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A consensus sequence for substrate hydrolysis by rhinovirus 3C proteinase

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Kinetic constants were determined for the hydrolysis of a series of synthetic peptide substrates by recombinant rhinovirus (HRV 14) 3C proteinase. Systematic removal or replacement of individual residues indicated that the minimum sequence required for effective cleavage by the viral cysteine proteinase was P_5-Val/Thr-P_3-P_2-Gln-Gly-Pro.

Rhinovirus proteinase; Synthetic substrate; Specificity; Minimum requirement

1. INTRODUCTION

The production of picornaviral proteins as 200 kDa polyproteins requires the action of virally-encoded proteinases to produce correct cleavage and assembly of the virus [1]. One of these proteinases (3C) has been shown to be responsible for most of the cleavages [2]. This enzyme from rhinovirus (HRV14 3C proteinase) has been cloned and expressed in *E. coli* [3,4] and in a previous report, its action on a series of long synthetic peptide substrates and susceptibility to inhibition was described [5]. The enzyme demonstrated complete specificity for Gln-Gly bonds and the presence of a proline residue in P_2' (i.e. in the sequence Gln-Gly-Pro) appeared to enhance the rate of substrate cleavage by approximately two orders of magnitude. The minimum requirements to maintain this effective rate of hydrolysis are described herein.

2. MATERIALS AND METHODS

Rhinovirus 14 3C proteinase was cloned and expressed in *E. coli* as described previously [4] and purified to homogeneity [4]. Peptides were synthesised by the solid phase method of Merrifield [6] on an Applied Biosystems Model 430A peptide synthesiser, utilising preformed symmetric anhydrides [5]. After reverse-phase FPLC/HPLC purification, certain peptides were acetylated by reaction with acetic anhydride as described before [7] and re-isolated by reversed-phase chromatography.

Incubations of proteinase with the various peptide substrates at a variety of concentrations was carried out under the optimum conditions elucidated previously [5] for the substrate.

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Tyr-Arg-Pro-Val-Val-Val-Gln-Gly-Pro-Asn-Thr-Glu-Phe

i.e. in 350 μl of 6 mM sodium citrate/94 mM sodium phosphate buffer containing 5 mM DTT, 2 mM EDTA and 10% glycerol, pH 7.6. From each reaction mixture, aliquots (80 μl) were withdrawn at 3 separate time points and the reaction was quenched by addition of trifluoroacetic acid to a final concentration of 0.1 % (v/v). Product analysis, identification and quantitation was carried out as described previously [5]. Initial rates of hydrolysis were calculated from each linear progress curve thus obtained and kinetic parameters (K_m and V_{max}) were derived from computerised least-squares fitting of the initial velocities to those predicted by the Michaelis-Menten equation in a similar manner to that report earlier [5].

3. RESULTS AND DISCUSSION

Determination of the kinetic parameters for the hydrolysis of the parent peptide 1 (table 1) derived from the 3B/3C junction in the HRV14 polyprotein confirmed the results reported previously [5] of rapid hydrolysis of this substrate.

Systematic truncation of this peptide from the N-terminal end was carried out whilst maintaining the C-terminus constant (peptides 2, 3, 4 and 6; table 1). Removal of the tyrosine, P_7 and arginine, P_6 residues in succession (peptides 2 and 3) had only very small effects on V_{max} and K_m. In an enzyme assay which, of necessity, was required to be based upon resolution of peaks by FPLC for quantitation, these small differences were not considered to be of significance particularly by comparison with the effect observed upon deletion of the next residue (proline, P_5) in turn (peptide 4, table 1). The rate of cleavage became so slow that it was less than 1% of that observed previously and deletion of a further residue (valine, P_4) to produce peptide 6

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Table 1

Kinetic parameters for rhinovirus proteinase 3C cleavage of synthetic peptide substrates with systematic *N*-terminal truncation

