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STEREOCHEMICAL RECOGNITION OF ENANTIOMERIC AND DIASTEREOMERIC DIPEPTIDES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ON A CHIRAL STATIONARY PHASE BASED UPON IMMOBILIZED α-CHYMOTRYPSIN

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SUMMARY

A series of 24 enantiomeric and diastereomeric dipeptides were chromatographed on a chiral stationary phase (CSP) based upon immobilized α-chymotrypsin (ACHT). The ACHT-CSP was able to resolve stereochemically a number of the enantiomeric D,D- and L,L-dipeptides as well as the diastereomeric D,D-/L,L- and L,D-/D,L-dipeptides. The solutes were also chromatographed on a N-tosyl-L-phenylalanine chloromethyl ketone-deactivated form of the ACHT-CSP, where stereochemical separations were also achieved. The results of this study suggest that binding interactions between the dipeptides and the ACHT-CSP occur at the active site of the ACHT and at other hydrophobic sites on the ACHT molecule.

INTRODUCTION

The stereochemical purity of small peptides is an important and difficult problem in the development of a number of new drugs[1]. The complexity of the problem is illustrated by the simplest case, a dipeptide, where the molecule can exist in four stereoisomeric forms, *i.e.* two diastereomeric molecules, L,L/D,D and D,L/L,D, which are themselves enantiomeric pairs. The number of possible stereoisomers for a given peptide is 2^n where n is the number of constituent amino acids.

At the present time, the determination of the stereochemical composition of a peptide has been mainly limited to dipeptides. This problem has been approached by using enantioselective chromatographic techniques that utilized chiral stationary phases (CSPs) developed for high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC).

The most comprehensive study of the application of HPLC CSPs to the separation of peptide stereoisomers was carried out by Florance *et al.*[2]. This work involved the use of three different types of commercially available HPLC CSPs, based upon β-cyclodextrin (CD-CSP), (*R*)-N-(3,5-dinitrobenzoyl)phenylglycine (PG-CSP), and an unspecified amino acid derivative (WE-CSP).

Using the CD- and WE-CSPs, Florance *et al.*[2] were able to achieve the chromatographic resolution of diastereomeric dipeptides, such as L-Leu–L-Tyr/D-Leu–L-Tyr.

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The resolutions were accomplished without derivatization of the dipeptides and by the use of aqueous mobile phases. Diastereomeric cyclic dipeptides were also resolved on the CD- and PG-CSPs, enantiomeric dipeptides, such as L-Leu–L-Tyr/D-Leu–D-Tyr, were not.

Hyun *et al.*[3] have also used an HPLC CSP to resolve a series of enantiomeric dipeptides. The CSP was synthesized with (*S*)-1-(6,7-dimethyl-1-naphthyl)-isobutylamine as the chiral selector. However, to accomplish the separations, the dipeptides had to be first converted to the corresponding N-3,5-dinitrobenzoyl methyl esters. Chromatography was then carried out with a mobile phase composed of hexane–2-propanol (90:10, v/v). Using this method, all four stereoisomers of Leu–Ala and fourteen other dipeptides were resolved.

Pirkle *et al.*[4] have accomplished similar separations of enantiomeric di- and tripeptides on HPLC CSPs related to the one reported by Hyun *et al.*[3]. The peptides also had to be converted to the corresponding N-3,5-dinitrobenzoyl methyl esters.

The stereochemical resolution of enantiomeric dipeptides without derivatization has been reported by Günther[5]. These separations were accomplished by using enantioselective TLC on a commercially available TLC CSP, based upon (2*S*,4*R*,2′*RS*)-N-(2′-hydroxydodecyl)-4-hydroxyproline. The dipeptides were chromatographed with a mobile phase composed of methanol–water–acetonitrile (50:50:200, v/v/v). Under these conditions, enantiomeric dipeptides such, as D-Ala–L-Phe/L-Ala–D-Phe, could be stereochemically resolved. This method can also be used to resolve diastereomeric dipeptides[6].

