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OPTIMIZATION OF THE ENZYMATIC SYNTHESIS OF AMINO ACID ESTERS. REACTION IN POLYPHASIC MEDIUM

J. L. VIDALUC, M. BABOULENE, V. SPEZIALE and A. LATTES
Laboratoire des Interactions Moléculaires et Réactivité Chimique et Photochimique, ERA-CNRS No. 264, Université Paul Sabatier, 31062 Toulouse Cédex, France

and

P. MONSAN*
Département de Génie Biochimique et Alimentaire, ERA-CNRS No. 879, I.N.S.A., Avenue de Rangueil, 31077 Toulouse Cédex, France

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Abstract—N-acetyl-L-Tyrosine Ethyl Ester (ATEE) was synthesized from N-acetyl-L-Tyrosine and ethanol, with immobilized chymotrypsin as catalyst. For this purpose, a biphasic liquid reaction mixture consisting of an organic chloroform phase and an aqueous phase was used. This system allows the shift of the reaction equilibrium towards ATEE synthesis by achieving continuous ATEE extraction in the organic phase. Synthesis rates of up to 90% were obtained by optimizing the reaction conditions and controlling the pH of the aqueous phase.

The industrial use of enzyme catalyzed reactions is becoming more and more important, especially for reactions involving immobilized enzyme derivatives, which operationally are more stable and allow the use of continuous reactors.[1-4] However, the reactions commonly treated by this technique belong mainly to hydrolysis or isomerization reactions. It would be extremely useful if this technique could be extended to a third group, synthetic reactions. This last group requires an energy input which is usually obtained by the coupling of an endergonic synthesis reaction with an exergonic reaction, involving in most cases the stoichiometric consumation of a co-catalyst, the coenzyme. However, at present it is not feasible to continuously regenerate the coenzyme molecules.

An alternative approach to the problem is to consider that most enzymatic reactions may be regarded as equilibria:

$$E + S \rightleftharpoons ES \rightleftharpoons E + P.$$

It is therefore possible, for example, to use a hydrolytic enzyme, such as α-chymotrypsin or papain, under suitable conditions, to achieve a synthesis reaction.[5-9]

When considering an equilibrium reaction such as:

$$A + B \rightleftharpoons C + H_2O$$

the equilibrium may be shifted to the right if either one of the products is continuously removed. Experimentally, this can be achieved by using a biphasic reaction mixture, consisting of an aqueous phase, and an immiscible organic phase which effectively extracts the reaction product C.

Using this technique, amino acid esters and peptide bonds have been synthesized by Klibanov *et al.*[8] who have synthesized N - acetyl - L - tryptophan ethyl ester in 100% yield. The synthesis in aqueous media gives only a yield of 0.01%. Tarquis *et al.*[7] only obtained a 40% yield for the synthesis of N - acetyl - L - tyrosine ethyl ester (ATEE) when using the same enzyme, α-chymotrypsin, as the catalyst but using a different method of immobilization for the enzyme.

We have therefore reinvestigated the enzymatic synthesis of ATEE from N - acetyl - L - tyrosine (AT) and ethanol, with the intention of identifying the rate limiting step and extending the work to the synthesis of amino acid esters and peptides having a given sequence.

Effect of the support porosity. The physico-chemical nature of the support used for enzyme immobilization has a pronounced effect on the efficiency of the resulting derivative. Tarquis *et al.*[7] used α-chymotrypsin covalently coupled to an amino porous silica activated with glutaraldehyde, with a specific area of $24\ m^2 \cdot g^{-1}$. Monsan[10] has shown that this parameter has a marked effect on the efficiency of trypsin immobilized onto porous silica, with optimal activity occurring at a specific area of $50\ m^2 \cdot g^{-1}$. We have therefore initially determined the effect of the porosity of the silica support on immobilized chymotrypsin efficiency, under the optimum conditions determined by Tarquis *et al.*[7] The results are shown in Fig. 1. A maximum yield is obtained when the specific area is in the range of $50–120\ m^2 \cdot g^{-1}$, and corresponds to 60–65% ester synthesis. Figure 2 shows the hydrolytic activity as a function of the specific area and it should be noted that the maxima occur for the same specific area. The optimal activity of immobilized chymotrypsin therefore appears to be identical for either the synthetic or the hydrolytic action of the enzyme. In both cases the efficiency of the catalyst is directly connected with the amount of active chymotrypsin coupled onto the support, as this amount is lowered for extremal specific areas[10] because of either a limitation by the available coupling area (low specific areas) or a limitation by the pore diameter (high specific areas).

