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SPECIFIC ESTERASE ACTIVITY OF SUBTILISIN TOWARD ESTERS OF α-HALOACIDS

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Abstract: *Esters of α-haloacids are specific substrates for subtilisin which catalyses their hydrolysis in aqueous media. The same esters undergo transesterifications in organic solvents in the presence of subtilisin immobilized on an alumina-phosphate complex.*

Carlsberg's subtilisin is an enzyme which aspecifically hydrolyses peptides and proteins. In addition to its proteolytic activity, the enzyme possesses esterasic activity toward synthetic derivatives of several amino acids such as N-acylated-L-amino acid esters (1). Subtilisins from different strains of *Bacillus subtilis* show very similar behaviours (2). Owing to this broad specificity the activity of subtilisin can be measured with a large number of substrates each of which also reacts with other hydrolytic enzymes thus hindering a specific detection of subtilisin activity within complex enzymatic mixtures. We found that esters of α-haloacids behave as specific substrates for subtilisin which catalyses their hydrolysis in water as well as transesterification reactions within organic solvents.

The kinetic data reported in Table 1 show that methyl chloroacetate is the best substrate for subtilisin; this is probably due to its small molecular size and higher solubility in water which also allows the measurement of the k_{cat}/K_m (302 M^{-1} sec^{-1}) not far from that of other substrates. The upper analogue methyl bromoacetate as well as upper homologues (at the alcohol or acyl portion) show a reactivity which decreases with increasing molecular weights which makes them less and less soluble in water. On one hand, this may explain their lower reactivity and, on the other hand is a serious barrier for determining second order rate constants without addition of organic cosolvents which anyway alter the Km values. However the low water solubility of higher α-haloacid esters cannot be the only cause of their weak reactivity since the same relative results have been observed during transesterifications within organic solvents (see Table 2) where all the components, except

the enzyme, were perfectly soluble. Nevertheless α–haloacid esters are much better substrates for subtilisin than esters of the parent fatty acids. The enzymatic hydrolysis of methyl butyrate and methyl valerate which are the most susceptible compounds among the lower fatty acid esters (3) takes place too slowly to be measured in the range of subtilisin concentration reported in Table 1. α–Haloacid esters, because of the incorporation of an electronegative substituent in the acyl portion, are more reactive toward nucleophiles and probably require less activation energy than the parent fatty acid derivatives also in the enzymatic reaction pathway.

Table 1: Kinetic data of subtilisin catalysed hydrolysis of α–haloacid esters: RCH(X)COOR'.
1: R=H; 2: R=CH_3; 3: R=CH_3CH_2; a: R'=CH_3; b: R'=CH_3CH_2; c: R'=$CH_3CH_2CH_2$;
d: R'=$(CH_3)_2CH$; e: R'=$CH_3CH_2CH_2CH_2$

Ester	X	μmoles min^{-1} per mg enzyme	Ester	X	μmoles min^{-1} per mg enzyme
1a	Cl	26	1c	Br	7.5
1b	Cl	14	1d	Br	1.6
1c	Cl	5.7	1e	Br	1.5
1d	Cl	0.6	(R)(+)2a	Cl	14.3
1e	Cl	1.3	(S)(-)2a	Cl	12.1
1a	Br	14.2	2a	Br	4.2
1b	Br	11.1	3a	Br	1.5

The reaction mixtures contained initially 10^{-2} moles of ester in 200 ml water which were treated with 1 to 5 mg subtilisin under vigorous stirring. In all cases the amount of ester was higher than its solubility in water. Hydrolysis of esters were followed by automatic titration at pH 6.5 and 30°C with 0.2 N NaOH.

A peculiar behaviour of subtilisin in the hydrolysis of an α-haloacid ester is the absence of enantioselectivity. Actually and beside α–haloacetic acid, esters of upper homologues exist as R and S enantiomers. As shown in Table 1, the R and S enantiomers of methyl chloropropionate are hydrolysed at quite similar rates while the racemic methylbromopropionate and methylbromobutyrate have been hydrolysed till completion without change in the reaction rate. This, together with the high reactivity of the symmetric α-haloacetic acid ester, suggests that the productive binding with the enzyme takes place through less than three simultaneous interactions. Those may be the hydrolysing ester function and the polar carbon-halogen site, while the alkyl moiety of the acyl group (R in Table 1) behaves as a sterically disturbing group. Other hydrolases such as chymotrypsin, trypsin, lipase, papain and pronase (a complex mixture of hydrolytic enzymes) were found to have no effect on these esters under the experimental conditions of Table 1. In this respect, α-haloacid esters can be considered to be specific substrates for detecting subtilisin activity in the presence of other hydrolytic enzymes.