Substrate	Structure	V_{max} (μmol/min/mg)	K_m (mM)
1	Y-R-P-V-V-V-Q-G-P-N-T-E-F	3.2	0.7
2	R-P-V-V-V-Q-G-P-N-T-E-F	1.4	0.4
3	P-V-V-V-Q-G-P-N-T-E-F	2.5	1.8
4	V-V-V-Q-G-P-N-T-E-F	0.01*	-
5	Acetyl-V-V-V-Q-G-P-N-T-E-F	0.004*	-
6	V-V-Q-G-P-N-T-E-F	0	-
7	Acetyl-V-V-Q-G-P-N-T-E-F	0	-

All measurements were carried out at 35°C in a buffer consisting of 6 mM sodium citrate/94 mM sodium phosphate, pH 7.6 containing 5 mM DTT, 2 mM EDTA and 10% glycerol. Samples were removed at 3 appropriate time points and quenched by addition of trifluoroacetic acid to a final concentration of 0.1 % before analysis by reversed phase HPLC. Each K_m and V_{max} value given is from a single determination and the estimated precision is in the range ± 20%. Replicate analyses were performed in some instances

*Hydrolysis was very slow; the value given is the rate of cleavage measured over 22 h at a substrate concentration of 0.5 mM

resulted in no hydrolysis whatsoever, even upon prolonged incubation.

Thus it would appear that the enzyme requires 5 residues to be present in P_5-P_4-P_3-P_2-P_1 upstream from the scissile Gln-Gly bond for efficient cleavage to occur. The requirements in the region of P_4 and P_5 were investigated further by neutralisation of the positive charge on the amino terminal valine residue occupying the P_3 position (peptide 6) through acetylation (to produce peptide 7). However, the resultant peptide was still totally resistant to enzymatic action (table 1). Similarly, acetylation of the terminal valine residue occupying the P_4 position (in peptide 4) generated peptide 5 (table 1). However, this modification had no effect either on the very slow rate of cleavage that had been observed with the unblocked peptide (peptides 4 and 5; table 1). Thus, it would appear that neutralisation of the positive charge on the N-terminus of these shorter peptides is not adequate to achieve effective hydrolysis of the substrate.

Further investigations were then performed by systematic truncation from the C-terminal end of the peptide with maintenance of the residues in P_5-P_1 plus an additional arginine residue in P_6 which, although it contributes little in the way of facilitating interaction with the enzyme (table 1) nevertheless confers greater solubility on the peptides. Once again, removal successively of Phe, Glu, Thr and Asn from the C-terminus (peptides 8, 9, 10 and 11) still permitted rapid cleavage of the diminishing (in size) peptides (table 2). Deletion of the Asn residue from the P_3' position (peptide 11) appeared to decrease the binding of the substrate since the observed K_m value for substrate interaction (table 2) increased by about 8-fold.

The importance of the Pro in P_2' has been indicated previously [5] so that the reduction (by 1–2 orders of magnitude) in the rate of hydrolysis measured upon removal of this residue (with replacement by an amide function; peptide 12) was not unexpected (table 2). Indeed, upon deletion of the proline and replacement with (the downstream sequence of) Asn-Thr, the resultant peptide (peptide 13) was totally resistant to hydrolysis.

In a further attempt to investigate the significance of the proline residue in P_2', peptide 14 was prepared which is an amidated version of peptide 11 (table 2). This modification resulted in an essentially linear increase in v with concentration of peptide 14 up to concentrations which exceeded the practical limitations on solubility of peptide 14. Thus, the already weak K_m observed for peptide 11 was made apparently worse by this C-terminal modification in peptide 14. However, from the estimated value of K_m obtained for peptide 14, it is evident that although it interacts less efficiently with the proteinase, it is nevertheless still cleaved quite readily (table 2).

Finally in this series, in order to confirm the stringent requirements of this proteinase, the Gln residue in the P_1 position in a good substrate (peptide 9) was substituted by glutamic acid. The resultant peptide [15] (table 2) was not cleaved even upon prolonged incubation.

From these data, it may be concluded that the minimum length of peptide required for efficient cleavage by rhinovirus 3C proteinase is seven residues spanning P_5-P_2'. However, the presence of an additional residue in P_3' appears to facilitate substrate binding such that peptide 10 was the best substrate (V_{max}/K_m = 5.4) devised in these experiments. Amidation of the Pro residue in P_2' did not produce a similar improvement.