The optimization of the porosity of the support used

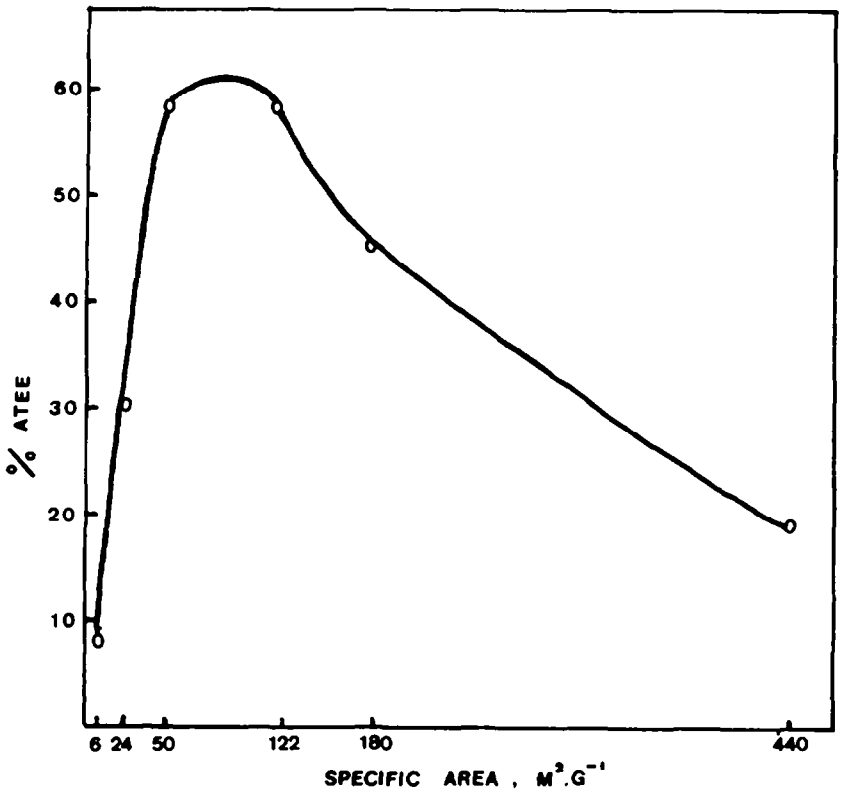


Fig. 1. Effect of the specific area of the silica support used for chymotrypsin immobilization on the ATEE synthesis yield. Results are expressed as percent of ATEE obtained.

