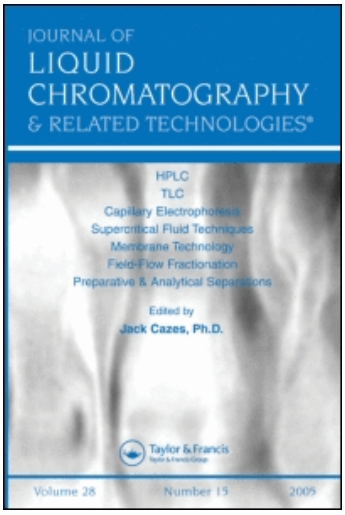

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Study of a Bioreactor Based upon α-Chymotrypsin

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Study of a Bioreactor Based upon α-Chymotrypsin

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ABSTRACT

α-Chymotrypsin (ACHT) was immobilized on an epoxide-derivatized silica gel by an in situ immobilization process. Several racemates has been resolved according an enzymatic recognition mechanism on this ACHT chiral stationary phase. The loadability of the ACHT-CSP, used as bioreactor, has been evaluated in both buffered and non-buffered mobile phases under the enzymatic process.

Key Words: α-Chymotrypsin; Enzymatic enantiomeric separations; Bioreactor; Loadability.

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INTRODUCTION

α-Chymotrypsin (ACHT) has been immobilized by different ways and the resulting CSP used for the separation of enantiomeric amino acids derivatives[1–3] and dipeptides.[4,5] On the other hand, ACHT immobilized on different kinds of solid phases is hydrolytically active and resolution of racemic amino acid esters or amides can be obtained by the separation of L-amino acids and intact D-amino acid esters or amides.[1,2,6,7] More recently, we have shown with a different immobilization technique used for the preparation of protein-CSP, (i.e. covalent immobilization via reactive epoxy by an in situ process)[8,9] that it can be apply to immobilize ACHT to give a CSP that can be used in structural enantiomer separations.[10]

We report here, the possibility to use ACHT-CSP both as a bioreactor using its enzymatic recognition properties and we have estimated the loading capacity of this type of bioreactor.

EXPERIMENTAL

Materials

HPLC was performed with a Hewlett–Packard 1050 quaternary solvent system equipped with a Hewlett–Packard 1050 multiwavelength detector and a Rheodyne 7125 manual injector with 20-μL sample loop. The pump and the detector were controlled by an HP ChemStation microcomputer.

Chemicals

The ACHT from bovine pancreas (type II), 3-glycidoxypropyltrimethoxysilane, amino acids, amino acid esters, and potassium dihydrogenophosphate, were purchased from Sigma-Aldrich-Fluka (St. Quentin-Fallavier, France). Kromasil 200 Å, 5 μm, with a specific surface of $200\,m^2/g$ was from Eka-Nobel (Bohus, Sweden). Solvents of HPLC grade were from ICS-Nationale (Belin-Beliet, France). Water was de-ionized by passing through an Elgastat UHQ II system.

Synthesis

The synthesis of ACHT-CSPs has been reported elsewhere.[10] In brief, the synthesis was accomplished in three steps: (1) silica gel was derivatized

by 3-glycidoxypropyltrimethoxysilane to give an epoxide silica gel. (2) The epoxide silica gel was then packed in $15.0 \times 4.6\,mm^2$ stainless tubes by a classical slurry technique (3) After equilibration of the column with the coupling solution, i.e., KH_2PO_4 (pH 8, 0.05 M) containing ammonium sulfate (1.9 M), 65 mL of the coupling solution containing 10 mg/mL of ACHT was passed through the column at 0.5 mL/min. The column was back flushed every 30 min until the solution was used up. The column was washed with 60 mL of KH_2PO_4 (pH 6, 0.01 M) and the remaining epoxide groups blocked with a solution of glycine, finally equilibrated with a solution of KH_2PO_4 (pH 6, 0.01 M).

The amount of ACHT immobilized on the silica gel was 93 mg ACHT/g of solid phase.