Another possible approach to this problem has been suggested by the development of a HPLC CSP based upon the enzyme α-chymotrypsin (ACHT), the ACHT-CSP[7]. The CSP was synthesized by the immobilization of ACHT on a silica-based support containing covalently bonded glutaraldehyde. The immobilization produced a stationary phase which contains an active enzyme capable of binding and hydrolyzing natural substrates of ACHT, such as L-amino acid amides and esters[8]. Stereochemical resolutions of a number of enantiomeric compounds, including free and derivatized amino acids, were observed on this "active" form of the ACHT-CSP[7,8].

Another form of the CSP can be produced by inactivating the ACHT. This can be accomplished by blocking the active site of the enzyme with N-tosyl-L-phenylalanine chloromethyl ketone (TPCK)[9,10]. Thus, the "active" ACHT-CSP was treated with TPCK, resulting in an "inactive" ACHT-CSP[8]. The "inactive" form of the CSP was unable to resolve stereochemically most of the substrates separated on the "active" form of the CSP, although some enantiomeric amino acid esters were separated with higher stereoselectivity[8].

ACHT is an endopeptidase which stereoselectively hydrolyzes peptides at bonds involving the carboxyl groups of aromatic L-amino acids, particularly phenylalanine (Phe), tyrosine (Tyr) and tryptophan (Trp)[11]. Therefore, it is likely that the ACHT-CSP will be able to discriminate between the stereoisomers of dipeptides containing Phe, Tyr, and Trp. To test this hypothesis, we have chromatographed on the ACHT-CSP a series of dipeptides composed of leucine (Leu) and one of the three aromatic amino acids, *i.e.* Phe, Tyr, Trp. The results demonstrate that both enantioselective and diastereoselective resolutions can be achieved on both "active" and "inactive" forms of the CSP.

EXPERIMENTAL

Apparatus

The HPLC experiments with the ACHT-CSP were performed with two modular liquid chromatographs. One system was composed of a Beckman 116 solvent module pump (Beckman Instruments, Houston, TX, U.S.A.), a Beckman 160 absorbance detector (wavelength fixed at 254 nm) and a Shimadzu C-R6A Chromatopac integrator (Shimadzu Scientific Instruments, Columbia, MD, U.S.A.). The other system consisted of the same pump, a multiwavelength Beckman programmable UV detector module 166 and a Shimadzu C-R5A Chromatopac integrator. The pumps and the second detector were controlled by a NEC PC-8300 microcomputer (NEC Home Electronics U.S.A., Wood Dale, IL, U.S.A.). The 10 cm × 4.6 mm I.D. and 25 cm × 4.6 mm I.D. stainless-steel columns used in this study were packed with the ACHT-CSP by J. T. Baker (Phillipsburg, NJ, U.S.A.).

Chemicals

The α-chymotrypsin (Type VII, TLCK-treated), amino acids, amino acid derivatives, and TPCK were purchased from Sigma (St. Louis, MO, U.S.A.). The L,L-dipeptides were also obtained from Sigma. The other dipeptides were synthesized in the Department of Virology and Molecular Biology of St. Jude Children's Research Hospital.

The hydrophilic polymer-bonded silicas (particle diameters: 5 and 15 μm, pore diameter: 300 Å), containing covalently bonded glutaraldehyde, were obtained from J. T. Baker.

Chromatographic conditions

The standard mobile phases were composed of pure buffers, usually at pH 5.5–6.0. For the comparison of the retention on active and TPCK-deactivated ACHT-CSP, the mobile phase was an aqueous solution of sodium phosphate (0.123 *M*, pH 6.0, $I = 0.140$). Flow-rates of 0.3 ml/min and 0.6 ml/min were used for the 15-μm and 5-μm packing, respectively. All experiments were carried out at ambient temperature. The elution order of the isomers was established by chromatographing the solutes separately.

Synthesis of the ACHT-CSP

The synthesis of the ACHT-CSP has been reported elsewhere[7]. In brief, the synthesis was accomplished in three steps.