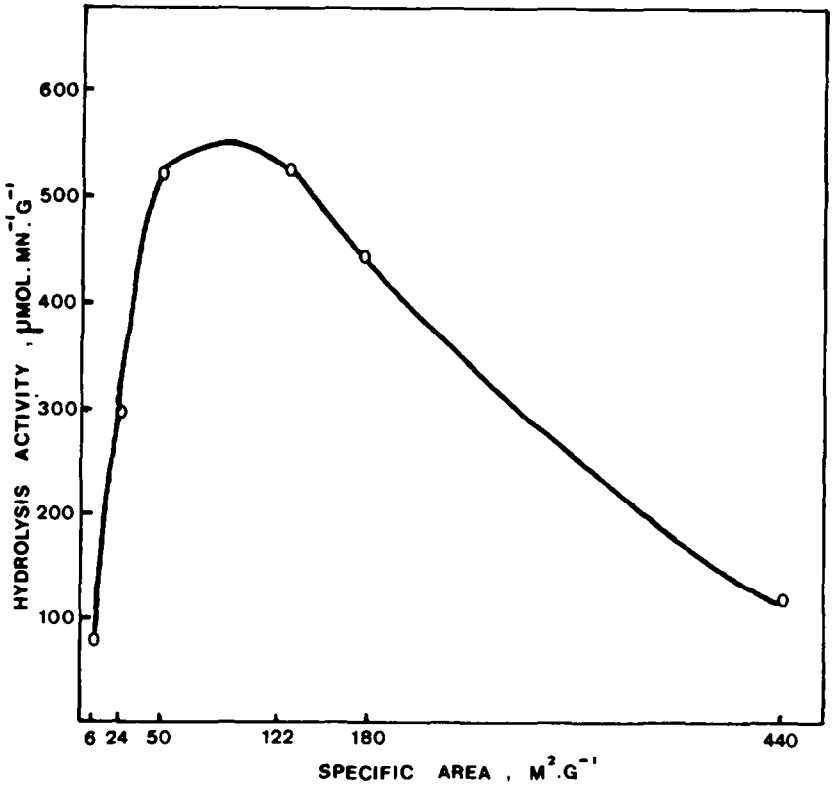


Fig. 2. Effect of the specific area of the silica support used for chymotrypsin immobilization on the ATEE hydrolysis rate. Results are expressed as ATEE μmol hydrolyzed per min. and per g of support (see Experimental).

for α-chymotrypsin immobilization results in a 20% increase in ATEE synthesis. Further experiments were thus carried out with a $50\,m^2 \cdot g^{-1}$ specific area silica support.

Effect of reaction time. Ester synthesis was studied as a function of time, to check that the conversion yields previously obtained corresponded to equilibrium points. From these determinations, the initial synthesis rate was deduced and found equal to $0.13\,\mu mol\ ATEE \cdot min^{-1}$. The equilibrium being reached after 30 h, and no hydrolysis of the ester was observed after this time. For all subsequent experiments, the same experimental scheme was used, in order to be sure that the conversion yields obtained were corresponding to equilibrium values.

Effect of reaction mixture. Modifying the nature of the organic solvent used modifies the yield of ATEE. As shown in Table 1, the highest yield is obtained with chloroform.

For a given denaturing effect, the partition coefficients of substrate (AT) and product (ATEE) between the two liquid phases determine the efficiency of the reaction, as does the partial miscibility of the organic solvent in the aqueous phase. The addition of 10% (V/V) dioxan, which is completely miscible with water, with the intention of increasing AT dissolution in pentane, results in a 14% yield instead of the 22% yield obtained when pure *n*-pentane is used (Table 1). Therefore the volume of the aqueous phase used is also an important parameter: the experimentally obtained optimum value corresponds to a 5.6% aqueous phase.

Effect of reactants. The biphasic liquid mixture must simultaneously allow the action of the enzyme in a buffered aqueous phase, the preservation of its catalytic activity, and the shift of the equilibrium towards ATEE synthesis by the continuous extraction of ATEE in the organic phase.

The influence of ethanol, which acts both as a reactant (mass effect) and as a solvent (effect on partition coefficients), was also studied. A 3 M ethanol concentration is the highest concentration which allows enzyme activity. Furthermore, lower ethanol concentrations decrease the conversion yield: a 0.1 M ethanol concentration gives only 31% ATEE. This result may be compared with that of Nakamoto *et al.*[13] who, operating in an aqueous phase containing 30% (V/V) ethanol, report an ATEE yield equal to that obtained by Tarquis *et al.*[7] for the same synthesis using a chloroform–water medium and a 1 M ethanol concentration. It is therefore possible to perform ATEE synthesis using initial AT concentrations higher than those usually reported in the literature. It can be seen from Fig. 3 that the initial AT concentration has a marked effect on the yield of ATEE: a relatively sharp maximum is observed, corresponding to *ca.* 1×10^{-2} M initial AT concentration. Even if the initial ascending part of this curve may correspond to enzyme saturation, the marked decrease in efficiency at AT concentration greater than 1×10^{-2} M cannot be attributed to a simple inhibition of chymotrypsin activity by excess substrate.

