NON CATIONIC SUBSTRATES OF TRYPSIN

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Among proteases trypsin has the highest specificity, cleaving peptides and esterases selectively at the carbonyl donated by the cationic amino acids lysine (I) and arginine (II). Hence, it was deduced that a substrate of trypsin must have a positive charge by which it is presumably anchored electrostatically to an anionic specificity site of the enzyme prior to the catalytic events.

Based on this assumption Lindley (1956) elegantly converted cysteinyl residues into lysine-like derivatives by aminoethylation of the thiol groups with β-bromoethylamine. The resulting compounds, S-β-aminoethylcysteine (thialysine) derivatives, were hydrolyzed by trypsin.

Recently Raftery and Cole (1963,1966) used ethyleneimine instead of bromoethylamine, thus speeding up the aminoethylation step. This method found application in determination of the amino acid sequences around crysteinyl residues in proteins by specifically cleaving the peptide at the cysteinyl bond after the appropriate modification of the thiol with ethylenimine. Thus Raftery and Cole (1963,1966) cleaved reduced insulin B chain at the cysteinyl residues after treatment with ethyleneimine and trypsin. By the same method Tsung and Fraenkel-Conrat (1966) cleaved TMV protein at the only cysteinyl residue. Similarly Tanaka _et_ _al_.(1964) sequenced ferredoxin which has high content of cysteine residues.

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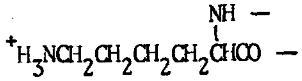
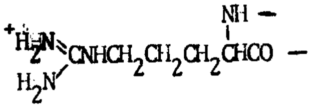
Shalitin (1961) has demonstrated that β-alanyl-glycine ethyl ester (IV) and N-benzoyl-O-glycyl-serine ethyl ester (V) are good substrates of trypsin.

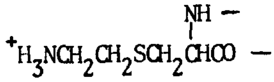
In all these cases the modified substrates are lysine analogs, carrying an ammonium group at the same distance from the susceptible bond as lysine. Replacing methylene groups in the side chain by a sulfur atom or by an ester or amide bond did not effect much the susceptibility of the substrates to tryptic digestion.

I 

$$^{+}H_3NCH_2CH_2CH_2CH_2\overset{\overset{NH-}{|}}{C}HCO-$$

II 

$$\genfrac{}{}{0pt}{}{^{+}H_2N}{H_2N}\!\!>CNHCH_2CH_2CH_2\overset{\overset{NH-}{|}}{C}HCO-$$

III 

$$^{+}H_3NCH_2CH_2SCH_2\overset{\overset{NH-}{|}}{C}HCO-$$

IV $^{+}H_3NCH_2CH_2CONHCH_2COOC_2H_5$

V $^{+}H_3NCH_2COOCH_2CH(NHCOC_6H_5)COOC_2H_5$

VI $(CH_3)_2\overset{+}{N}HCH_2CH_2CH_2CH_2CH(NHCOCH_3)COOC_2H_5$

VII $H_2NCOCH_2CH_2CH_2CH(NHCOCH_3)COOC_2H_5$

VIII $H_2NCOCH_2SCH_2CH(NHCOCH_3)COOC_2H_5$

Recently we have noticed that α-N-acetyl-ε-N-dimethyl lysine methyl ester (VI) is completely stable towards trypsin attack. This compound is structurally similar to arginine. VI has a cationic group at the end of side chain, the N atom of which is surrounded by three C atoms, while in arginine the guanidinium C atom is surrounded by three N atoms. It is therefore surprising that the ε-N-dimethyl lysine ethyl ester failed to react with trypsin.

The ε ammonium group of VI contains only one H atom directly bonded to the N atom, which is surrounded by methyl groups. On the other hand lysine and arginine have several H atoms at their side chain cationic group. We suspected that the lack of H-bondable atoms at the end of the side chain renders VI unsusceptible to tryptic attack. To test this hypothesis we prepared some non cationic lysine analogs containing at the end of the side chain an amide group which can donate hydrogens and form H bond.

Thus the ester of α-acetamido-adipamic acid (VII) (acetyl homoglutamine ester) was found to be a very good substrate of trypsin in spite of the fact that it lacks a positive charge. Also histidine methyl ester and α-N-carbobenzoxy histidine methyl ester were very good substrates of trypsin at neutral pH, where practically there is no imidazolium form. On the other hand the dimethylamido derivative of VII, lacking H bondable hydrogen atoms, was completely resistant to tryptic hydrolysis.

The data recorded in Table 1 shows that the neutral substrates are good substrates of trypsin although not as good as the cationic ones.

This rules out the current assumption that only positively charged compounds can serve as good substrates for trypsin.

An interesting application of the above finding is the conversion of cysteine derivatives to neutral trypsin substrates. N-acetyl cysteine ethyl ester was reacted with an equimolar amount of iodoacetamide (IAA) at pH range 6-8. The alkylation reaction is fast and can be readily followed in the pH stat by titrating the released protons. At pH 8 with 0.01 M of each component the reaction is over within three minutes. This alkylation rate is about 100 fold faster than that of the thiol with ethyleneimine under comparable conditions. The resulting compound N-acetyl-S-carboxamidomethyl cysteine ester (VIII) was readily split by trypsin (see Table 1).

Table 1

Kinetic parameters[a] of some cationic and neutral trypsin substrates

Substrates	Km (mM)	k(min^{-1})
Benzoyl arginine ethyl ester [b]	0.01	504
Lysine methyl ester	9.3	820
α-N-acetyl lysine ethyl ester	0.28	4800
α-N-acetyl thialysine ethyl ester (III)	1	1120
Methyl α-acetamido adipamate (VII)	4.5	192
N-acetyl-S-carboxamidomethyl cystein ethyl ester (VIII)	40	625
Histidine methyl ester	30	330
α-N-carbobenzoxy-histidine methyl ester	4.2	415

[a] Evaluated from Lineweaver-Burk plot. pH=80, 25° in 0.1 M NaCℓ

[b] From Inagami, T. and Sturtevant, J.M., J. Biol. Chem. 235, 1019 (1960)

This method may be applied to cleave peptides at their cysteinyl residues after their modification to S-carboxamidomethyl derivatives. Indeed when glutathione ester was treated with IAA and then incubated with trypsin, cleavage of the cysteinyl-glycyl bond took place.

The reaction of the above neutral substrates with trypsin was found to be competitively inhibited by cationic trypsin specific inhibitors like benzamidine, butylamine and benzylamine, with K_I values similar to those obtained in the reaction between the classical substrate benzoyl-arginine ethyl ester and the enzyme in the presence of the inhibitors. This indicates that the neutral lysine analogs bind to the same site of trypsin as the "conventional" cationic substrates and inhibitors.

It is of interest to note that while the above mentioned primary ammonium compounds are efficient competitive inhibitors of trypsin, their conversion to tertiary ammonium compounds is accompanied by loss of inhibitory power. Thus benzylamine has $K_I = 10^{-4}M$, while benzyl dimethylamine is not inhibitory. On the other hand amides like benzamide and caproamide were found to inhibit trypsin activity, although their binding to the enzyme was weaker than that of the corresponding amines. Thus K_I of benzamide was found to be $13 \times 10^{-3}M$.

This again indicates the role that H-bonding may have in binding substrates or inhibitors to trypsin.

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