyme, both during the course of the reaction and at equilibrium (Tables 3 and 4). The increase in the ratio *G2*/*G3* and of *G1* production paralleled the decrease in specific activity on immobilization. These

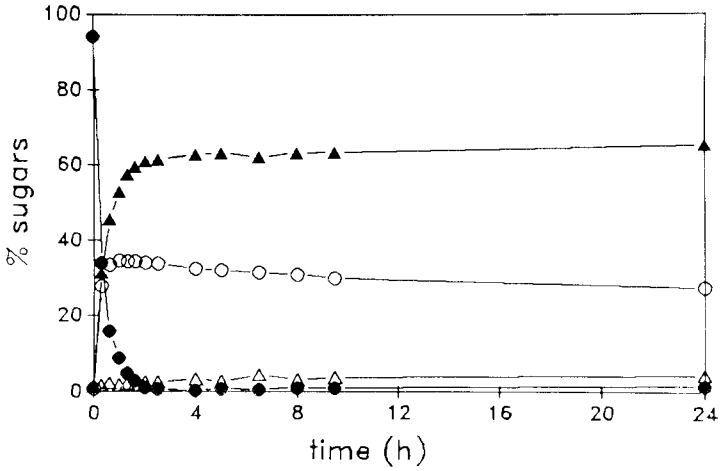

Fig. 2. Distribution of the products of maltotetraose degradation by free alpha-amylase. Fungamyl 800 L (20 mm^3) diluted 200 times with distilled water are added to 2 cm^3 substrate (a solution of 20 mg cm^{-3} maltotetraose in 0·02 mol dm^{-3}; pH 4·7, acetate buffer); the temperature is 37 C. (△) *G1*, (▲) *G2*, (○) *G3*, (●) *G4*.

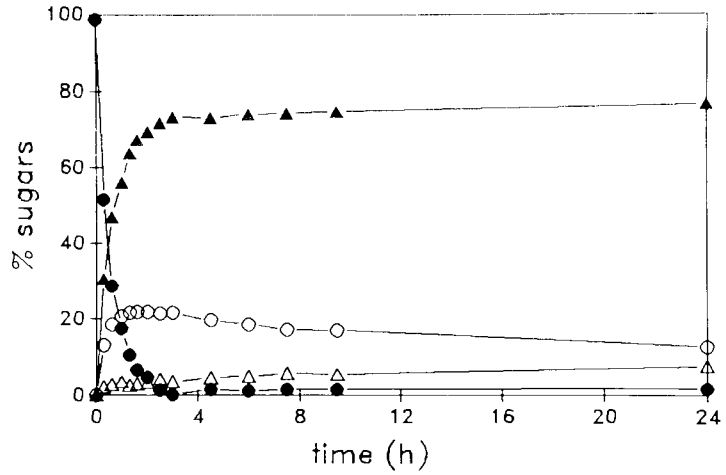

Fig. 3. Distribution of the products of maltotetraose degradation by alpha-amylase immobilized onto corn grits. Alpha-amylase (100 mg) bound to corn grits are added to 20 cm^3 substrate (a solution of 20 mg cm^{-3} maltotetraose in 0·02 mol dm^{-3}, pH 4·7, acetate buffer); the temperature is 37 C. (△) *G1*, (▲) *G2*, (○) *G3*, (●) *G4*.

two effects could both be related to decreasing substrate accessibility after immobilization. To investigate this hypothesis, diffusional resistances with *G4* as substrate were studied.