(1) The support was washed four times with phosphate buffer (0.1 *M*, pH 7.0).

(2) The support was added to a sodium borate solution (0.1 *M*, pH 8.7) at a rate of 2.5 ml per gram of support; the solution contained ACHT in an amount of at least 60 mg per gram of support. The mixture was stored at 5–6°C for at least 18 h.

(3) The mixture was filtered, and the solid phase was washed four times with phosphate buffer (0.1 *M*, pH 7.0).

The calculated concentration of ACHT on the support was 50–53 mg ACHT per gram of solid phase.

Deactivation of the ACHT-CSP

The deactivation of the ACHT-CSP (10 cm long column) was carried out by injecting 3.5 ml of a solution of TPCK [5.5 m*M* in phosphate buffer (0.1 *M*, pH 6.0)–acetonitrile (50:50, v/v)]. This volume of 3.5 ml corresponds to 35 injections with a 100-μl loop. The injection of D,L-tryptophanamide into this deactivated support showed a lack of hydrolysis and stereoselectivity[8].

RESULTS

The structure of the dipeptides used in this study are presented in Fig. 1. Table I summarizes the chromatographic results obtained with the "active" and "inactive" form of the ACHT-CSP. Some examples of chromatograms, for the pairs L,D-/L,L-Tyr–Leu, D,D-/L,L-Leu–Trp and L-Trp–D-Leu/L-Leu–D-Trp, are presented in Fig. 2a, b and c, respectively. The results of this study indicate that the relative retention of the dipeptides, *i.e.* the capacity factor, k', is a function of the hydrophobicity of the constituent aromatic amino acid, and of the interactions of this moiety with the active site of the enzyme. This is demonstrated by the retention of Leu–Phe and Leu–Tyr dipeptides on the "active" and "inactive" forms of the ACHT-CSP. Phe is more hydrophobic than Tyr[12] while Tyr has a higher affinity for ACHT[13]. On the active form of the CSP, the binding to the active site of the ACHT played an important role in the retention[7,8], and Tyr-containing dipeptides had higher k' values than the corresponding Phe-containing dipeptides. When the active site was blocked by TPCK, hydrophobic interactions played a dominant role in the retention[8], and the Phe-containing dipeptides had the higher k' values.

In addition, the observed retentions of the dipeptides were not the arithmetic sum of the k' values of the constituent amino acids. This was clearly demonstrated by the k' values for the dipeptides composed of Leu–Tyr and Leu–Trp. With regard to the stereoselectivity of the "active" and "inactive" forms of the ACHT-CSP, the following results were observed.

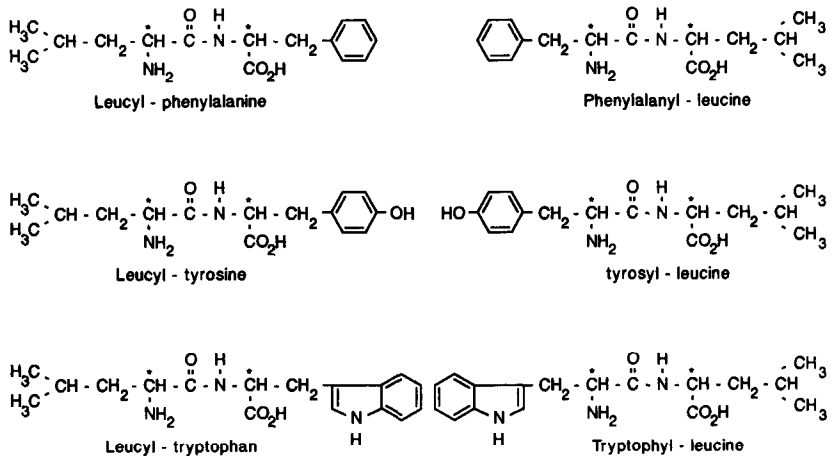

Fig. 1. The structure of the solutes used in this study.

