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Differentiation of tryptic enzymes based on enantiomeric specificity at the deacylation step

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The chiral specificity of tryptic enzymes at their deacylation step has been determined for the first time by virtue of 'inverse substrates' carrying optically active acyl groups. Differentiation of tryptic enzymes was also successful with these substrates. The stability of acyl-thrombin is substantially higher than those of trypsin and plasmin when the (*S*)-dihydrocoumarilyl group is applied. This is in contrast to the result with its (*R*)-antipode in which all three enzymes are not differentiated. The use of chiral *p*-amidinophenyl esters is proposed as a versatile methodology for the design of specific inhibitors capable of discriminating among tryptic enzymes.

Trypsin; Thrombin; Plasmin; Deacylation rate; Acyl enzyme

1. INTRODUCTION

Catalyses by thrombin and plasmin are directly related to physiological processes, such as coagulation and fibrinolysis, respectively. It is of great value for clinical purposes to design specific compounds which can distinguish between these enzymes. The development of such compounds is difficult because these enzymes have similar substrate specificity. Inverse substrates, *p*-amidinophenyl esters, appear promising for this objective by virtue of their kinetic characteristics [1–3]. They react commonly with tryptic enzymes to produce a variety of acyl enzymes specifically, and the stabilities of the resultant acyl enzymes were expected to be diverse depending on the structure of acyl groups and on the enzymes. Our interest is also focused on the response of tryptic enzymes toward chiral acyl groups at their deacylation steps, which was not capable of being analyzed previously.

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2. EXPERIMENTAL

2.1. *Materials*

Bovine trypsin was obtained from Worthington (lot TRL). Human plasmin and human thrombin were prepared as in [4,5]. Concentrations of enzyme preparations were determined on the basis of titrated normality [6] or reported rate constant [7]. Optically active inverse substrates, **Ia–h**, were prepared as reported [8].

2.2. *Determination of kinetic parameters*

Hydrolyses of **Ia–h** by trypsin, thrombin and plasmin were determined in 0.05 M Tris-HCl (pH 8.0) at 25°C by monitoring the liberation of *p*-amidinophenol at 305 nm. The rapid acylation process was analyzed by a stopped-flow spectrophotometer under either condition [S] $\gg$ [E] ([S], 20–100 μM; [E], 1 μM), or [S] $\ll$ [E] ([S], 0.5 μM; [E], 1.5–10 μM). A very slow deacylation rate for the thrombin-catalyzed hydrolysis of (*S*)-**Ic** was determined from the reactivation of the isolated acyl enzyme. All the kinetic analyses were performed as reported [1,8].

3. RESULTS AND DISCUSSION

Optically active inverse substrates, **Ia–h**, were synthesized from highly purified optically active carboxylic acid and *N*-carbobenzyloxy-*p*-amidinophenol [8]. Their structures are shown in fig.1. Catalytic properties of bovine trypsin, human thrombin and human plasmin towards these

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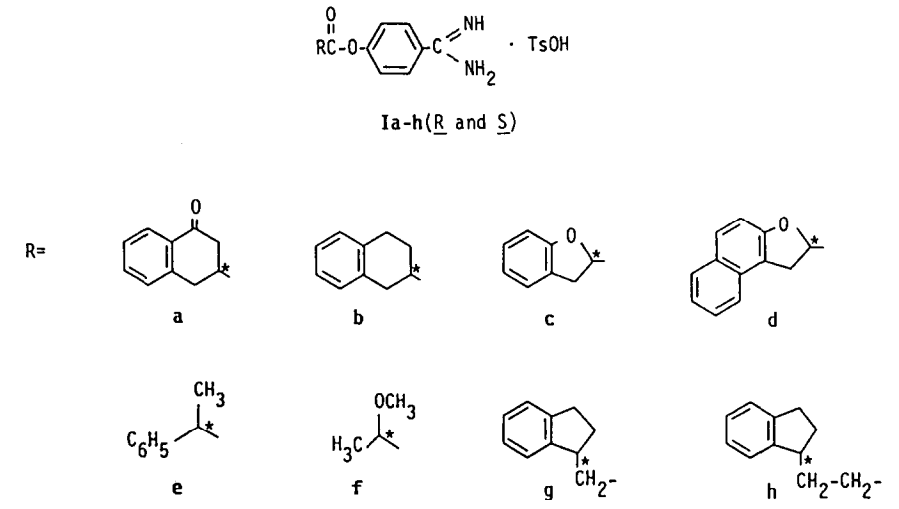

Fig.1. Structure of optically active inverse substrates.

substrates were analyzed. It was substantiated that all the esters were susceptible to the enzymatic catalyses in a specific manner. Every substrate was analyzed and shown to have a strong affinity to these three enzymes with a K_s value ranging from 10^{-5} to 10^{-7} M. The reactions proceeded through the formation of an acyl enzyme intermediate, exhibiting efficient acylation and slower deacylation steps as discussed in [8]. It was shown that the acylation rate constants, k_2, were greater than the deacylation rate constants, k_3, in every case, thus producing ratios k_2/k_3 which ranged from 1.4 to 7970. Our interest is focused on the deacylation process which reflects the stability of the resultant