The effect of the buffer must therefore be considered, as using the results reported by Tarquis *et al.*[7] we have obtained an optimal pH value for ATEE synthesis of 6.8 when using 0.1 M citrate–phosphate buffer. We have, however, observed that in such conditions the pH of the aqueous phase was modified with increasing the initial AT concentration. Figure 4 shows the effect of the concentration of the citrate–phosphate buffer both on the pH of the aqueous phase and on the ATEE yield: a 1 M buffer concentration is necessary to obtain a stabilized pH value for the aqueous phase in the presence of an initial 1×10^{-2} M AT concentration. Furthermore, a lower pH value gives a higher yield. This result may also be compared with the results presented on the initial part of Fig. 3, where increasing initial AT concentration from 0.25×10^{-2} M to 1.0×10^{-2} M increased the ATEE yield. This may simply be a consequence of a decrease in the pH of the aqueous phase from 5.9 at 0.25 M to 4.5 at 1 M initial AT concentrations which favors ATEE synthesis (Fig. 4). We have therefore determined the effect of the pH of 1.0 M citrate–phosphate buffer on ATEE synthesis (Fig. 5): an optimal value is obtained at pH 4.2, corresponding to a 85–90% ester yield. The reaction efficiency is rapidly decreased on moving from this optimum. This point will be discussed further on.

The importance of the pH of the aqueous phase has been underlined by determining the ester yield using 1 M citrate–phosphate buffer at pH 4.2 with various initial AT concentrations. A constant conversion rate is observed for AT concentrations ranging from 0.25×10^{-2} M to 1.0×10^{-2} M (Fig. 6).

DISCUSSION

From our results, it appears that two parameters are of prime importance for achieving ATEE synthesis from AT with α-chymotrypsin as the catalyst:

(a) The equilibrium between dissociated and undissociated acid AT as a function of medium characteristics,

(b) The value of the partition coefficient of each reactant between the two immiscible liquid phases.

These parameters cannot be easily dissociated. Taking into account only the principle of reactions occurring within this biphasic liquid medium, the following Scheme may be suggested.

The ester synthesis reaction must obviously be located within the aqueous phase. The organic phase extracts continuously the ATEE as it is produced, thus allowing the shift of the reaction equilibrium towards synthesis. The substrate involved must of course be soluble within the aqueous phase. AT is very soluble in the buffered aqueous phase and, under our reaction conditions, at least 90% of it remains in the aqueous phase whereas ATEE is dissolved uniquely in the chloroform phase.

Table 1. Effect of solvent at ATEE synthesis

Solvent	n-Pentane	Toluene	Ethyl Acetate	Chloroform
ATEE Synthesis yield (%)	22	24	33	60

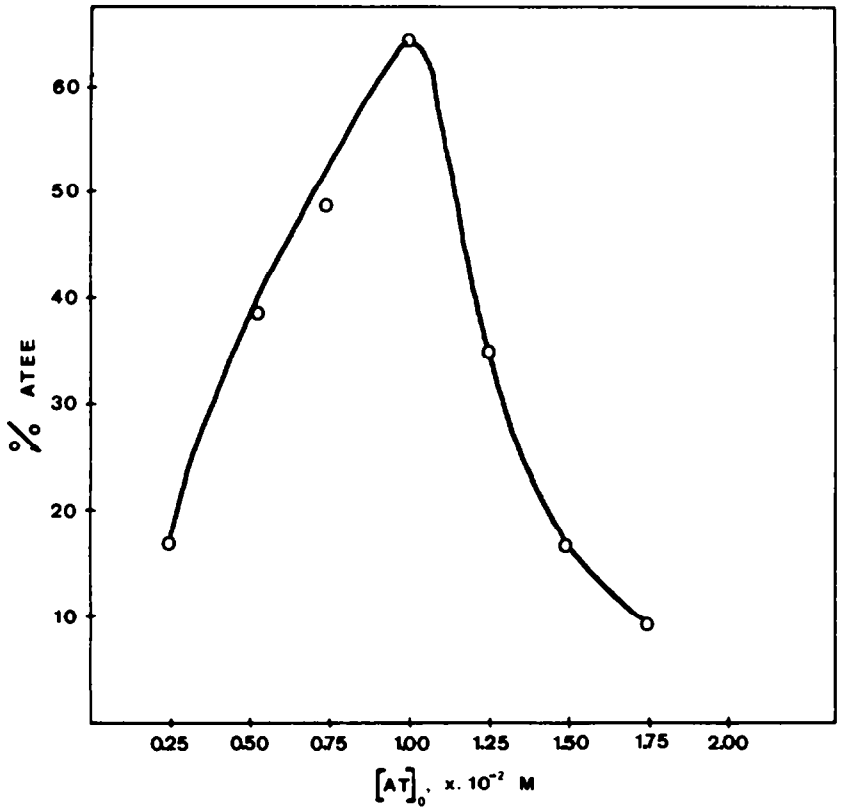


Fig. 3. Effect of total initial AT concentration $(AT)_0$ on ATEE synthesis yield. Experimental conditions: aqueous phase is 5.6% (V/V) 0.1 M citrate–phosphate buffer pH 6.8 containing 300 mg chymotrypsin–silica derivative; ethanol initial concentration in the organic phase: 1 M.

