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ON THE SPECIFICITY OF PORCINE ELASTASE

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Summary

The specificity of porcine elastase (EC 3.4.4.7) has been studied. Ethyl esters derived from benzoyl amino acids with straight side chains are better substrates than those with branched side chains; the best substrate is norvaline ester. In the series of benzoylalanine alkyl esters the alcohol moiety markedly affects the susceptibility. The benzyl ester was found to be the best nonactivated substrate derived from monomeric amino acid.

With elastase acylation is rate limiting, in contrast to chymotrypsin and trypsin where deacylation is generally the rate determining step with specific ester substrates.

Elastase (EC 3.4.4.7) has been the subject of extensive studies in recent years. The specificity of the enzyme was studied [1—15], its sequence solved [1, 2, 16, 17] and the tridimensional structure determined [18—21].

Geneste and Bender [5] studied the kinetics of hydrolysis of active p-nitrophenyl esters derived from carbobenzoxy amino acids. As these substrates are sparingly soluble in water only in a few cases the kinetic parameters could be determined. It was found that the alanine derivative was the best in the series. Kaplan and Dugas [3] demonstrated that benzoyl L-alanine methyl ester was a good ester substrate for elastase. Kaplan et al. [4] studied a series of *N*-carbalkoxy alanine methyl esters and found that the nature of the carbalkoxy moiety only slightly affected the kinetic parameters. Gertler and Hofmann [6] demonstrated that acetyl trialanine methyl ester was an excellent substrate for the enzyme, indicating that residues not directly linked to the scissile bond can have a tremendous effect on the enzymic reaction rate. Berger and collaborators [7—9] and Blout et al. [10—15] extended the study

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Abbreviations: Abu, α-amino butyric acid; Cys(CH_3), *S*-methyl cysteine; C_4H_9, normal butyl; C_6H_{11}, cyclohexyl; $CH_2C_6H_5$, benzyl; Z, carbobenzoxy = C_7H_7OCO; Ac, acetyl; Bz, benzoyl.

and investigated elastase catalyzed hydrolysis of peptides. They found that the nature of the peptide residues can enhance or depress the susceptibility of the alanine ester (or peptide) bond due to the interaction between the residues and binding subsites on the enzyme.

In this work we extended the study of the specificity of elastase and centered our research on two substrate families: (a) methyl esters derived from amino acids having non-branched side chains and (b) alanine esters having different alcohol moieties.

Elastase was purchased from Sigma, and amino acids of highest purity from Fluka and Sigma. Carbobenzoxy alanine and carbobenzoxy alanyl alanine were products of Fluka.

Benzoylation of amino acids was conducted by the Schotten Baumann reaction [22]. Esterification was carried out by refluxing the acyl amino acid in the respective alcohol using a few drops of sulfuric acid as a catalyst. The esterification course was followed by titration in ethanol, with 0.1 M sodium hydroxide, of a small sample using thymol blue as indicator. After the reaction was complete the alcohol was evaporated, the residue dissolved in ethylacetate, washed with bicarbonate solution and water, dried over sodium sulfate and the solvent evaporated. The resulting compounds did not show any acid content, indicating that the benzoyl amino acid was completely esterified.

Carbobenzoxyalanine phenyl ester was prepared by coupling the acid with an equivalent amount of phenol in ethylacetate using dicyclohexyl carbodiimide as the coupling agent. The urea was filtered off, the solution washed with bicarbonate and water, dried and evaporated.

Hydrolysis reactions were carried out in Radiometer PM26 pH meter in conjunction with Radiometer titrator. The reaction course was monitored by a recorder. Standard 0.05 M NaOH was used to titrate the released protons. The reaction solution contained substrate, generally in the concentration range 0.5—5 mM, (except for the substrates with lowest K_m where the concentration was 0.1—1mM), 5% dioxane and 0.1M sodium chloride in a final volume of 5.0 ml. The temperature was kept at 25°C and the pH at 8.0.

The kinetic parameters of the enzymic reactions were calculated from Lineweaver-Burk plot [24], which generally yielded straight lines.

Among the benzoyl common amino acid methyl esters that of alanine is the best substrate for elastase [4]. However Table I shows that elongation of the side chain markedly decreases K_m, namely increases affinity, whereas k_{cat} is affected to a lesser degree. The reactivity constant k_{cat}/K_m is maximum with norvaline.

Branching of side chain causes a dramatic fall, more than 300-fold decrease, in the reactivity constant values (compare norvaline and norleucine substrates with those derived from valine and leucine). These findings imply that the binding site in elastase contains a narrow somewhat elongated groove which can readily accommodate substrates with straight side chains but repelling bulky side chain groups.

The higher susceptibility of $ZAla_2OMe$ compared with that of ZAlaOMe is due to the additional alanine residue which interacts with another subsite of the enzyme. This is consistent with the findings of Gertler and Hofmann [6], Berger et al. [7—9] and Blout et al. [10, 13, 14] who showed that esters of oligoalanines are excellent substrates for elastase.