Changes in the proportion of reaction products with immobilized alpha-amylase, that correspond to a more exoenzymic action mechanism than seen with free enzyme, have been observed in studies with various substrates, e.g. starch,[3,5]

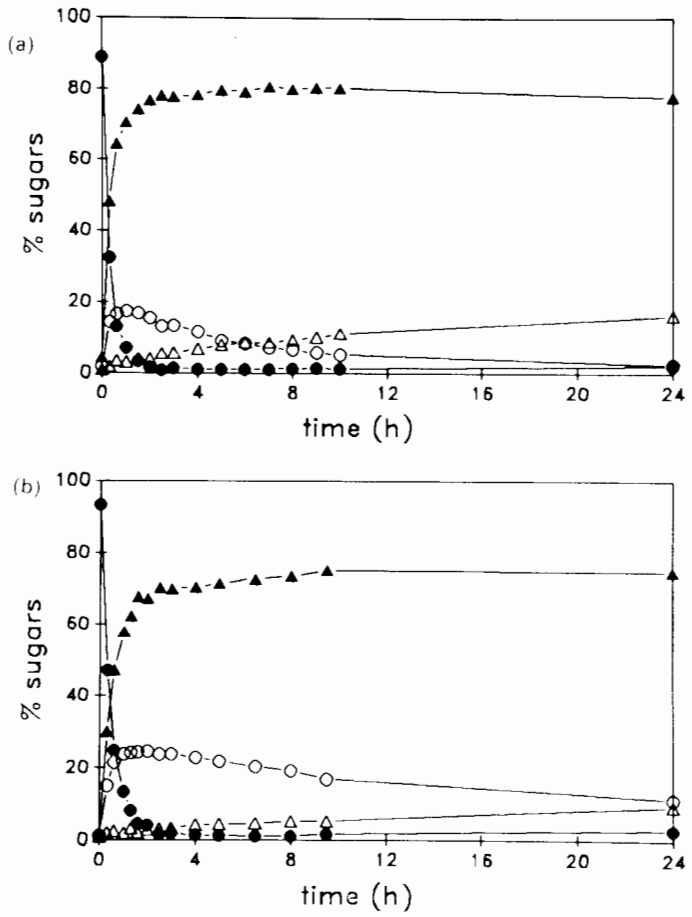

Fig. 4. Distribution of the products of maltotetraose degradation by alpha-amylase immobilized onto porous silica. (a) silica 180, (b) silica 440. Alpha-amylase silica 180 (50 mg) and alpha-amylase silica 440 (90 mg) are added to 20 cm^3 substrate (a solution of 20 mg cm^{-3} maltotetraose in 0·02 mol dm^{-3}, pH 4·7, acetate buffer); the temperature is 37 C. (△) *G1*, (▲) *G2*, (○) *G3*, (●) *G4*.

amylose[4,18,19] and amylopectin.[4,19] In general, immobilized alpha-amylase produced more oligosaccharides of low molecular weight (polymerization degree 1 8, mainly *G1* and *G2*) than the free enzyme. These results were attributed to a steric hindrance effect which was a function of both the porous nature of particle support (the hypothesis of diffusional resistances was rejected, because increased agitation of the reaction mixture had no effect on the overall rate[5,18]) and the extent of branching of the macromolecular substrate.[4,19]

3.2.2 Transglycosylation products

In Fig. 5 are shown transglycosylation products having a polymerization degree higher than 4, resulting from the action of free and immobilized alpha-amylase on *G4*. Profiles of transglycosylation products of free and silica 440 immobilized alpha-

TABLE 3
Hydrolysis of Maltotetraose by Free and Immobilized Alpha-amylase: Product Distribution (%) at 50% Substrate Hydrolysis[a]

	G1	*G2*	*G3*	*G4*	*G>4*	*G2/G3*
Free enzyme	2·0	22·0	20·0	50·0	6·0	1·1
Enzyme linked onto corn grits	2·5	30·5	13·5	50·0	3·5	2·3
Enzyme linked onto silica 180 $m^2 g^{-1}$	2·0	33·0	11·5	50·0	3·5	2·9
Enzyme linked onto silica 440 $m^2 g^{-1}$	2·0	27·5	14·5	50·0	6·0	1·9

[a] Experimental conditions: 20 cm^3 of a solution of 20 mg cm^{-3} maltotetraose in 0·02 mol dm^{-3} pH 4·7 acetate buffer is used as substrate; the amounts of immobilized alpha-amylase used are 100 mg for alpha-amylase bound on corn grits, 50 mg for enzyme-silica 180 and 90 mg for enzyme-silica 440; the temperature is 37 C.