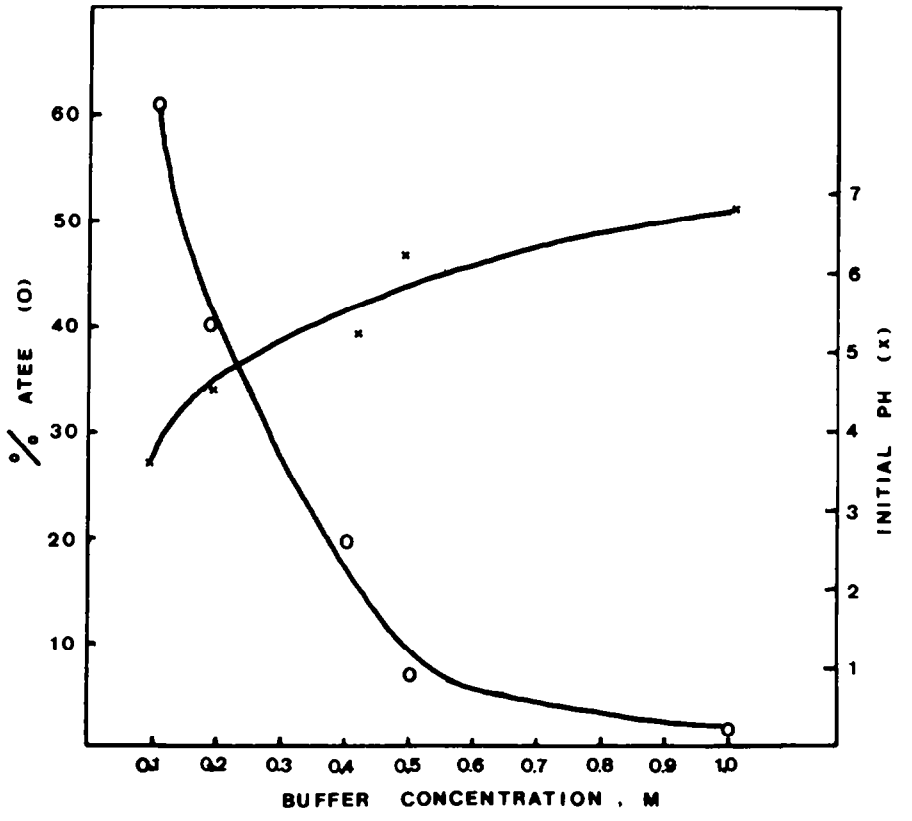


Fig. 4. Effect of the concentration of citrate–phosphate buffer pH 6.8 on ATEE synthesis yield. Initial AT concentration is 1×10^{-2} M in the experimental mixture.