TABLE I

CHROMATOGRAPHIC RETENTION, ENANTIOSELECTIVITY α_1 AND DIASTEREOSELECTIVITY α_2 OF AMINO ACIDS AND DIPEPTIDES ON ACTIVE AND TPCK-DEACTIVATED ACHT-CSP

Column, 100 × 4.6 mm I.D.; stationary phase, α-chymotrypsin-bonded silica, 15 μm; mobile phase, phosphate buffer (0.123 *M*, pH 6.0); flow-rate, 0.30 ml/min; temperature, ambient; detection, UV at various wavelengths.

Solutes	*ACHT-CSP (active)*[a]			*ACHT-CSP (TPCK)*[b]		
	k'	α_1[c]	α_2[d]	k'	α_1[c]	α_2[d]
D,L-Leu	0.10	1.00		0.18	1.00	
L-Leu	0.10			0.19		
D,L-Phe	0.20	1.00		0.39	1.00	
L-Phe	0.23			0.39		
D-Tyr	0.34	1.03		0.36	1.00	
L-Tyr	0.35			0.36		
L-Trp	1.00	1.16		1.02	1.03	
D-Trp	1.16			0.99		
L-Leu–D-Phe	0.39	1.00		0.55	1.07	
D-Leu–L-Phe	0.39		1.08	0.59		1.07
L-Leu–L-Phe	0.41	1.05		0.59	1.05	
D-Leu–D-Phe	0.43			0.62		
L-Phe–D-Leu	0.37	1.06		0.58	1.02	
D-Phe–L-Leu	0.35		1.17	0.59		1.03
D-Phe–D-Leu	0.40	1.10		0.63	1.11	
L-Phe–L-Leu	0.44			0.57		
L-Leu–D-Tyr	0.47	1.00		0.44	1.02	
D-Leu–L-Tyr	0.47		1.47	0.45		1.20
L-Leu–L-Tyr	0.69	1.00		0.53	1.02	
D-Leu–D-Tyr	0.69			0.54		
L-Tyr–D-Leu	0.40	1.03		0.40	1.05	
D-Tyr–L-Leu	0.41		1.74	0.42		1.59
D-Tyr–D-Leu	0.68	1.07		0.60	1.17	
L-Tyr–L-Leu	0.73			0.70		
L-Leu–D-Trp	1.47	1.03		1.49	1.02	
D-Leu–L-Trp	1.51		2.09	1.46		1.32
L-Leu–L-Trp	2.20	1.83		1.85	1.11	
D-Leu–D-Trp	4.03			2.05		
L-Trp–D-Leu	1.05	1.04		1.18	1.02	
D-Trp–L-Leu	1.09		1.80	1.20		1.33
D-Trp–D-Leu	1.91	1.02		1.57	1.01	
L-Trp–L-Leu	1.95			1.59		

[a] Active form of the ACHT.

[b] Inactive form of ACHT after treatment with TPCK.

[c] $\alpha_1 = (t_{Rb} - t_0)/(t_{Ra} - t_0)$ where t_{Ra}, t_{Rb} and t_0 are the retention times of the less retained compound, of the more retained compound, and the dead time, respectively.

[d] $\alpha_2 = [(t_{RD,D} + t_{RL,L})/2 - t_0]/[(t_{RD,L} + t_{RL,D})/2 - t_0]$.

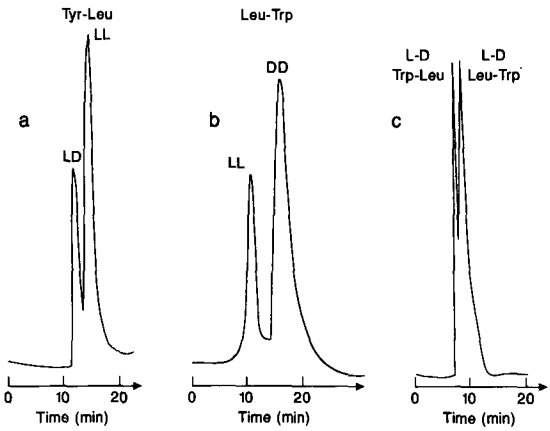

Fig. 2. Representative chromatograms for two of the solutes (a) L,D-/L,L-Tyr–Leu; (b) D,D-/L,L-Leu–Trp; (c) L-Trp–D-Leu/L-Leu–D-Trp. Chromatographic conditions: column, 250 × 4.6 mm I.D.; stationary phase, α-chymotrypsin-bonded silica, 5 μm; mobile phase, phosphate buffer (0.070 *M*, pH 5.5); flow-rate, 0.60 ml/min (optimal); temperature, ambient; detection, UV at 254 nm.