TABLE 4
Hydrolysis of Maltotetraose by Free and Immobilized Alpha-amylase: Product Distribution (%) at Reaction Equilibrium[a]

	G1	*G2*	*G3*	*G4*	*G>4*	*G2/G3*
Free enzyme	4·4	65·5	27·6	1·7	0·8	2·4
Enzyme linked onto corn grits	7·6	76·7	12·8	1·8	1·1	6·0
Enzyme linked onto silica 180 $m^2 g^{-1}$	16·0	77·4	2·3	2·1	2·2	33·6
Enzyme linked onto silica 440 $m^2 g^{-1}$	9·3	76·2	11·1	2·2	1·2	6·8

[a] Experimental conditions: conditions are the same as in Table 3.

amylase are very similar, reaching concentrations higher than 6%. This maximum value decreases significantly to less than 4% when enzyme is grafted onto corn grits or silica 180. These products appeared only in early steps of the reaction, their hydrolysis occurring thereafter (Tables 3 and 4).

3.3 Study of diffusional resistances

The possible influence of external and/or internal diffusional limitations on the kinetics of the immobilized enzyme with maltotetraose as substrate, has been investigated. The results and their interpretation are given below.

The time scale of transfer processes relative to time concentration change enables us to use the quasi-stationary hypothesis.

3.3.1 External diffusion

The mass transfer equation is:

$$N = k(C_B - C_S) = k\,\Delta C \qquad (1)$$

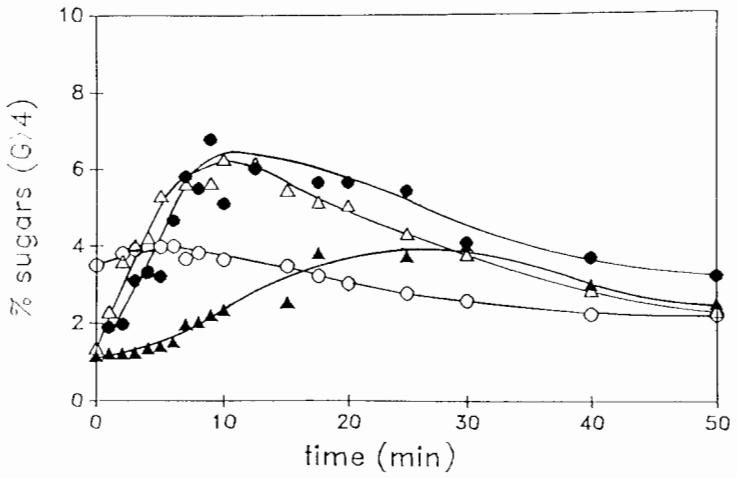

Fig. 5. Transglycosylation products of maltotetraose degradation by free and immobilized alpha-amylase. The conditions are the same as for Figs 2, 3 and 4. (△) Free enzyme, (▲) enzyme onto corn grits, (○) enzyme onto silica 180, (●) enzyme onto silica 440.

where N is defined per unit of external surface of support, k is the external mass transfer coefficient and C_B and C_S are, respectively, the substrate or product concentration in the bulk phase and at the external surface of the support. The values of k were obtained as a function of both propeller rotation speed (800 rev min^{-1}) and support particle diameter.[20] Selected values are:

$$k_{silica} = 2{\cdot}8 \times 10^{-4}\ m\ s^{-1}$$

$$k_{corn\ grits} = 1{\cdot}8 \times 10^{-4}\ m\ s^{-1}$$

N may also be expressed as:

$$N = rV/S \tag{2}$$

where r is the observed reaction rate per volume of solution, V is the reactive volume (0·02 dm^3) and S is the external catalyst surface. The external catalyst surface is:

$$S = 6V_c/d_p \tag{3}$$

where d_p is the average support particle diameter and V_c is the catalyst volume. V_c is obtained from dividing total mass of support in the reactor (mass of corn grits = $0{\cdot}117 \times 10^{-3}$ kg, mass of silica $180 = 0{\cdot}055 \times 10^{-3}$ kg, mass of silica 440 = $0{\cdot}095 \times 10^{-3}$ kg) by the apparent volumic mass of the supports (ρ) experimentally measured, assuming a nonporous void fraction of 0·4. Thus:

$$\rho_{silica} = 0{\cdot}8133 \times 10^3\ kg\ m^{-3}$$

$$\rho_{corn\ grits} = 0{\cdot}8646 \times 10^3\ kg\ m^{-3}$$

The observed reaction rates (r) are obtained by measuring the slopes of the curves at the point of origin. Reaction products resulting from a secondary attack of the enzyme can thus be neglected. r is taken as positive when the substrate

TABLE 5
Observed Reaction Rates of Immobilized Alpha-amylase Using Maltotetraose as Substrate[a]

	r_{G4} ($g\ liter^{-1}\ s^{-1}$)	r_{G3} ($g\ liter^{-1}\ s^{-1}$)	r_{G2} ($g\ liter^{-1}\ s^{-1}$)
Corn grits	$1{\cdot}67 \times 10^{-2}$	$-3{\cdot}33 \times 10^{-3}$	$-8{\cdot}33 \times 10^{-3}$
Silica 180 $m^2\ g^{-1}$	$1{\cdot}64 \times 10^{-2}$	$-4{\cdot}37 \times 10^{-3}$	$-1{\cdot}20 \times 10^{-2}$
Silica 440 $m^2\ g^{-1}$	$9{\cdot}97 \times 10^{-3}$	$-3{\cdot}37 \times 10^{-3}$	$-7{\cdot}90 \times 10^{-3}$

[a] Experimental conditions: conditions are the same as in Table 3.

TABLE 6
Hydrolysis of Maltotetraose by Immobilized Alpha-amylase: Effect of External Diffusional Resistances on Substrate Concentration in the Microenvironment of the Immobilized Enzyme during the Initial Stage of Reaction[a]

	G2		*G3*		*G4*	
	ΔC	%	ΔC	%	ΔC	%
Corn grits	−0·9	−107·0	−0·4	−161·0	2·5	13·0
Silica 180 $m^2\ g^{-1}$	−0·3	−35·0	−0·1	−31·0	0·4	2·5
Silica 440 $m^2\ g^{-1}$	−0·1	−33·0	−0·1	−21·0	0·1	0·8

[a] Experimental conditions: conditions are the same as in Table 3.

concentration decreases and as negative when the products concentration increases. Values are given in Table 5.

These data enabled the calculation of the values of ΔC given in Table 6 as well as their percentages in relation to the corresponding values of C_B. They are positive for the substrate, i.e. substrate concentration is higher in the bulk solution than at the catalyst surface and negative for the products as these are accumulated near the enzyme. The values of the percentages show that for the three types of support, external diffusional resistances are important for the products (*G2* and *G3*), while they become negligible for the substrate in the case of the enzyme bound on silica supports. The greatest influence of external diffusional factors was with enzyme immobilized onto corn grits; this is probably due to a small outer interfacial area between solid catalyst and liquid phase. Only in this case, ΔC obtained for the substrate is important.

3.3.2 Internal diffusion

The Thiele modulus (ϕ)[21] is the most convenient expression for quantifying the importance of internal diffusion in the case of heterogeneous reactions with porous catalysts, although it is restricted to simple irreversible reactions. Utilization of this concept is not suitable, in the present case, because its evaluation requires the knowledge of the intrinsic kinetic parameters, which are difficult to obtain from experimental data of the overall rate. Thus, the modified modulus defined by Weisz & Prater,[22] that involves only measured overall reaction rates, has been used. It is

defined by:

$$\phi_m = R^2 r V / D_s^{eff} C_S V_c \quad (4)$$

where R is the radius of the particle and D_s^{eff} is the effective diffusivity of the substrate in the porous support. C_S is the concentration at the surface of the particle and depends on external diffusional limitations. The magnitude of substrate depletion in the pores of the enzymatic catalyst increases with increasing values of ϕ_m. Weisz & Prater[22] have shown that diffusional effects become significant for $\phi_m > 1$; for $\phi_m < 1$ the reaction is essentially kinetically controlled.

