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Sequence-Specific Inhibition of a Nonspecific Protease

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Supporting Information

ABSTRACT: A nonspecific exopeptidase, aminopeptidase N (APN), is inhibited sequence-specifically by a synthetic host, cucurbit[7]uril (Q7), which binds with high affinity and specificity to N-terminal phenylalanine (Phe) and 4-(aminomethyl)phenylalanine (AMPhe) and prevents their removal from the peptide. Liquid chromatography experiments demonstrated that in the presence of excess Q7, APN quantitatively converts the pentapeptides Thr-Gly-Ala-X-Met into the dipeptides X-Met (X = Phe, AMPhe). The resulting Q7-bound products are completely stable to proteolytic digestion for at least 24 h. Structure–activity studies revealed a direct correlation between the extent of protection of an N-terminal amino acid and its affinity for Q7. Therefore, Q7 provides predictable sequence-specificity to an otherwise nonspecific protease and enables the production of a single peptide product. Conversely, APN uncovers a high-affinity epitope that is subsequently bound by Q7, and thus this approach should also facilitate the molecular recognition of peptides.

Methods for selective enzymatic digestion of peptides and proteins are crucial to many processes in molecular biology and biotechnology. Proteases are widely used to process polypeptides for sequencing[1] and other applications in proteomics[2] and medicine.[3] Endopeptidases, which cleave the peptide backbone at nonterminal sites, typically have well-defined sequence specificity. Exopeptidases, which remove amino acids sequentially from a terminus, can be specific for a small set of amino acids or generally nonspecific, which allows them to digest a peptide completely into amino acids.[4,5] Developing methods that change the substrate specificity of proteases would broaden the scope of their applications. Here we show that a synthetic receptor, cucurbit[7]uril (Q7), can be used to impart specificity to an otherwise nonspecific exopeptidase, porcine aminopeptidase N (APN), by binding to a specific residue and inhibiting its removal from the peptide.

Q7 is a highly stable container molecule that can associate noncovalently with a wide range of cationic organic guests in aqueous media with equilibrium association constant (K_a) values of up to 10^{15} M^{-1}.[6−8] We and others have studied the capacity of Q7 to bind to amino acids, peptides, and proteins and found that Q7 prefers to bind N-terminal phenylalanine (Phe), tyrosine (Tyr), and tryptophan (Trp) residues by incorporating the side chain within the nonpolar Q7 cavity and chelating the N-terminal ammonium group with Q7 carbonyl oxygens.[9−14] Nau and co-workers have shown that Q7 can slow the activity of an endopeptidase, trypsin, and an exopeptidase, leucine aminopeptidase (LAP), by binding to their respective substrates.[15,16] In both cases, they observed only partial and short-lived inhibition. We hypothesized that this approach could be used to crop a complete sample of peptide down to the first Phe residue if the aminopeptidase could be inhibited exclusively at this position.

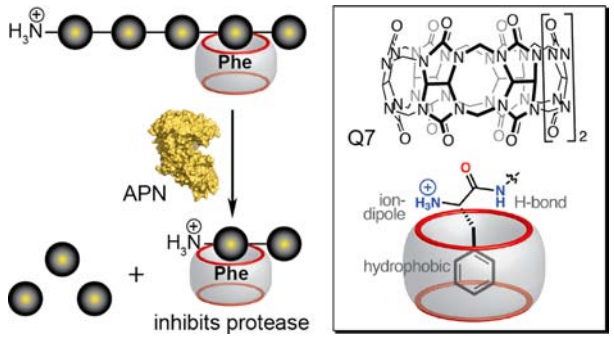

Figure 1. (left) Schematic illustration of the inhibition of APN-mediated peptide digestion at a Phe residue using Q7. (right) Chemical formula of Q7 and schematic illustration of the molecular recognition of N-terminal Phe.

LAP was not completely inhibited by Q7,[15,16] and therefore, we chose to test a different aminopeptidase. APN was chosen for its lack of specificity,[4] medicinal relevance,[17] and commercial availability. The pentapeptide Thr-Gly-Ala-Phe-Met (**1**) was chosen as the first substrate for APN digestion because it contains five different amino acid residues, including hydrophilic and hydrophobic side chains, and it contains a nonterminal Phe residue, which becomes the N-terminal residue after APN removes the Thr, Gly, and Ala residues (Figure 1). The entrance to the catalytic site of APN is highly constricted, and specific interactions are made with the side chain of the terminal residue.[18] Q7 should protect an N-terminal Phe by encapsulating its side chain and interacting directly with the terminal ammonium group (Figure 1). Peptide **1** and the predicted digestion product, Phe-Met (**2**), were synthesized. These peptides and the others used in this study had C-terminal amides because they were synthesized on Rink amide resin.