The "active" ACHT-CSP

D,L-/L,D-dipeptides, retention and enantioselectivity. For all the dipeptides studied, the k' values were higher when the aromatic amino acid was at the carboxyl end of the chain. The difference in retention between the two forms increased with increasing hydrophobicity, *i.e.* there was a 5% difference in k' between D-Leu–L-Phe and L-Phe–D-Leu and a 44% difference between D-Leu–L-Trp and L-Trp–D-Leu.

No significant enantioselectivity was achieved for the twelve L,D- and D,L-dipeptides studied. However, when some enantioselectivity was observed, the stereoselectivity (α) was higher when the aromatic amino acid was at the amino end of the chain. In addition, except for Phe–Leu, the L,D-form of the dipeptide was eluted before the D,L-form.

L,L-/D,D-dipeptides, retention and enantioselectivity. For the D,D-forms of the dipeptides composed of Leu and Phe or Tyr, there was little difference in k' between the molecules with the aromatic amino acid at the carboxyl end or at the amino end of the chain. However, the k' values for L-Phe–L-Leu and L-Tyr–L-Leu were about 6% higher than the corresponding dipeptides, L-Leu–L-Phe and L-Leu–L-Tyr. As a result, there was a change in the elution order, *i.e.* L,L- was eluted before D,D- when the aromatic amino acid was at the carboxyl end and D,D- was eluted before L,L- when the position of Phe or Tyr was reversed.

The position of the aromatic amino acid also affected the enantioselectivity between the L,L-/D,D-enantiomers. When the aromatic amino acid was at the carboxyl end of the chain, the stereoselectivity (α) was lower than when that moiety was at the amino end of the dipeptide.

Among the enantiomers of Leu–Trp, L-Leu–L-Trp was eluted before the D,D-isomer while the elution order was reversed for the Trp–Leu enantiomers. This was similar to the results observed with the Phe- and Tyr-containing dipeptides, but the cause of this reversal was different. In this case, the k' values of both the D,D- and

L,L-enantiomers decreased when the aromatic amino acid was shifted from the carboxyl end to the amino end of the chain. However, there was a 53% difference between the retention of D-Leu–D-Trp and D-Trp–D-Leu, and only a 11% difference between the corresponding L,L-isomers.

Unlike the Phe- and Tyr-containing dipeptides, the L,L-/D,D-Leu–Trp enantiomers were better resolved than the Trp–Leu isomers. When Trp was at the carboxyl end of the dipeptide $\alpha = 1.83$, and when it was at the amino terminus the stereoselectivity was almost lost ($\alpha = 1.02$).

D,L-/L,D- and D,D-/L,L-diastereomers, retention and resolution. Among the six sets of diastereomeric dipeptides used in this study, the D,L-/L,D-pairs were eluted before the corresponding D,D-/L,L-pairs. The difference in the k' of the diastereomers, and therefore their stereochemical resolution, increased with the hydrophobicity of the aromatic amino acid. In addition, the stereochemical resolution achieved for the diastereomers with the aromatic amino acid at the amino end of the dipeptide was greater than that observed for the isomers with the aromatic amino acid at the carboxyl terminus.

