The Susceptibility of *N*-Acetyl-*S*-alkyl- and *N*-Acetyl-*S*-aryl-cysteine Ethyl Esters to Chymotryptic Hydrolysis

By A. P. DAMOGLU, H. LINDLEY AND I. W. STAPLETON

Division of Protein Chemistry, C.S.I.R.O., Parkville (Melbourne), Vic. 3052, Australia

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1. The preparation of the ethyl esters of some *N*-acetyl-*S*-alkyl- and *N*-acetyl-*S*-aryl-cysteine derivatives is described. 2. Kinetic parameters for the hydrolysis of these esters by chymotrypsin are reported. 3. The results are discussed in terms of the mechanism of action of chymotrypsin and also with reference to the possible application to sequence studies of cysteine-containing proteins.

The increase in our knowledge of the specificity and mode of action of proteolytic enzymes has led to the possibility of modifying substrate proteins so as to influence their susceptibility to enzymic attack. This is especially true of tryptic digestion, where a number of techniques have been used to inhibit hydrolysis adjacent to lysine residues and hence restrict breakdown (Redfield & Anfinsen, 1956; Anfinsen, Sela & Tritch, 1956; Merigan, Dreyer & Berger, 1962; Goldberger & Anfinsen, 1962). Conversely the susceptibility of proteins to digestion by trypsin has been extended by *S*-aminoethylation of cysteine side chains to form the lysine analogue, 2-aminoethylcysteine (thialysine) (Lindley, 1956). The side chain of this residue resembles that of lysine sufficiently closely for the adjacent peptide bond to be split by trypsin. It has also been shown that the corresponding serine derivative (oxalysine) can act as a substrate for trypsin (Tesser, Nivard & Gruber, 1964), but exploitation of this finding awaits satisfactory procedures for the *O*-aminoethylation of serine in proteins. However, the demonstration by Patchornik, Wilchek & Zioudrou (1969) that serine can be converted directly into thialysine (and presumably into other *S*-substituted cysteine derivatives) suggests a valuable alternative extension of the principle. It would seem probable that any further extension of this general principle of converting amino acid side chains into enzyme substrate analogues will follow most simply from consideration of other proteolytic enzymes. Chymotrypsin is an obvious choice not only because its specificity has been more thoroughly studied than that of any other proteolytic enzyme but also because its three-dimensional structure is known (Blow, 1969). In consequence detailed theories about its mechanism of action have been proposed (Blow, Birktoft & Hartley, 1969), so that observa-

tions on novel substrates are especially relevant at this time. Consideration of the probable type of side-chain modification required to satisfy the specificity of chymotrypsin by reference to the review of Knowles (1965a) suggests that modification of cysteine is once again the simplest choice. This paper describes the preparation of five substituted cysteine derivatives and a study of their behaviour as chymotryptic substrates.

EXPERIMENTAL

Preparation of cysteine derivatives. (a) Alkyl and aryl cysteines. *S*-Benzyl-L-cysteine (Wood & du Vigneaud, 1939), *S*-phenyl-L-cysteine (Zbarsky & Young, 1943), *S*-ethyl-L-cysteine (Armstrong & Lewis, 1951), *S*-*n*-propyl-L-cysteine (Armstrong & Lewis, 1951) and *S*-isopropyl-L-cysteine (Stoll & Seebeck, 1949) were prepared by published procedures. The compounds gave *R_F* values of 0.71, 0.71, 0.59, 0.82 and 0.68 respectively on silica gel t.l.c. with the solvent system butan-2-ol-acetic acid-water (5:1:4, by vol.). Satisfactory N contents were obtained in each case.

(b) Amino acid ethyl ester hydrochlorides. The ethyl ester hydrochlorides of the five substituted cysteines were prepared by the standard Fischer procedure and crystallized from ethanol-ether. Preparative data are shown in Table 1 for the three new compounds. *S*-Benzyl-L-cysteine ethyl ester hydrochloride (Harington & Pitt-Rivers, 1944) has been previously reported and gave m.p. 156–157°C (lit. m.p. 156–157°C) as well as *S*-ethyl-L-cysteine ethyl ester hydrochloride (Enz & Cecchinato, 1961), which gave m.p. 137–138°C (lit. m.p. 138–139.5°C).