RESULTS AND DISCUSSION

Enzymatic Catalysis in Buffered Mobile Phase

Jadaud et al. have shown that the separation of chiral aromatic amino acid ester or amide derivatives results from the enzymatic activity.[2] The L enantiomer is hydrolyzed and then the D enantiomer is separated from the L acid form by the enzyme achiral interactions. The hydrolysis of some amino acid esters have been carry out under normal conditions [room temperature, flow rate 0.5 mL/min, mobile phase: KH_2PO_4, (pH 6.5, 0.1 M)], the results are presented in Table 1. On this ACHT-CSP a good selectivity is obtained between the ester and the acid forms giving baseline resolution for the compounds (Fig. 1). These results show that all the L-tryptophane methyl ester is hydrolyzed at the beginning of the column with no hydrolysis of the D-tryptophane methyl ester. By using a C_3 reversed phase column[a] as post column, the enzymatic activity involved in the chiral separation can be verified easily and in this way, if necessary, the selectivity of the separation can be increased as shown in Table 2 on tryptophane esters.

Enzymatic Catalysis in Non-buffered Mobile Phase

The quasi-totality of solvents has been studied in enzymology with very variable results in regards to the enzymatic activity, stereospecificity, and

[a]The C_3 stationary phase is slightly hydrophobic, so able to separate the ester and the acid forms with a mobile phase totally buffered.

Table 1. Separation of amino acid derivatives.

Racemates	K_1	α
D,L-Tryrosine methyl ester	0.07	6.35
D,L-Tryptophane methyl ester	1.11	3.27
D,L-Tryptophane butyl ester	1.23	6.55
D,L-Phenylalanine methyl ester	0.35	7.14

Note: Mobile phase: KH_2PO_4, (pH 6.5, 0.1 M). Flow rate of 1 mL/min.

stability of the enzymes.[11,12] A lot of solvents obey these three parameters with a very precise percentage of water. But in HPLC the solvents must satisfy several other conditions.

The speed of reaction must be compatible with HPLC.

The stereoselectivity must be significant.

The elution of the ester and acid form must be obtained in a reasonable time.

The viscosity of the mobile phase must be sufficiently low to not damage the chromatographic system by a too high pressure.

The mobile phase must not absorb in UV to allow a reasonable detection threshold.

These conditions drastically decrease the number of solvents and from the results previously published by Kise et al. on native ACHT[13–15] only acetonitrile is acceptable. These authors have obtained the hydrolysis of D,L-tyrosine ethyl ester with 49% yield and an enantiopurity better than 99%, in a mixture of acetonitrile/water (90/10, v/v) at 30°C. If the same conditions are applied to HPLC, the hydrolysis of aromatic amino acids esters can be obtained (Table 3, Fig. 2). The injection of pure enantiomers shows that the hydrolysis is complete and no trace of D-tryptophane can be detected. No decrease in yield and stereoselectivity has been observed by using between 5% and 25% water as previously obtained with native ACHT.[13]

An organo-aqueous mobile phase has the advantage to decrease the microbial contamination[12] and to inhibit the non-enzymatic hydrolysis.[13] The solubility of some substrates in the mobile phase is easier and the absence of buffer facilitates the recovery of compound when the column is used on a preparative scale.