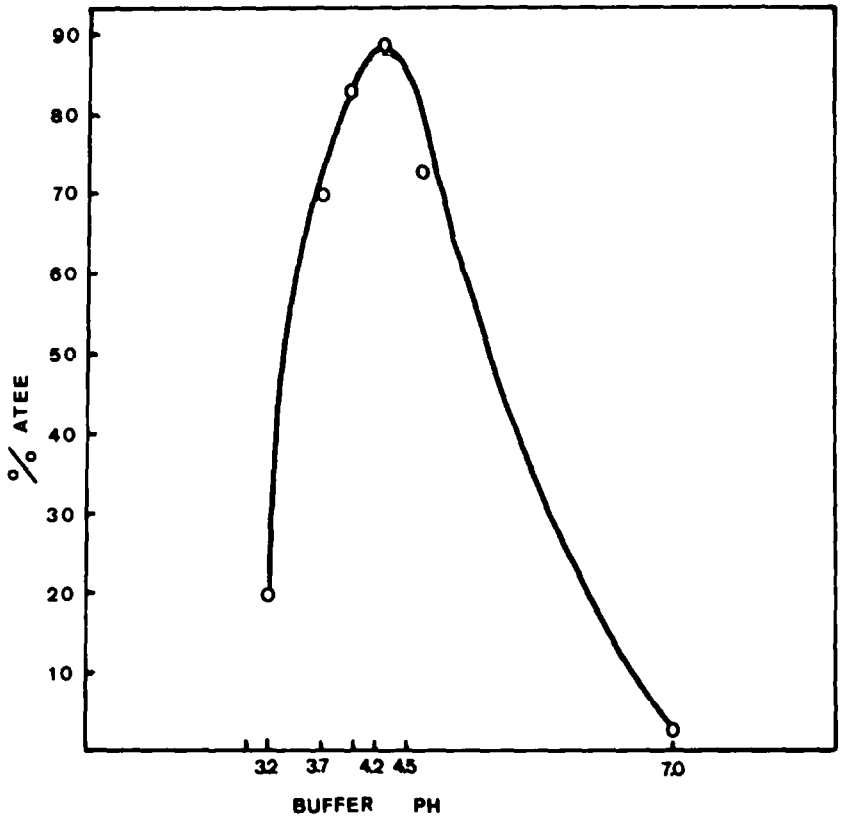


Fig. 5. Effect of the pH of the buffered aqueous phase on ATEE synthesis yield. Citrate–phosphate buffer concentration is 1 M.

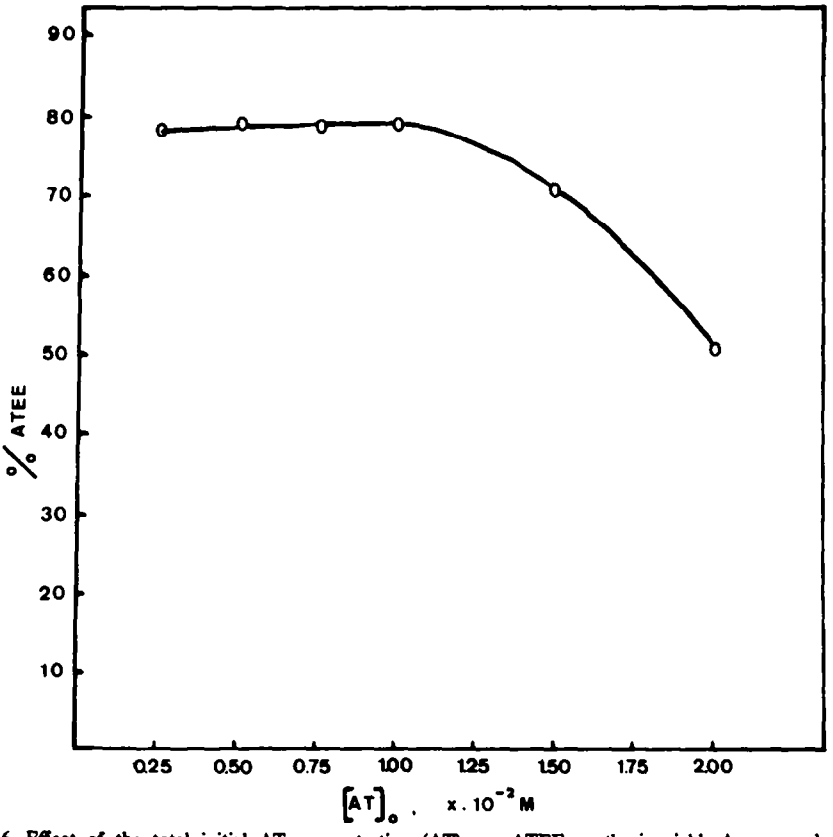
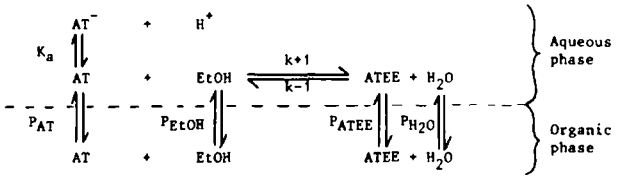


Fig. 6. Effect of the total initial AT concentration $(AT)_0$ on ATEE synthesis yield. Aqueous phase is 1 M citrate–phosphate buffer pH 4.2.