(c) Acetyl amino acid ethyl esters. The amino acid ester hydrochloride (9 mmol) was dissolved in chloroform (70 ml) containing triethylamine (9 mmol) and the resulting solution stirred vigorously with a solution of Na₂CO₃ (1 g) in water (6 ml). Acetyl chloride (11 mmol) was added in one portion and the stirring continued for 2–3 h. The organic layer was separated and washed successively with *N*-HCl, water, 5% NaHCO₃, water and finally dried over Na₂SO₄. The solvent was removed

Table 1. *Analytical data on S-substituted cysteine ethyl esters*

Amino acid ester hydrochloride	Yield (%)	m.p. (°C)	$[\alpha]_D^{21}$	Analysis†					
				Found			Calc.		
				C	H	S	C	H	S
<i>S</i> -Phenyl-L-cysteine	62	107–108	+85.4°*	49.8	6.2	12.3	50.5	6.2	12.2
<i>S</i> - <i>n</i> -Propyl-L-cysteine	58	150–151	–6.3°†	42.2	7.8	14.1	42.4	7.8	13.4
<i>S</i> -Isopropyl-L-cysteine	49	161–162	–15.0°†	42.2	7.8	14.1	41.6	7.9	14.4

* *c* 4 in ethanol.† *c* 1 in *m*-HCl.

‡ Analyses carried out by Australian Microanalytical Laboratory, C.S.I.R.O., Melbourne, Vic., Australia.

Table 2. *Analytical data on N-acetyl ethyl esters of S-substituted cysteine derivatives*

The compounds listed as oils ran as single spots on t.l.c. n.d., Not determined.

<i>N</i> -Acetylamino acid ethyl ester	Yield (%)	m.p. (°C)	$[\alpha]_D^{21}$ *	Analysis							
				Found				Calc.			
				C	H	N	S	C	H	N	S
<i>S</i> -Benzyl-L-cysteine	73	65–66	–60.6°	59.6	7.0	4.9	n.d.	59.8	6.8	5.0	n.d.
<i>S</i> -Phenyl-L-cysteine	52	54–55	–7.5°	58.2	6.7	n.d.	12.3	58.4	6.4	n.d.	12.0
<i>S</i> - <i>n</i> -Propyl-L-cysteine	74	Oil	–26.9°	51.1	8.1	6.5	13.7	51.5	8.2	6.0	13.7
<i>S</i> -Isopropyl-L-cysteine	73	Oil	–32.0°	51.1	8.1	6.2	13.3	51.5	8.2	6.0	13.7
<i>S</i> -Ethyl-L-cysteine	68	Oil	–27.5°	48.7	7.9	6.4	14.6	49.3	7.8	6.4	14.6

* *c* 1 in ethanol.Table 3. *Substrate and enzyme concentrations used in kinetic experiments*

Ester side chain	Concn. of substrate (mM)	Concn. of enzyme (μM)
<i>S</i> -Ethyl	0.3 –2.7	0.338
<i>S</i> - <i>n</i> -Propyl	0.09–2.3	0.28
<i>S</i> -Isopropyl	0.2 –1.35	0.28
<i>S</i> -Benzyl	0.1 –1.6	0.304
<i>S</i> -Phenyl	0.09–0.9	0.02275

in vacuo and the oily product crystallized from ether-light petroleum (b.p. 40–60°C). The *S*-alkylcysteine derivatives could not be induced to crystallize and were purified by passage through a short silica gel column in chloroform. Analytical and other data are recorded in Table 2.

α-Chymotrypsin (EC 3.4.4.5). This was isolated from bovine pancreas and used as bought from Mann Research Laboratories (3× crystallized, salt-free; cat. no. 05300-4767).

Reaction kinetics. The hydrolysis of these esters by chymotrypsin to yield an acid and an alcohol was followed by alkali titration in a pH-stat (Wood, 1960) of the acid produced. The titrations were carried out at pH 7.9, 25°C and in 0.1 *M*-NaCl under an atmosphere of N₂ with the substrate and enzyme concentrations shown in Table 3. The substrate and salt were equilibrated at the temperature and pH of the digest and the enzyme solution (50–

100 μl) was added rapidly from a syringe. The initial velocity of the reaction was found from a computer analysis of the graph of alkali addition against time. A least-squares regression programme was used fitting polynomials of increasing degree and testing the significance of each added term. The 'observed' kinetic parameters were found by averaging the values obtained by plotting 1/*v* against 1/*s*, *s/v* against *s* and *v* against *v/s* and in each case calculating the best straight line by the method of least squares.