ϕ_m has been calculated only for alpha-amylase immobilized onto silica 180. With the other two supports, internal diffusional resistances have no justification, because the enzyme is not immobilized inside silica 440 pores, and macropores of corn grits are large enough for it to be considered that all enzyme molecules are equally accessed by substrate.

The Thiele modulus can be used only for substrate and no complete theoretical studies have been developed for product diffusion.

Calculus of ϕ_m for the substrate was performed using values for D_s^{eff} estimated by a procedure described in the appendix, a value for ϕ_m of 20·6 was obtained for the diffusion of *G4* in porous silica 180. Thus it is possible to state that, in this instance, internal diffusional resistances play a significant role for the substrate. Although diffusion effects are not easily quantified in the case of products, it can be postulated that such effects are present, because diffusion coefficients are of the same order of magnitude (see appendix), and they induce an accumulation of products in the vicinity of the enzyme. The high product concentration favours the degradation of *G3* into *G2* and *G1* which explains the difference in experimental ratio of these products at equilibrium.

It is demonstrated here that the coupling of complex kinetic and non-negligible diffusional effects gives rise to an apparently different activity pattern for the immobilized and free enzymes.

From the study of external and internal diffusional resistances, it is possible to propose an explanation for the apparent differences in the action pattern of alpha-amylase, induced by immobilization onto porous supports. For the three types of support, substrate depletion and product accumulation in the microenvironment of the enzyme have been identified. The magnitude of these effects has been quantified. The more pronounced the effects are, the more important is the apparent modification of the action pattern of the immobilized enzyme in relation to the free enzyme. The production of low polymerized sugars (*G1*, *G2*) increases for the enzyme bound to silica 180 compared with enzyme immobilized onto corn grits and silica 440. This apparent increase of hydrolysis (or decrease of transglycosylation) is in direct relation to the increase in diffusional limitations. These mass transfer resistances lead to an accumulation of *G2* and *G3* and a depletion of *G4* in the microenvironment of the enzyme. Thus, for alpha-amylase bound to silica 180, which produces the highest *G2*/*G3* ratio and the less transglycosyled oligosaccharides, it can be postulated that there must be an important substrate and product concentration gradient between the surface of the support and the interior of the pores. Moreover, external transfer limitations for products are added to these

internal diffusional resistances. For corn grits immobilized enzyme, external diffusional resistances lead to a relatively high gradient of substrate and product concentration between the bulk solution and the surface of the support, and hence to the observed high *G2/G3* ratio. Finally, for alpha-amylase bound to silica 440, where the distribution of reaction products differs only slightly from that of free enzyme, external diffusional limitations have been identified, but only for products.

Although the complexity of the heterogeneous enzyme system is widely recognized, most theoretical results have been obtained with relatively simple models. However, the mode of action of alpha-amylase includes reaction schemes involving multiple products and substrates. As the enzyme shows both hydrolysis and transglycosylation activities, the same oligosaccharide can act simultaneously as both substrate and product and the observed reaction-rates result from both enzyme activities. Despite their unavoidable simplifications, these models have nevertheless been useful in aiding interpretation of kinetic data.

3.4 Reactions with soluble starch as substrate

Studies on the mode of action of immobilized alpha-amylase preparations were extended by analysis of the hydrolysis of soluble starch by both free and immobilized enzyme. The results presented in Table 7 correspond to the HPLC analysis of equilibrium products, having a polymerization degree of less than 9. More highly polymerized products represent limit dextrins for the enzyme and constitute 40% of the initial dextrin concentration. By comparing these results with those obtained with maltotetraose as substrate (Table 4), it can be concluded that the influence of immobilization support on reaction products distribution is similar and that *G1* production and *G2/G3* ratio increase in the same order of magnitude for both substrates. Thus, it can be postulated that the effects of diffusional limitations on alpha-amylase catalysed hydrolysis of starch are similar to those for hydrolysis of maltotetraose; an effect suggested by Eadie–Hofstee plots shown in Fig. 1.