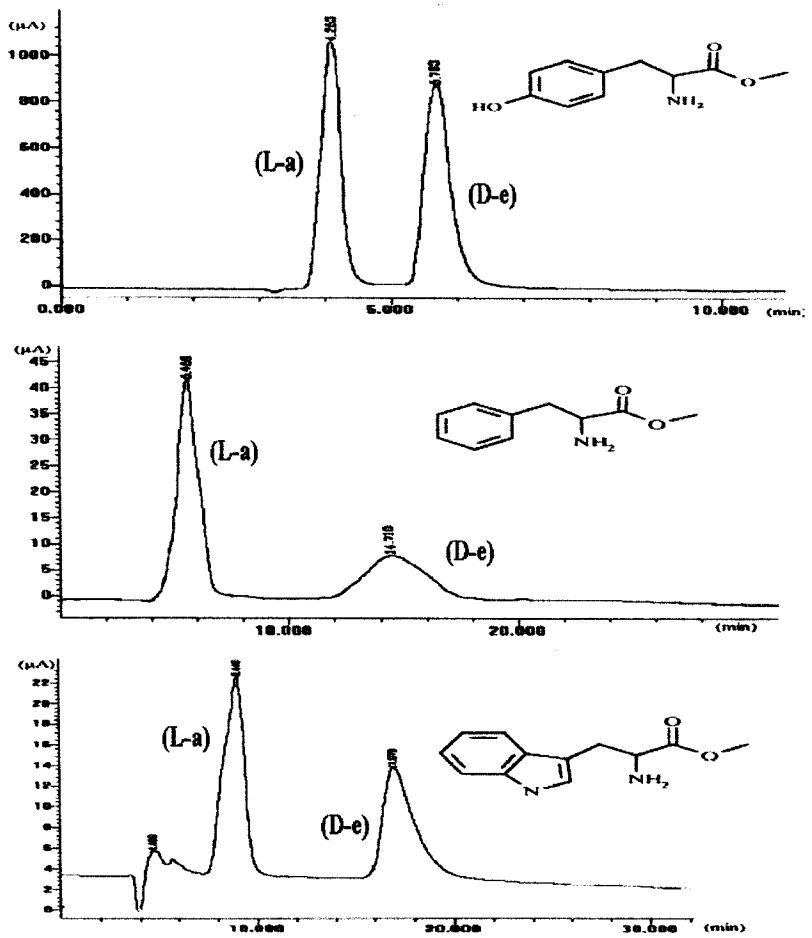

Figure 1. Separation by enzymatic resolution of three amino acid esters in buffered mobile phase. Mobile phase: KH_2PO_4, (pH 6.5, 0.1 M). Flow rate 1 mL/min; (L-a): L-acid enantiomer; (D-e): D-ester enantiomer.

Loading Capacities

In preparative chromatography, several papers have shown that the use of protein-CSPs is limited because of their low capacity loading.[16–20] The use of enzymes as bioreactor can be a good alternative to this handicap, for this purpose, we have studied the loading behavior of an ACHT column used on a semi-preparative scale for the enantiomeric enzymatic resolution

Table 2. Retention times in minutes on a C_3, an ACHT-CSP and an ACHT-CSP + C_3.

Racemates	C_3	ACHT	ACHT + C_3
L-Tryptophane	1.00	2.54	3.58
L-Tryptophane methyl ester	2.10	2.61	3.64
D-Tryptophane methyl ester	2.10	4.56	6.80

Note: Mobile phase: KH_2PO_4 (pH 6.5, 0.1 M). Flow rate 1 mL/min.

of racemates in buffered and non-buffered mobile phases. The different conditions of mobile phases and solutes are listed in Table 4. To observe the influence of the increase of the loading, we have studied the change in retention factors vs. the sample loading.

In buffered mobile phase, during the study of the separation of D,L-tryptophane methyl ester the retention time of the acid form stayed constant (Fig. 3), showing that the ester form is hydrolyzed at the beginning of the column otherwise the retention time would increase.

However, the more interested result is in the estimation of the enantiomeric purity (Fig. 4). The hydrolysis of L-tryptophane is complete up to 4 μmol. Beyond this, very small amounts of the ester form are eluted (1% for 8 μmol injected). With the D ester form the achiral hydrolysis decrease up to 6 μmol and increases after this, showing that the stereoselectivity of the enzyme decreases when the loading increases. Figure 5 shows the change in the separation of D,L-tryptophane methyl ester with the increase of the loading.

In an organo-aqueous phase, the retention times of the acid and ester forms decrease when the loading increases (Fig. 6). This is probably due to a decrease of the enzymatic activity and in consequence the hydrolysis of the ester takes place, not at the beginning of the column as in the buffered mobile phase, but along the column, also decreasing the retention of the acid form.