K_a : dissociation constant

k+1, k-1 : reaction rate constants

P_i : partition coefficient of reactant or product i

This partition of both substrate and product is of fundamental importance to obtain high yields. For example, the 22% ester yield obtained when using *n*-pentane as an organic phase may be attributed to the low solubility of ATEE in this solvent. The effect of the other components of the mixture may be explained in a similar way. The increased buffer concentration at acidic pH favors AT and ATEE partition in their respective phases. Furthermore, 37% of the ethanol is found in the aqueous phase, although this alcohol is perfectly miscible with both solvents. This explains the effect of ethanol concentration and in particular its inhibitory effect on enzyme activity due to its high concentration within the aqueous phase. A 5.6% aqueous phase volume gives the highest ester yield. This optimal ratio may be related to the partition of water, as the water miscibility within the chloroform phase may interfere with the AT ⇌ ATEE equilibrium shift by modifying the volume of the aqueous phase. In any case, the water ratio affects the dissociation constant K_a of the substrate, according to the Ostwald dilution law. If we consider this parameter, and acidic reaction medium will limit AT dissociation. This is consistent with an aqueous phase buffered at pH 4.2, even if neutral media have been previously reported. Such a result is in agreement with the observations made when adding a phase transfer chemical catalyst to the reaction mixture: an alkaline salt (benzyltriethylammonium hydroxyde) decreases the reaction efficiency while an acidic salt (benzyltriethylammonium chloride) gives a synthesis yield similar to the yield obtained with our usual experimental conditions. Furthermore, in the optimal synthesis conditions reported by Tarquis *et al.*[7] the aqueous phase pH is in the range 3.5–4.5 during the reaction.

In addition, our data are in agreement with the results recently reported by the Martinek group[8,9,15] on the synthesis of N - benzoyl - L - tryptophane ethyl ester using as a catalyst α-chymotrypsin adsorbed onto porous glass.

CONCLUSION

Our results must be linked with the more general aim of the synthesis of optically active amino acid esters. A good knowledge of the reaction mixture was thus sought. We were able to determine experimental conditions yielding 85–90% ATEE synthesis yield (Experimental), by operating with an aqueous phase at pH 4.2. The optimal pH for ester synthesis is consequently significantly lower than the optimal pH for the enzymatic hydrolysis reaction, which is about pH 8.0. This approach opens a new field of application of enzymatic catalysis in organic synthesis.

†Experimental sample XOB 030.

EXPERIMENTAL

(1) *Enzyme immobilization.* 2 g amino Spherosil (Rhône Poulenc Ind.†) was kept in contact with 50 ml of a 5% glutaraldehyde soln in 0.05 M pyrophosphate buffer pH 8.6, for 2 h at 25° under rotative agitation. Excess glutaraldehyde was eliminated by repeated washings with the pyrophosphate buffer. The activated silica support reacted with 50 ml of a $8\,g \cdot l^{-1}$ α-chymotrypsin (Sigma Chem. Co.) soln in 0.05 M pyrophosphate buffer pH 8.6 for 16 h at 4°. The compound thus obtained was repeatedly washed with the pyrophosphate buffer and a 1 M NaCl soln was added and kept for 1 h at 4° to wash off adsorbed chymotrypsin. The support was then washed with 10^{-3} M HCl, then with distilled water, and lyophilized. Immobilized chymotrypsin was stored at −20°.

(2) *ATEE synthesis.* 300 mg immobilized chymotrypsin were suspended in 1.12 ml 1 M citrate-phosphate buffer pH 4.2. An organic AT soln prepared by dissolving 42.15 mg AT in 1.1 ml dry EtOH (1 M) and 17.78 ml $CHCl_3$ was added to the aqueous phase. AT final concentration was 1.10^{-1} M. The biphasic mixture was agitated for at least 30 h at 25°. The immobilized enzyme was then separated by filtration and washed with a water/MeOH/MeCN (50/40/10) mixture. The filtrate was evaporated until completely dry and redissolved in a 5 ml water/MeOH/MeCN mixture.

(3) *Analysis.* The determination of synthesized ATEE and residual AT was achieved by hplc (Waters 440) using a μ-Bondapack C_{18} column eluted with the water/MeOH/MeCN mixture and a UV detection at 254 nm.

For the kinetic studies, samples were directly withdrawn from the organic phase.

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