CHANGES OF SUBSTRATE CONFIGURATION DURING TRYPSIN HYDROLYSIS OF ARGININE AND LYSINE 2-PHENYL-THIAZOL-5-ONES.°)

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SUMMARY - Arg and Lys 2-phenyl-thiazol-5-ones behave as chromophoric specific substrates for trypsin. Although they are racemic compounds their enzymatic hydrolysis is complete and the resulting product, thiobenzoyl amino acid, is the L-enantiomer in both cases.

Synthetic substrates for trypsin, as well as for other proteolytic enzymes usually are suitable amino acid derivatives susceptible to be hydrolyzed by enzymatic catalysis. Because of the marked preferentiality of this enzyme towards one antipode, when asymmetric derivatives are concerned, only one half of a racemic mixture is transformed, the other half normally being an inhibitor of the reaction (1).

We wish to present in this paper that racemic mixtures of 2-phenyl-thiazol-5-ones related to Arg and Lys behave as chromophoric specific substrates for trypsin. The most striking properties of these racemic compounds are that their enzymatic hydrolysis to TBz amino acids°°) results in a complete inversion of configuration of the D enantiomer.

EXPERIMENTAL

2-Phenylthiazol-5-one of Arginin (PTA Arg)

A solution of L-Arg·HCl (10^{-3} moles)in 1N NaOH (1 ml) is mixed with

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°°) Abbreviations used :
TBz = Thiobenzoyl
PTA = 2-phenyl-thiazol-5-one
TFA = Trifluoroacetic acid

a solution of carboxymethyldithiobenzoate (10^{-3} moles) (2)in 1N NaOH (1 ml) and the mixture is allowed to stand overnight at room temperature. The TBz-L-Arg·HCl, which separates as pale yellow crystals, is collected by filtration and dryed over P_2O_5. Yield g 0.25, m.p. 205-7 °C, $[\alpha]_{546}^{20}$ = +30, c = 0.1 in phosphate buffer 0.2 M, pH 6. TBz Arg was dissolved into anhydrous TFA (3-5 ml) and the solution heated 1 hr at 60°C (3). The TFA was removed under reduced pressure and the residue dissolved in HCl saturated methanol (about 1 ml), which is immediately removed. The racemic PTA Arg HCl is crystallized in methanol-ethyl ether. Yield g 0.23, m.p. 177-9 °C.

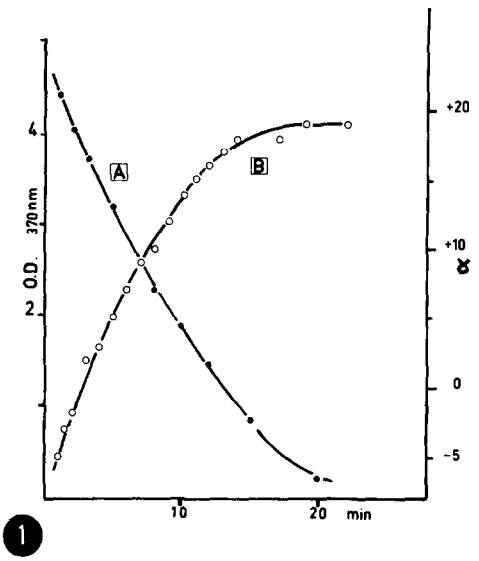
2-Phenylthiazol-5-one of Lysin (PTA Lys)

A solution of ε-carbobenzoxy-L-lysin (10^{-3} moles) is treated with one equivalent of carboxymethyldithiobenzoate as described for TBz Arg. After about 24 hr at room temperature, the solution is acidified till pH 3 with HCl and extracted with ethyl acetate. The organic layer is washed with 20 % NaCl in water, dried over Na_2SO_4 and evaporated. The oily residue of α-TBz, ε--carbobenzoxy-L-Lys ($[\alpha]_{546}^{20}$ = +37, c = 0.4 in methanol) is dissolved in TFA (about 5 ml) and heated 1 hr at 60°C to bring about both the cyclodehydratation reaction between the thioamide and the carboxyl group to yield the thiazolinone ring and the removal of the ε-protecting carbobenzoxyl group. The TFA is removed and the racemic PTA Lys is collected as hydrochloride, previous HCl/methanol treatment, and crystallized from methanol-ethyl ether. Yield g 0.145, m.p. 142-4°C.

Enzymatic hydrolysis

Kinetics of hydrolysis were followed spectrophotometrically using a Beckman DB and a Cary Model 15 equipped with a thermostated cell compartment. The hydrolysis of PTA was measured at 370 nm (Fig. 1, curve A) with a substrate concentration 0.5 to 1 x 10^{-4}M : enzyme concentration was 10^{-5} to 10^{-8}M, the higher concentrations being used at the extreme pHs were the rate constants are smaller (Fig. 2). The kinetic constants were calculated from Lineweaver-Burk plots (4) : only the linear derivative (TBz amino acid) was present at the end-point of the reaction with both PTAs.