TABLE 7
Hydrolysis of Starch by Free and Immobilized Alpha-amylase: Product Distribution (%) at the Reaction Equilibrium[a]

	G1	*G2*	*G3*	*G4*	$4 < G < 9$	*G2/G3*
Free enzyme	5·0	59·3	33·9	0·2	1·6	1·7
Enzyme linked onto corn grits	8·5	66·9	18·8	2·0	3·8	3·6
Enzyme linked onto silica 180 $m^2\ g^{-1}$	20·6	68·5	3·4	4·8	2·7	20·1
Enzyme linked onto silica 440 $m^2\ g^{-1}$	11·9	70·4	12·4	2·1	3·2	5·7

[a] Experimental conditions: 20 ml of a solution of 50 mg cm^{-3} starch in 0·02 mol dm^{-3} pH 4·7 acetate buffer is used as substrate; the amounts of immobilized alpha-amylase used are 100 mg for alpha-amylase bound to corn grits, 50 mg for enzyme-silica 180 and 90 mg for enzyme-silica 440; the temperature is 37°C.

4 CONCLUSION

Immobilization of *A. oryzae* alpha-amylase results in a change of enzyme activity from an endo- to a more exo-type of action, as seen from analysis of reaction products of free and immobilized alpha-amylase activity on maltotetraose and soluble starch. This means that the proportion of low polymerization degree products increases when using immobilized enzyme. This effect results from substrate and product transfer limitations, induced by the porous nature of the immobilization supports. The diffusional resistances lead to substrate depletion and product accumulation in the microenvironment of the enzyme. In consequence there is a lower degree of product polymerization giving an apparent increase of hydrolysis in relation to transglycosylation reactions. The greater the diffusional resistances, the alpha-amylase activity becomes more exoenzymic.

A similar explanation has been proposed for immobilized glucoamylase.[2,3] Computer simulation of the system has demonstrated that the change in reaction products distribution results from diffusional limitations in the pores. Diffusional resistances thus seem to be the principal cause for the apparent modification of the mode of action induced by the immobilization of enzymes catalysing reactions that involve intermediates and multiple products.

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APPENDIX

D_s^{eff} is related to the substrate diffusivity in the bulk solution (D_S) by the following expression[24]:

$$D_s^{eff} = \varepsilon D_s / \tau \tag{A1}$$

where ε is the void fraction in the porous catalyst ($\varepsilon = 0{\cdot}525$ for silica) and τ the tortuosity factor that takes into account the pore geometry and its value is usually taken between 1·5 and 5 ($\tau = 3{\cdot}25$ as an average value).

The values of D_S at 20°C are obtained from the one of glucose ($D_S = 6{\cdot}7 \times 10^{-10}\ m^2\ s^{-1}$) given by Engasser[24] and the formula of Wilke & Chang.[25] The effect of temperature on D_S is then accounted for by assuming:

$$D_S \eta_w / T = \text{constant (Ref. 25)} \tag{A2}$$

where η_w is the viscosity of water (cP) (at 20°C, $\eta_w = 1{\cdot}04$ and at 37°C $\eta_w = 0{\cdot}71$) and T the temperature (K). The values of D_S and D_s^{eff} ($\times 10^{10}\ m^2\ s^{-1}$) calculated for substrate and products are:

for *G2*, D_S at 20°C = 4·56, D_S at 37°C = 7·07, D_s^{eff} at 37°C = 1·14

for *G3*, D_S at 20°C = 3·61, D_S at 37°C = 5·59, D_s^{eff} at 37°C = 0·90

for *G4*, D_S at 20°C = 3·05, D_S at 37°C = 4·73, D_s^{eff} at 37°C = 0·76