Table 3. Separation of amino acid derivatives.

Racemates	K_1	α
D,L-Tryrosine methyl ester	0.88	13.1
D,L-Tryptophane methyl ester	0.85	9.50
D,L-Tryptophane butyl ester	0.77	11.7
D,L-Phenylalanine methyl ester	2.63	2.40

Note: Mobile phase: acetonitrile/water—90/10 (v/v). Flow rate 1 mL/min.

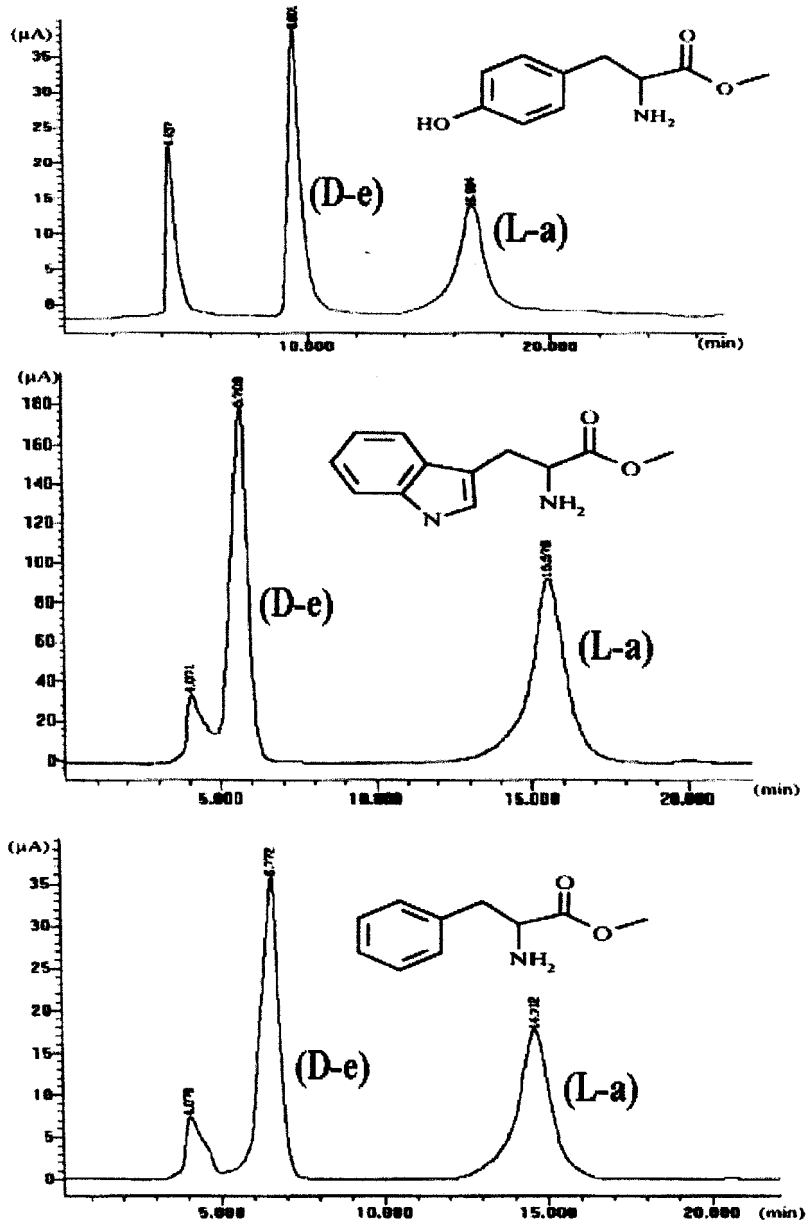

Figure 2. Separation by enzymatic resolution of three amino acid esters in organic mobile phase. Mobile phase: acetonitrile/water—85/15 (v/v). Flow rate 1 mL/min; (L-a): L-acid enantiomer. (D-e): D-ester enantiomer.