RESULTS

Table 4 shows the kinetic parameters we have found for the chymotryptic hydrolysis of the *N*-acetyl ethyl esters of *S*-ethyl-, *S*-propyl-, *S*-isopro-

Table 4. *Kinetic parameters of chymotryptic hydrolysis of* $\text{CH}_3 \cdot \text{CO} \cdot \text{NH} \cdot \text{CH}(\text{CH}_2 \cdot \text{S} \cdot \text{R}_s) \cdot \text{CO} \cdot \text{OEt}$

Hydrolysis was at 25°C, pH 7.9, in 0.1 M-NaCl.

R_s	k_0/K_0 ($\text{s}^{-1}\text{M}^{-1}$)	K_0 (mM)	k_0 (s^{-1})
$\text{CH}_2 \cdot \text{CH}_3$	786	3.04	2.39
$\text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}_3$	1720	6.00	10.33
$\text{CH} \cdot (\text{CH}_3)_2$	1542	1.46	2.23
6280	0.48	3.0
67000	0.61	41.2

Table 5. *Kinetic parameters of chymotryptic hydrolysis of* $\text{CH}_3 \cdot \text{CO} \cdot \text{NH} \cdot \text{CH}(\text{R}) \cdot \text{CO} \cdot \text{OMe}$

Hydrolysis was at 25°C, pH 7.9, in 0.1 M-NaCl. Taken from the review of Knowles (1965a). References to individual results are: (a) Jones & Niemann (1962); (b) Jones, Kunitake, Niemann & Hein (1965); (c) C. Niemann (unpublished work); (d) Knowles (1965b).

Amino acid	R	k_0/K_0 ($\text{s}^{-1}\text{M}^{-1}$)	K_0 (mmol)	k_0 (s^{-1})	Reference
Norvaline	$\cdot (\text{CH}_2)_2 \cdot \text{CH}_3$	265	10.2	2.7	(a)
Norleucine	$\cdot (\text{CH}_2)_3 \cdot \text{CH}_3$	1250	6.7	8.39	(b)
Methionine	$\cdot (\text{CH}_2)_2 \cdot \text{S} \cdot \text{CH}_3$	2300	8.1	18.6	(c)
α -Aminoheptanoic acid	$\cdot (\text{CH}_2)_4 \cdot \text{CH}_3$	8770	1.64	13.4	(b)
Phenylalanine		42000	1.25	52.5	(b)
Tyrosine		365000	0.32	117	(c)
Tryptophan		420000	0.117	49	(d)

pyl-, *S*-benzyl- and *S*-phenyl-L-cysteine. Obviously the compounds are substrates for chymotrypsin, the *S*-propylcysteine derivative being the best of the *S*-alkyl cysteines. However, this is easily surpassed by the *S*-benzylcysteine compound and even this is a poor substrate compared to the *S*-phenyl derivative.

Table 5 shows the same kinetic parameters for a range of related compounds: the data have been taken from a review by Knowles (1965a). Comparison of these data with our results show that in fact *S*-phenyl-L-cysteine ranks comparably with phenylalanine as a substrate for chymotrypsin.

DISCUSSION

The data in Table 4 and 5 enable us to compare two pairs of compounds in which a sulphur atom

has replaced a methylene group, namely (1) *S*-ethylcysteine and norleucine and (2) *S*-propylcysteine and α -aminoheptanoic acid. In both these instances the simple aliphatic compound is a better substrate than the corresponding *S*-alkyl cysteine and hence would suggest that a methylene group provides a better fit than a sulphur atom. However, Table 5 itself contains another pair of compounds for which a direct comparison can be made, i.e. methionine and norleucine. For this pair it would appear that the replacement of a methylene group by a sulphur atom has led to an increase in reactivity. We thus have the interesting trio of analogues, methionine, norleucine and *S*-ethylcysteine, which rank as substrates in that order. An examination of the structure of the side chain (I, II and III) shows that the location of the sulphur atom in the side chain is possibly an important factor and may

be related to the number of Van der Waal's interactions. In view of the very small alteration in shape and size introduced by the replacement of a methylene group by a sulphur atom it would seem likely that the explanation of these effects will require a very detailed knowledge of the mechanism of action of chymotrypsin. Now that the complete three-dimensional structure of the enzyme is known (Blow, 1969) it should be possible by the use of models to account for these results.

From the practical viewpoint of increasing peptide bond susceptibility to chymotrypsin in proteins, S-phenylation would seem to be the only chemical modification worth considering, especially if substantially complete hydrolysis of bonds adjacent to the modified cysteine residue is required. Unfortunately S-phenylation is not a simple reaction to carry out, especially on materials as complex as proteins, and preliminary results have not been very encouraging.

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