Analytical HPLC was used to monitor the digestion of 0.50 mM **1** by 0.20 μM APN in the absence of Q7 and in the presence of a substoichiometric quantity (0.25 mM), a stoichiometric quantity (0.50 mM), and a 2-fold excess (1.0 mM) of Q7 (Figure 2). Samples were analyzed at reaction times of 5 min, 3 h, and 24 h. At the 5 min time point, we observed substantial degradation of the starting material

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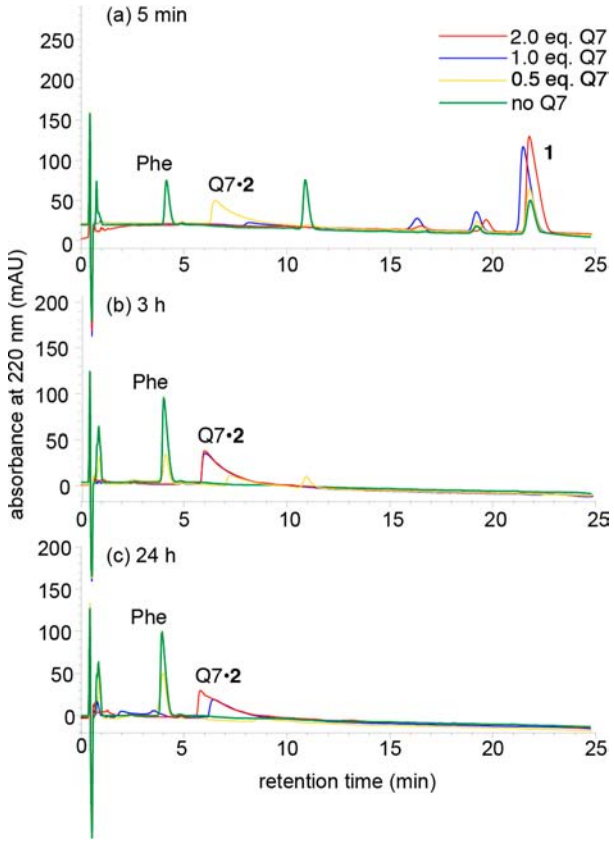

Figure 2. Analytical HPLC traces of the digestion of 0.5 mM Thr-Gly-Ala-Phe-Met (**1**) with 0.20 μM APN in the presence of 0, 0.5, 1.0, or 2.0 molar equiv of Q7 at 37 °C in 10 mM ammonium phosphate (pH 7.2) for reaction times of (a) 5 min, (b) 3 h, and (c) 24 h.

(retention time 21−22 min) in the absence of Q7 and at 0.25 mM Q7 but substantially slower degradation at 0.50 and 1.0 mM Q7.

At the 3 h time point (Figure 2b), all of the samples showed complete disappearance of the starting material. Remarkably, only one peak (at ∼6 min) remained in the HPLC traces of the samples containing 0.50 and 1.0 mM Q7. The broad shape and retention time of this peak corresponded exactly to those of a standard sample of the Q7·**2** complex, and electrospray ionization mass spectrometry (ESI-MS) confirmed the digestion product to be the Q7·**2** complex (see the Supporting Information). The conversion of pentapeptide **1** to the Q7·**2** complex was quantified by comparing the area of the product peak with that of a standard sample of Q7·**2**. We observed quantitative conversion (101 ± 1.1% and 98 ± 1.3%) in the presence of 1.0 and 0.50 mM Q7, respectively. With substoichiometric Q7 (0 or 0.25 mM), however, there was no substantial formation of the Q7·**2** product. Instead, we observed a peak at ∼4 min corresponding to free Phe.

At the 24 h time point (Figure 2c), there was no change in the HPLC trace of the sample containing 1.0 mM Q7. This result indicates that the Q7·**2** complex is highly stable under these conditions. In the sample containing 0.5 mM Q7, we observed a 37% reduction in the area of the Q7·**2** peak.[19] Therefore, an excess of Q7 is needed to protect the Q7·**2** product over longer periods of time.

It is interesting to consider how the increased concentrations of Q7 impeded the initial degradation of the substrate at the 5 min time point. In the samples containing 0.50 and 1.0 mM Q7, appreciable quantities of the Q7·**2** complex had not yet formed, but the enzymatic activity was clearly retarded. Higher Q7:**1** ratios were needed to form and protect the Q7·**2** product at 3 and 24 h. These results suggest that Q7 also has a small inhibitory effect by binding to the enzyme but that inhibition of proteolysis is primarily governed by binding of Q7 to the substrate, as observed for LAP.[15]

Table 1. Peptide Protection versus Binding Constant[a]

peptide	residual peptide (%)	K_a (M^{-1})[d]
Phe-Met (**2**)	93.9 ± 1.1[b]	(1.4 ± 0.1) × 10^7
Phe-Met (**2**)	100.4 ± 1.4[c]	
Tyr-Met (**3**)	19.8 ± 3.5[b]	(6.4 ± 0.4) × 10^5
Trp-Met-Gly (**4**)	1.5 ± 0.1[b]	(2.3 ± 0.1) × 10^5
AMPhe-Met (**5**)	101.6 ± 1.5[b]	(5.3 ± 1.1) × 10^8

[a]HPLC experiments were performed after reaction at 37 °C in 10 mM ammonium phosphate (pH 7.2); residual peptide values were determined from integrated peak intensities in the HPLC traces of samples after 24 h. ITC experiments were performed at 27 °C in 10 mM ammonium phosphate (pH 7.2). [b]Fraction of 0.50 mM peptide remaining after 24 h in the presence of 1.0 mM Q7 and 0.20 μM APN. [c]Fraction of 0.10 mM **2** remaining after 24 h in the presence of 1.0 mM Q7 and 0.040 μM APN. [d]Reported values are means ± standard deviations measured from at least three ITC experiments.