Table 4. Loading conditions.

Study	Solute	Mobile phase
Buffered	D,L-Tryptophane methyl ester	KH_2PO_4 (pH 6.7, 0.1 M)
Organic	D,L-Tryptophane methyl ester	Water/acetonitrile 18/82 (v/v)

Note: Flow rate 1 mL/min.

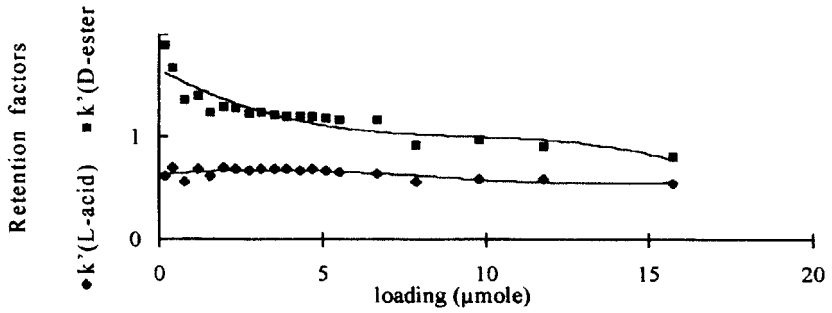

Figure 3. Influence of the loading on the retention of L-acid and D-ester form of tryptophane in buffered mobile phase. Conditions: see Table 4.

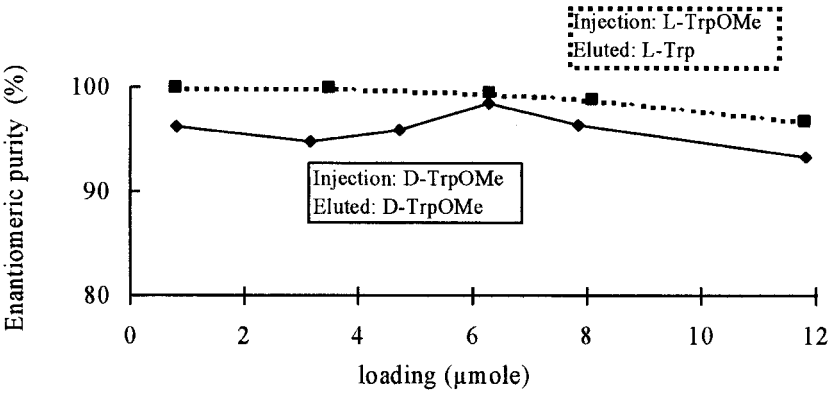

Figure 4. Influence of the loading on the stereoselectivity of D,L-tryptophane methyl ester in buffered mobile phase. Conditions: see Table 4.

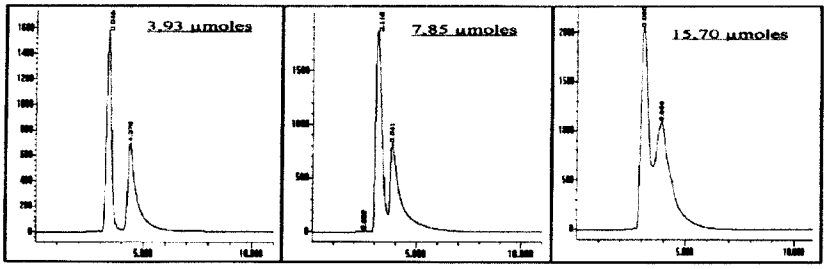

Figure 5. Examples of the increase of the loading in enzymatic resolution of D,L-tryptophane methyl ester in buffered mobile phase. Conditions: see Table 4.

Figure 7 shows that there is no hydrolysis of the D enantiomer. Thus, we have elution of L-tryptophane methyl ester for a loading of 3 µmol and a dramatic fall of the hydrolysis yield with a loading greater than 4 µmol. The influence of the loading on the separations is presented in Fig. 8.