Kinetics of optical rotation changes were followed at 546 nm using a Roussel-Jouan polarimeter digital type 71, equipped with a thermostated cell compartment (Fig. 1, curve B). Optical rotation comparison with synthetic TBz-L-Arg showed that the product resulting from trypsin action on PTA-



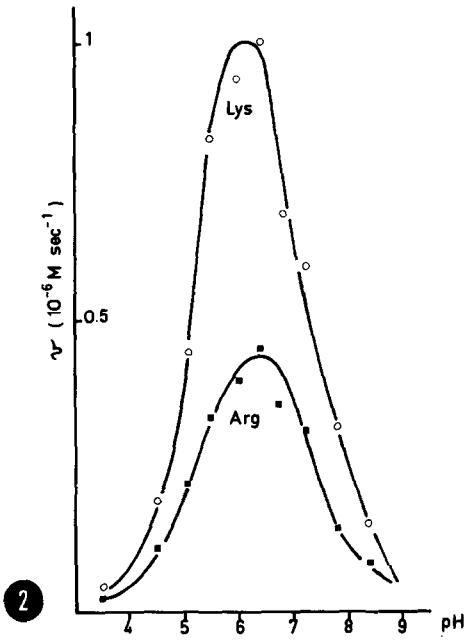


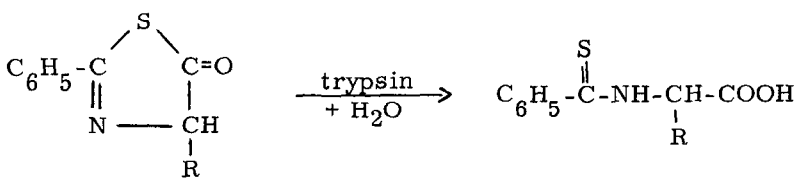
Fig. 1 : Decrease of absorbance at 370 nm (-• —• - , curve A) and increase in optical rotation, 1 = 0.7 (-○ —○- , curve B) during hydrolysis of phenyl-DL-Arg-thiazolinone (4 x 10^{-3}M) catalyzed by trypsin (5 x 10^{-6}M) at pH 6 and 25°C.

Fig. 2 : The trypsin catalyzed hydrolysis of PTA Arg and PTA Lys at 25°C in aqueous solutions. $[E]$ = 1 x 10^{-6}M.

-DL-Arg was in its L-form. In the case of PTA Lys, the product at the end point of the enzymatic reaction was carbobenzoxylated in ε-position, extracted from the reaction mixture and identified as α-TBz, ε-carbobenzoxy-L-Lys by comparison with the synthetic product.

RESULTS AND DISCUSSION

The Arg and Lys-DL-PTA are hydrolyzed by trypsin to the corresponding TBz-L-amino acids.

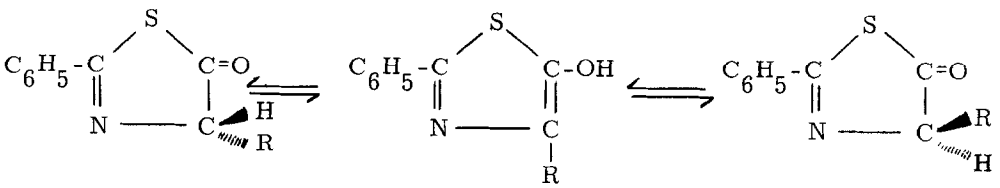


λmax (log ε) = 370 (3.98)

λmax (log ε) = 280 (3.96)
250 (4.06)

The cleavage of the chromophoric thiazolinone ring results in a complete suppression of absorbance at 370 nm, so that the reaction can be easily measured spectrophotometrically without any interference from the enzyme or the reaction product (Fig. 1, curve A). Steady state kinetic constants were determined in a pH range from 3 to 9 and plots of the initial velocity vs pH were bell shaped curves (Fig. 2) showing a maximum around pH 6.5. No spontaneous hydrolysis of the substrate is detectable in the described experimental conditions.

The k_{cat}/K_m values reported in Table show that Arg and Lys PTA behave as specific substrates for trypsin in spite of the absence of a conventional leaving group. This is not surprising if thiazolinones are viewed as internal esters of TBz Arg and Lys, being the linear benzoyl alkyl esters derivatives of the same amino acids the classical synthetic substrates of trypsin (5). Nevertheless the special molecular architecture of the thiazolinone molecules confers to these substrates additional properties when compared to the corres ponding oxygen-analogous linear compounds. Considering that solutions of asymmetric thiazolinones are optically inactive because they undergo to rapid racemization through a keto-enolic equilibrium (6)



while their hydrolysis products are much more stereostable, the simplest mechanism to account for the results reported here is that only the L-enan-

PTA derivatives of :	Arg	Lys	Phe
$k_{cat} / K_m \times 10^3$ $M^{-1}\ sec^{-1}$	8.810	13.600	0.012

TABLE : The kinetics of trypsin catalyzed hydrolysis of PTA derivatives at pH 6.5 , 25°C.

tiomer is the enzyme-susceptible molecule, being the racemisation equilibrium continuously shifted from D- to the L-form. In fact, Arg and Lys PTA are completely hydrolyzed by trypsin despite they are in racemic mixture and are entirely converted to the corresponding TBz amino acid in their L-form. This peculiar behavior, which has already been observed in the case of α-chymotrypsin (7) allows to follow the enzymatic hydrolysis by polarimetric measurements and constitutes an additional example of substrate participation to enzymatic action.

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