Subtilisin was immobilized on a complex of a ceramic porous carrier alumina (CPC Alumina, Fluka) with phosphoethanolamine (4) chemically activated by cyanuric chloride. In a typical procedure, CPC alumina (5 g) was suspended in 0.07 M phosphoethanolamine (15 ml) at pH 7. After 60 minutes of gentle stirring, the solid phase was washed with acetone, dried at 50 °C under vacuum and treated with a solution of cyanuric chloride (20 mg) in dry acetone (15 ml) under gentle stirring for 10 minutes. Activated alumina was washed with acetone and added to a solution of subtilisin (50 mg) in water (15 ml). Coupling proceeds rapidly (20 minutes) by gentle agitation at pH 7, and the final yield is about 60 %. Excess of reactive chlorine on the support was quenched by 15 ml of n-butylamine (0.3 M equilibrated at pH 7) overnight. The immobilized enzyme was thoroughly washed with water and stored at 4°C.

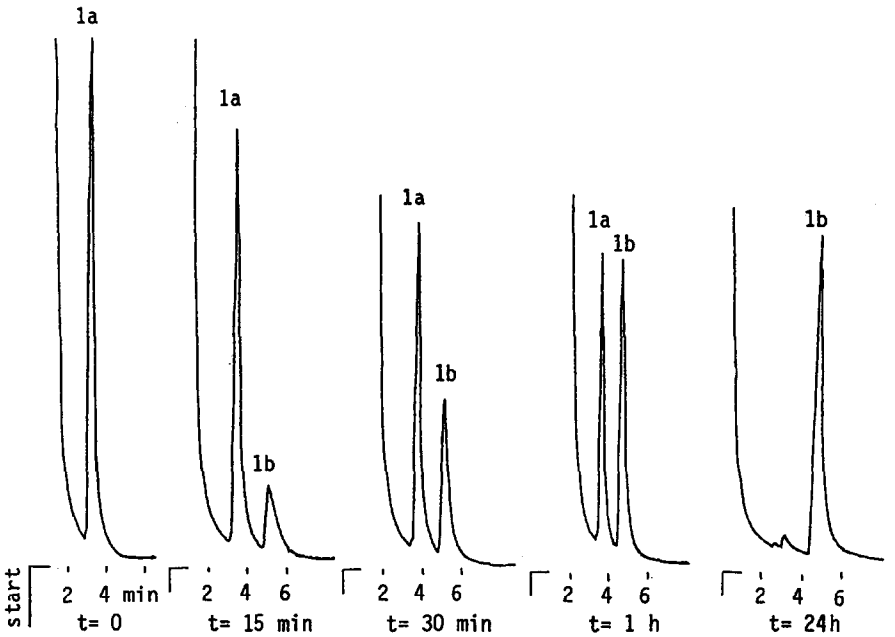

Fig 1: Progress of subtilisin catalysed transesterification: methyl-chloroacetate (1a) + ethanol -> ethyl-chloroacetate (1b) + methanol. Gas chromatographic analysis (2% XE60 chromosorb W, column temp. 70-100°C).

The immobilized enzyme was active in water (190 U/g dry support on N-acetyl-L-tyrosine ethyl ester) as well as in n-heptane where it catalyses transesterifications between esters of α–haloacids and alcools as shown in Fig. 1. The examples reported in Table 2

show that the esters of α–haloacetic acid are the most reactive, as previously observed during their enzymatic hydrolysis. The transesterification takes place more easily when methyl esters react with higher primary alcohols, yielding upper esters homologues which are less reactive toward the enzyme and can then accumulate during the reaction.

Table 2: Initial rate of transesterifications catalysed by immobilized subtilisin in n-heptane.

Substrate ester*	Alcohol	Product ester*	Initial rate µmoles/min g dry support
1a	ethanol	1b	13.6
1a	n-propanol	1c	8.6
1a	n-butanol	1e	8.4
1a	isopropanol	1d	3.3
1b	methanol	1a	1.6
1b	propanol	1c	2.6
2a	ethanol	2b	1.3

1 g of immobilized enzyme was collected from aqueous suspension on sintered glass, drained by suction and placed in 5 ml n-heptane containing 0.2 M α-haloacid ester and 1 M alcohol. The suspension was gently shaked at 22°C. The course of the reaction was followed by a gas chromatographic analysis on suitable aliquots of the supernatant.

* for structural formulas of α-haloacid esters, see Table 1.

These results are additional examples showing that enzymes covalently linked to an alumina phosphate complex are especially suitable for catalysing chemical reactions in organic media (6).

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