CONCLUSION

From these studies, for a resolution of 0.8 and an enantiomeric purity of 99% we can determine the production of enantiopure compounds in a 24 hr period (Table 5). The maximum loading is low compared with several others CSPs.[21] The ACHT-CSP supports higher loading capacity than the protein-CSPs, for example HSA,[19] but this difference is also a function of immobilization rate and the separation (selectivity and resolution) and is, therefore, a function of the structure of the racemate. The results are better

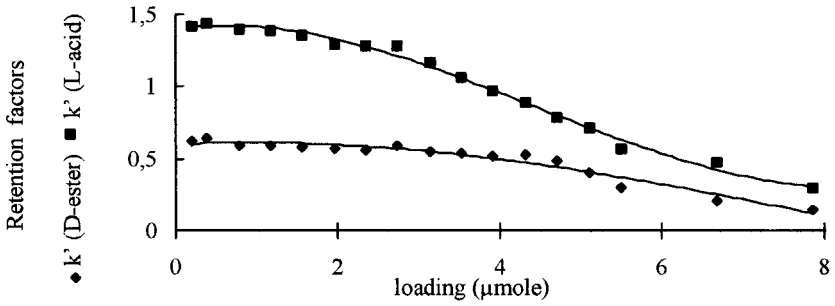

Figure 6. Influence of the loading on the retention of L-acid and D-ester form of tryptophane in organic mobile phase. Conditions: see Table 4.

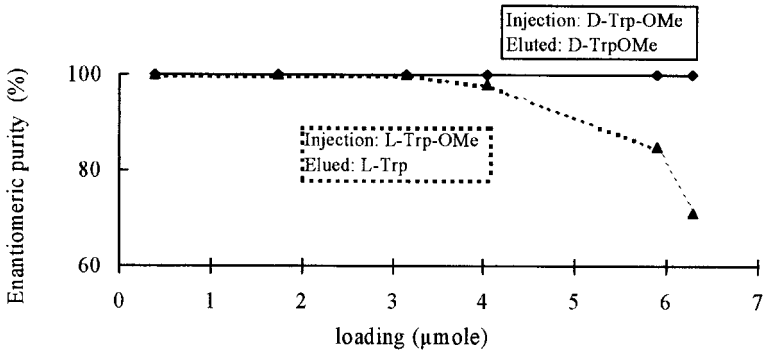

Figure 7. Influence of the loading on the stereoselectivity of D,L-tryptophane methyl ester in organic mobile phase. Conditions: see Table 4.

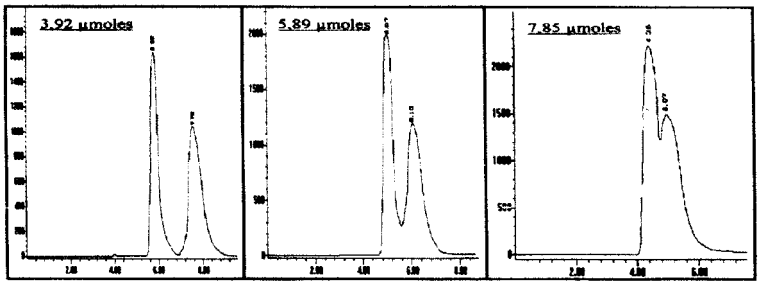

Figure 8. Examples of the increase of the loading in enzymatic resolution of D,L-tryptophane methyl ester in organic mobile phase. Conditions: see Table 4.

Table 5. Loading capacities of ACHT column bioreactor.

Study	Time separation (min)	Loading by injection (mg)	Loading by day (mg)
Buffered	10	2.0	290
Organic	12	1.0	120

in buffered mobile phase because the enzymatic activity is highest, but the use of an organo-aqueous mobile phase offers a better enantiomeric purity of recovered solutes. This kind of bioreactor does not seem useful for production units, but can be used in the laboratory to rapidly produce a few grams of pure chiral compound as a step in asymmetry synthesis or for pharmacologic tests.

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