Table 1

Comparison of the deacylation rate constants

Inverse substrate	Trypsin[a]		Thrombin[a]		Plasmin[b]		k_3(plasmin)/ k_3(thrombin)
	k_3 (s^{-1})	k_3 ratio (*R*)/(*S*)	k_3 (s^{-1})	k_3 ratio (*R*)/(*S*)	k_3 (s^{-1})	k_3 ratio (*R*)/(*S*)	
(*R*)-**Ia**	5.5×10^{-1}	1.2	3.0×10^{-1}	7.5	1.1×10^{-1}	1.0	0.37
(*S*)-**Ia**	4.6×10^{-1}		4.0×10^{-2}		1.0×10^{-1}		2.5
(*R*)-**Ib**	1.3×10^{-1}	1.6	1.1×10^{-2}	0.32	1.3×10^{-2}	0.31	1.2
(*S*)-**Ib**	7.9×10^{-2}		3.4×10^{-2}		4.3×10^{-2}		1.3
(*R*)-**Ic**	2.6×10^{1}	56	5.4	42000	1.0×10^{1}	50	1.9
(*S*)-**Ic**	4.6×10^{-1}		1.3×10^{-4}		2.0×10^{-1}		1500
(*R*)-**Id**	5.0×10^{1}	41	8.2	1.6	3.4	3.4	0.41
(*S*)-**Id**	1.2×10^{1}		5.2		1.0		0.19
(*R*)-**Ie**	8.2×10^{-4}	0.32	2.7×10^{-4}	0.25	1.7×10^{-3}	0.29	6.3
(*S*)-**Ie**	2.6×10^{-3}		1.1×10^{-3}		5.8×10^{-3}		5.3
(*R*)-**If**	8.1×10^{-2}	3.9	3.3×10^{-2}	3.0	5.6×10^{-2}	2.9	1.7
(*S*)-**If**	2.1×10^{-2}		1.1×10^{-2}		1.9×10^{-2}		1.7
(*R*)-**Ig**	1.1×10^{-2}	0.14	6.8×10^{-3}	2.7	9.6×10^{-3}	0.48	1.4
(*S*)-**Ig**	8.1×10^{-2}		2.5×10^{-2}		2.0×10^{-2}		0.8
(*R*)-**Ih**	1.1×10^{-1}	0.20	7.3×10^{-3}	0.05	1.0×10^{-2}	0.07	1.4
(*S*)-**Ih**	5.4×10^{-1}		1.4×10^{-1}		1.4×10^{-1}		1.0

[a] In 0.05 M Tris, 0.02 M $CaCl_2$ (pH 8.0) at 25°C
[b] In 0.05 M Tris, 0.1 M NaCl, 0.02 M lysine (pH 8.0) at 25°C

acyl enzymes. In table 1 deacylation rate constants for the 16 enantiomers in the catalysis by these three enzymes are listed. Enantiomers were classified on the basis of the sequence rule and denoted as *R* and *S*. The enantiomeric preferences proved to be variable for each case. For the compounds with an ether linkage (**Ic,d,f**) the preference is towards the *R* series. The greatest preference occurred in the thrombin-catalyzed reaction of **Ic**. In this case the *R/S* ratio is 4.2×10^4, whereas the ratios for trypsin and plasmin are only 56 and 50, respectively. Compound (*S*)-**Ic** led to a remarkable discrimination between the tryptic enzymes. The deacylation rate decreased dramatically only in the case of the thrombin-catalyzed reaction, whereas (*R*)-**Ic** indicated little differentiation. We feel that this anomalous behavior of (*S*)-**Ic** relative to thrombin catalysis can be explained on the basis of the spatial structures of the tryptic enzymes and the orientation of the oxygen atom of the benzofuran ring. Molecular modeling [9] of acyl trypsin suggests that the oxygen atom of (*R*)-**Ic** is oriented toward His-57 in the active site whereas that of (*S*)-**Ic** is oriented toward Gln-192. It should be noted that a mutation of an amino acid residue occurred at position 192 of the thrombin molecule, i.e. Gln for trypsin and plasmin is replaced by Glu for thrombin [10,11]. It is recognized that the instability ratio of acyl-plasmin to acyl-thrombin is 1500 for (*S*)-**Ic**, but only 1.9 for (*R*)-**Ic**. Apparently, (*S*)-**Ic** inhibits thrombin activity by producing the long-lived acyl-thrombin (half-life 1.5 h), irrespective of the presence of plasmin. Plasmin activity has virtually no effect due to the short-lived nature of acyl-plasmin (half-life 3.5 s). Discrimination of enzymes of similar specificity is possible by locating the amino acid mutation in the region near the active sites. Specific introduction of a chiral acyl group is feasible for sensitive differentiation of tryptic enzymes due to the temporary blocking of the enzyme activity. This approach will provide a new methodology for the development of clinically useful specific inhibitors of tryptic enzymes.

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