TABLE I

KINETIC PARAMETERS FOR ELASTASE CATALYZED HYDROLYSIS OF ESTERS*

	Ester substrate	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)	Ref.
1	C_6H_5CO-Ala-OCH_3	23	13	1 770	
2	C_6H_5CO-Abu-OCH_3	7.2	1.3	5600	
3	C_6H_5CO-Norval-OCH_3	20	2.4	8 300	
4	C_6H_5CO-Val-OCH_3			19	[4]
5	C_6H_5CO-Norleu-OCH_3			5 800	
6	C_6H_5CO-Leu-OCH_3			21	[4]
7	C_6H_5CO-Ile-OCH_3			16	[4]
8	C_6H_5CO-Cys(CH_3)-OCH_3	34	20	1 700	
9	C_6H_5CO-Ala-OC_4H_9	24	2.7	8 900	
10	C_6H_5CO-Ala-OC_6H_{11}	7.7	0.42	18 500	
11	C_6H_5CO-Ala-$OCH_2C_6H_5$	89	0.6	148 000	
12	C_7H_7OCO-Ala-OCH_3	3	6.25	480	[4]
13	C_7H_7OCO-Ala-OC_6H_5	45	0.53	80 300	
14	C_7H_7OCO-Ala-$OC_6H_4NO_2$ (*para*)	110	0.6	185 000	[5]
15	CH_3CO-AlaAla-OCH_3	49	22	2 300	[10]
16	C_7H_7OCO-AlaAla-OCH_3	130	6.2	21 000	
17	CH_3CO-AlaAlaAla-OCH_3	73	0.43	170 000	[6]
18	CH_3CO-AlaOCH_3	6.7	153	44	[6]

*Amino acids were of L configuration.

Comparing the kinetic parameters of ZAlaOMe with those of AcAlaOMe, or those of $ZAla_2OMe$ with those of $AcAla_2OMe$, demonstrates the noticeable effect the blocking group may have upon substrate binding, K_m, and catalytic constant, k_{cat}.

Inspection of the kinetic parameters of the alkyl esters series derived from benzoylalanine indicates that the nature of the alkyl moiety affects the susceptibility of the substrate, and the more hydrophobic is the alcohol the higher is k_{cat}/K_m resulting generally from better affinity and higher k_{cat}. The outstanding reactivity of the benzyl ester is noteworthy. This is the best substrate derived from non-activated ester of monomeric amino acid, and is comparable with the excellent tripeptide substrate $AcAla_3OCH_3$. It seems that the interaction of the benzyl group with the corresponding binding subsite of the enzyme has a pronounced effect on the reaction rate. Thus it is demonstrated that not only subsite S on the acyl moiety side, but also subsite S', on the leaving group side, contributes to binding and reactivity.

A similar conclusion has been made by Atlas and Berger [8] who found that *p*-nitrobenzyl esters of alanine peptides are comparable in activity to that of methyl esters, but the former bind better to elastase.

It is generally accepted that in elastase, like in chymotrypsin and other serine enzymes, the catalytic pathway involves the formation of an acyl enzyme intermediate according to the following scheme:

$$EOH + RCOOR' \overset{K_s}{\rightleftharpoons} EOH \cdot RCOOR' \xrightarrow{k_2} R'OH + EOCOR \xrightarrow{k_3} EOH + RCO_2^- + H$$

where EOH denotes the enzyme, $EOH \cdot RCOOR'$ the enzyme substrate complex, EOCOR the acyl enzyme intermediate, k_2 and k_3 the acylation and deacylation rate constants and K_s the enzyme-substrate complex dissociation constant. For such a mechanism the following relationships hold [25, 26].

$$k_{cat} = \frac{k_2 k_3}{k_2 + k_3} \qquad K_m = K_s \frac{k_3}{k_2 + k_3} \qquad \frac{k_2}{K_s} = \frac{k_{cat}}{K_m}$$

For a series of ester substrates derived from the same acid a common intermediate should form and if its deacylation is rate determining ($k_2 > k_3$) then $k_{cat} \approx k_3$ for all substrates irrespective of their alcohol moiety. The four-fold difference in k_{cat} between the benzyl and methyl esters of benzoylalanine implies that at least in the methyl, butyl and cyclohexyl esters acylation is rate-determining ($k_2 < k_3$), for which case $k_{cat} \approx k_2$ and $K_m \approx K_s$, namely the Michaelis constant K_m is the equilibrium dissociation constant of the enzyme-substrate complex.

From the different k_{cat} values for methyl, phenyl and *p*-nitrophenyl esters of carbobenzoxyglycine a similar conclusion is derived, namely that for methyl and phenyl esters acylation is rate-determining. The ester susceptibilities in this case are in the order of their intrinsic reactivities. This is in contrast to the case of chymotrypsin and trypsin where specific ester substrates derived from the same acid but from different alcohols undergo enzyme catalyzed hydrolysis with the same rate constant due to the rate determining deacylation of the common acylenzyme intermediate formed [27].

As elastase and chymotrypsin are homologous and related enzymes [1, 2] it was of interest to study salt effects on enzyme activity. With chymotrypsin it has been found that high salt concentration enhances enzyme activity [28]. When elastase was allowed to react with BzAlaOMe in the presence of NaCl rate enhancement was observed. The results are listed in Table II.

TABLE II

SALT EFFECT ON REACTIVITY OF ELASTASE

Benzoylalanine methyl ester was used as a substrate.

NaCl [M]	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)
0.1	23	13	1770
1.0	52	17.5	3000
2.0	66	16	4100

The higher the salt concentration the faster the reaction. The effect is expressed mainly by increasing k_{cat}, acylation rate, whereas K_m is only slightly affected. These results differ from those of Hartley and Shotton [2] who found that at 1.2 M sodium sulfate K_m rather than k_{cat} was affected. The reaction rate enhancement in salt may be caused by salt induced conformation change which enhances the reaction rate, as in the case of chymotrypsin [28].

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