The "inactive" ACHT-CSP

D,L-/L,D-dipeptides, retention and enantioselectivity. The deactivation of the ACHT-CSP by TPCK had very little effect on the retention and stereoselectivity of the Tyr- and Trp-containing dipeptides used in this study. However, the deactivation resulted in a significant increase in the retention of the Phe-containing solutes. When Phe is at the carboxyl end of the dipeptide, the k' values on the "inactive" ACHT-CSP increased by over 40% relative to the results on the "active" ACHT-CSP, and a stereochemical resolution of the L,D-/D,L-enantiomers was observed ($\alpha = 1.07$).

The k' values for the Phe–Leu enantiomers also increased. In this instance, the k' for the L,D-isomer increased by 57% and the k' for the D,L-isomer increased by 69%. This resulted in a net loss in enantioselectivity, $\alpha = 1.06$ and 1.02, on the "active" and "inactive" CSPs, respectively.

L,L-/D,D-dipeptides, retention and enantioselectivity. As observed with the D,L-/L,D-dipeptides, deactivation of the ACHT-CSP resulted in a significant increase in the k' values of the Phe-containing dipeptides. However, there was no change in the stereoselectivities.

When the Tyr- and Trp-containing dipeptides were chromatographed, there was a significant decrease in the observed k' values for all of the compounds and an increase in α for the Leu–Tyr and Tyr–Leu solutes, $\alpha = 1.02$ and 1.17, respectively. For Leu–Trp there was a decrease in the stereoselectivity relative to the separation observed on the "active" ACHT-CSP ($\alpha = 1.87$ on the "active" CSP and 1.11 on the "inactive" CSP). The chiral resolution of the Trp–Leu enantiomorphs was unchanged.

D,L-/L,D- and D,D-/L,L-diastereomers, retention and resolution. On the "inactive" ACHT-CSP, the relative retention order of the six sets of diastereomeric dipeptides was identical to that found on the "active" form of the CSP, *i.e.* the D,L-/L,D-pairs were eluted before the D,D-/L,L-pairs. All of the diastereomeric pairs were resolved, although the stereochemical resolution was lower on the "inactive" CSP.

DISCUSSION

As stated above, ACHT primarily catalyzes the hydrolysis of amide bonds of proteins and peptides adjacent to the carbonyl group of the aromatic L-amino acid residues of Trp, Tyr and Phe[11]. The catalytic activity takes place at a single site on the enzyme, which is composed of a hydrophobic pocket and a hydrolytically active cavity. In the hydrolytic cavity, the probable contacts are between Ser-195 and His-57 and the carboxylic group of the substrate, Met-192 with C^{α} and the group bonded on it, and the hydrophobic cavity (Met-192, Cys-191, Ser-190, Gly-216, Ser-217, ...) with the aromatic system of the solute[14]. With a D-amino acid, the interactions with Ser-195 may be replaced by interactions with Ser-214[14].

The chromatographic results obtained on the "active" form of the ACHT-CSP can be explained, in part, by binding at the active site of the enzyme. This is particularly clear for the L,L-/D,D-dipeptides, especially the series composed of Trp–Leu and Leu–Trp.

The Trp–Leu dipeptide is in the "normal" configuration for insertion into the active site of the enzyme, *i.e.* the amide bond is formed with the carboxyl group of the aromatic amino acid. In this configuration, the natural L,L-substrate should be more tightly bound than the unnatural D,D-isomer. The enantiomeric elution order (the D,D-enantiomer is eluted before the L,L-) is consistent with this view.

The Leu–Trp dipeptide is not in the "normal" configuration for insertion into the active site of the enzyme, *i.e.* the amide bond is formed with the carboxyl group of the aliphatic amino acid. In this configuration, the best fit between the enzyme and dipeptide would be accomplished by rotating the peptide 180° relative to the position assumed by the Trp–Leu molecule. This should reverse the enantiomeric elution order, since the enzyme now sees the mirror image of the Trp–Leu dipeptide. The observed enantiomeric elution order (the L,L-enantiomer is eluted before the D,D-) is consistent with this interpretation.