Table 2

Kinetic parameters for rhinovirus 3C proteinase cleavage of synthetic peptide substrates with systematic C-terminal truncation

Substrate	Structure	V_{max} (μmol/min/mg)	K_m (mM)
2	R-P-V-V-V-Q-G-P-N-T-E-F	1.4	0.4
8	R-P-V-V-V-Q-G-P-N-T-E	2.1	0.9
9	R-P-V-V-V-Q-G-P-N-T	1.7	0.5
10	R-P-V-V-V-Q-G-P-N	2.0	0.4
11	R-P-V-V-V-Q-G-P	5.0	3.1
12	R-P-V-V-V-Q-G-NH_2	~0.07*	-
13	R-P-V-V-V-Q-G-N-T	0	
14	R-P-V-V-V-Q-G-P-NH_2	~10	>10
15	R-P-V-V-V-E-G-P-N-T	0	

Conditions were as described in the legend to table 1
*Reaction rate very slow; the value given is the rate of cleavage measured over 6 h at a substrate concentration of 1.1 mM

In a final series of investigations then, peptide 10 was mutated in order to examine the importance of the nature of the residue occupying the P_4 position (table 3). Substitution of the valine residue by an isosteric threonine resulted in a reduction in the rate of cleavage (peptide 16; table 3). The K_m value for this peptide (2.0 mM) was also considerably poorer than the corresponding value (0.4 mM; table 2) measured for the valine-containing peptide 10. This effect was relatively minor, however, in comparison with the reduction in sensitivity to hydrolysis observed upon replacement of the Val in P_4 with a bulkier, aromatic tyrosine residue (peptide 17; table 3). Substitution with a positively charged Arg residue (peptide 18) abolished hydrolysis completely. None of the shorter peptides lacking residues in P_6 and P_5 (peptides 19–22) were hydrolysed to any significant extent irrespective of the nature of the residue occupying the P_4 position (table 3).

These results further substantiate our earlier findings [5] of rapid hydrolysis of (larger but variable) peptides containing Val, Thr or Ala in the P_4 position. Thus a residue with a small relatively hydrophobic side chain would appear to be required in this (relatively) distant position before the rhinovirus 3C proteinase can carry out effective cleavage at the P_1–P_1' bond.

Although it has not been examined directly in this report, the nature of the residues occupying the P_5, P_3 and P_2 positions would appear to be less crucial since our previous work [5] has indicated effective cleavage of (larger) peptides with a much wider variety of residues (P_5 = Glu, Phe or Pro). (P_3 = Leu, Gln or Val), (P_2 = Phe, Thr or Val) in these positions. Therefore, this enzyme with its absolute primary specificity towards the Gln-Gly bond in the sequence

P_5-Val/Thr-P_3-P_2-Gln-Gly-Pro

requires that these positions be occupied in order to ensure rapid cleavage but the detailed atomic interactions may be less critical. This binding could involve specific spatial or conformational properties. Unfortunately, no detailed analysis of the three-dimensional structure of such viral enzymes is currently available to facilitate

Table 3

Cleavage of synthetic peptide substrates varying in the P_4 position by rhinovirus 3C proteinase

Substrate	Structure	V_{max} (μmol/min/mg)
10	R-P-V-V-V-Q-G-P-N	2.0
16	R-P-T-V-V-Q-G-P-N	0.3
17	R-P-Y-V-V-Q-G-P-N	0.03*
18	R-P-R-V-V-Q-G-P-N	0
19	V-V-V-Q-G-P-N	~0
20	T-V-V-Q-G-P-N	0.05**
21	Y-V-V-Q-G-P-N	0
22	R-V-V-Q-G-P-N	0

Conditions were as described in the legend to table 1
*Cleavage rate measured at the highest substrate concentration (0.5 mM) that could be achieved in practice
**Cleavage rate measured at the highest substrate concentration (16.8 mM) that could be attained

these interpretations. However, these requirements will be the subject of further investigations.

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