The highest-affinity binding sites for Q7 on natural peptides are at N-terminal Phe, Tyr, and Trp residues (K_a ∼ 10^5−10^7 M^{-1}); other potential binding sites have much weaker affinities.[9] To assess the extent to which Q7 can protect these residues from APN digestion, we prepared a series of peptides with different aromatic N-termini and measured the extent of their protection by Q7 in the presence of APN. Phe-Met (**2**), Tyr-Met (**3**), and Trp-Met-Gly (**4**), each at 0.50 mM, were incubated for 24 h with 1.0 mM Q7 and 0.20 μM APN and then analyzed by HPLC. Table 1 gives the residual quantities of peptides remaining after 24 h and compares these values to their binding affinities for Q7 as determined by isothermal titration calorimetry (ITC). These data reveal a direct correlation between the binding affinity and the extent of protection by Q7 and demonstrate that Q7 inhibits APN degradation most effectively at a Phe residue. On the basis of the clear substrate-inhibition mechanism, we should be able to increase the ratio of Q7:**2** in order to increase the extent of protection by Q7. To test this hypothesis, we increased the ratio of Q7:**2** to 10:1 and in fact observed complete retention of peptide **2** after 24 h (Table 1).

Considering the correlation between the extent of peptide protection and its binding affinity to Q7, we were interested in examining a stronger binding site. N-terminal Phe is the preferred epitope in native peptides, but we recently found that Q7 can bind with *nanomolar* affinity to peptides containing a 4-(aminomethyl)phenylalanine (AMPhe) residue at the N-terminal position (Figure 3 inset).[20] Indeed, we observed quantitative protection of AMPhe-Met (**5**) (K_a = 5.3 × 10^8 M^{-1}) from APN digestion in the presence of 2 equiv of Q7 (Table 1).

In view of the strong protection of **5** by Q7, we wanted to test the ability of Q7 to inhibit the complete digestion of a peptide containing a nonterminal AMPhe residue. Therefore, we synthesized the pentapeptide Thr-Gly-Ala-AMPhe-Met (**6**) and followed its degradation by APN using analytical HPLC (Figure 3). We observed substantial retention of the starting material until at least 3 h, with complete disappearance of the starting material by 16 h. Up to 3 h, three new peaks in the 2−5

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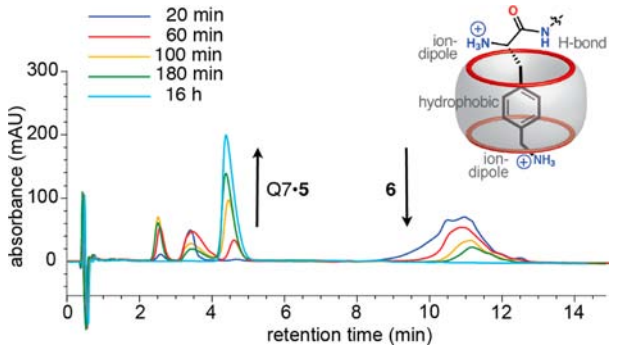

Figure 3. Analytical HPLC traces of the reaction of 0.5 mM Thr-Gly-Ala-AMPhe-Met (**6**) with 0.20 μM APN in the presence of 0.83 mM Q7 at 37 °C in 10 mM ammonium phosphate (pH 7.2).

min range grew, but at 16 h only a single peak remained (at ~4 min). This remaining peak correlated exactly with a standard sample of the Q7·**5** complex and was confirmed to be the Q7·**5** complex by ESI-MS (see the Supporting Information). In the absence of Q7, the peptide was digested completely into amino acids.

These results demonstrate that Q7 can completely inhibit the removal of Phe and AMPhe residues from the N-terminal position of peptides by APN. When such inhibition occurs, a peptide containing an internal Phe or AMPhe is cropped down to that residue. Therefore, Q7 imparts predictable sequence specificity to an otherwise nonspecific exopeptidase and enables the production of a single peptide product. This constitutes a novel approach to peptide processing that is specific, quantitative, and effective under mild conditions (pH 7.2 buffer, 37 °C). Proteolysis is inhibited only when APN reaches a target residue. Conversely, when APN is inhibited, a rare high-affinity epitope (e.g., the N-terminal Phe) is uncovered, and the resulting peptide product is bound by Q7. Therefore, this method may also facilitate the recognition and labeling of peptides at a single site.[12,21−24] It remains to be seen whether the method will work with longer polypeptides or with other receptor/protease pairs. Work in this area is ongoing and will be reported in due course.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental details and HPLC, ESI-MS, and ITC data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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