However, this mechanism does not explain the results obtained for D,D-/L,L-dipeptides on the TPCK-deactivated ACHT-CSP. If the dipeptides only interact with the enzyme at the active site, there should be a significant loss in retention and stereoselectivity when the active site is blocked with TPCK. Although there were some significant decreases in k' (for example, a 49% decrease in k' for D-Leu–D-Trp), there were also some increases (for example, a 58% increase in k' for both Phe–Leu isomers).

As discussed above, the chromatographic retention on the ACHT-CSP appears to be due to binding at the active site of the enzyme and at one or more additional hydrophobic sites on the molecule. These sites must also possess some enantioselectivity, since there is an increase in the stereochemical resolution of some of the D,D-/L,L-dipeptides on the "inactive" CSP (Tyr–Leu, for example), no change in the resolution of others (Phe–Leu, for example) and a reduced but still significant resolution of Leu–Trp. These results are consistent with the results obtained with amino acid esters on TPCK-deactivated ACHT-CSP[8].

The results for the series of enantiomeric D,L-/L,D-dipeptides are not as easily understood. No significant stereochemical resolutions were obtained on either the "active" or "inactive" forms of the CSP, although the "inactive" ACHT-CSP appeared to be slightly more enantioselective.

TABLE II

NUMBER OF THEORETICAL PLATES (N)[a] FOR EACH CHROMATOGRAPHED DIPEPTIDE ON ACHT-CSP

Chromatographic conditions: see Fig. 2.

Solute	*N*	*Solute*	*N*
D,L-Phe	1035	L,D-Tyr–Leu	1153
L-Tyr	1952	D,L-Tyr–Leu	1221
L-Trp	1591	D,D-Tyr–Leu	323
		L,L-Tyr–Leu	317
L,D-Leu–Phe	1045		
D,L-Leu–Phe	1015	L,D-Leu–Trp	585
L,L-Leu–Phe	519	D,L-Leu–Trp	571
D,D-Leu–Phe	418	L,L-Leu–Trp	265
		D,D-Leu–Trp	139
L,D-Phe–Leu	971		
D,L-Phe–Leu	781	L,D-Trp–Leu	622
D,D-Phe–Leu	362	D,L-Trp–Leu	621
L,L-Phe–Leu	366	D,D-Trp–Leu	94
		L,L-Trp–Leu	141
L,D-Leu–Tyr	1113		
D,L-Leu–Tyr	1085		
L,L-Leu–Tyr	1192		
D,D-Leu–Tyr	389		

[a] $N = 5.54\ (t_R/\delta)^2$ where t_R = retention time and δ = peak width at half-height.

These results suggest that the primary interactions between the D,L-/L,D-dipeptides and the ACHT-CSP do not take place at the active site of the enzyme, but at other hydrophobic sites on the ACHT. This may be due to the existence of molecular conformations of the D,L- and L,D-dipeptides that do not form stable complexes with the active site.

In addition, although the enantiomeric resolutions involving the D,L-/L,D-dipeptides were low, the diastereomeric resolutions of the D,L-/L,D- and D,D-/L,L-dipeptides were significant on both forms of the ACHT-CSP. This may also be a reflection of the fact that the two diastereomeric forms have different primary sites of interaction with the ACHT-CSP.

This possibility may be correlated with the influence of the structure of the dipeptide on the efficiency of its chromatographic peak, as reflected by the calculated theoretical plate number (N) (Table II). The stronger the interactions with the support, the slower the desorption of the solute and the lower the efficiency. As shown in Table II, there are two groups of solutes: (1) the L,D-/D,L-enantiomers with a "high" N; and (2) the D,D-/L,L-enantiomers, with an N which is one-half or less of the corresponding L,D-/D,L-solute. This is consistent with the existence of two types of binding sites with different affinities.

CONCLUSION

The ACHT-CSP is capable of the enantiomeric and diastereomeric resolution of the dipeptides derived from phenylalanine, tyrosine and tryptophan. These resolutions appear to be due to interactions at both the active site of the ACHT and at other hydrophobic sites on the ACHT molecule. The existence of two or more interaction sites is under further investigation through studies of the effect of temperature, mobile-phase composition and solute structure on retention and stereoselectivity.

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