Enzymatic Cyclization of Linear Peptide Esters Using Subtiligase

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Cyclic peptides play an important role in the drug discovery process due to their conformational rigidity[1] and resistance to degradation.[2] The development of small molecule peptide or peptidomimetic inhibitors of protein–protein interactions usually proceeds through a series of linear and cyclic peptides.[3,4] Several strategies exist for synthesizing cyclic peptides including side chain to side chain, side chain to terminal group, and terminal group to terminal group (head to tail) cyclizations.[5] Head to tail cyclizations can be accomplished either in solution[6] or while attached to a solid phase resin[7] using carbodiimide or some other form of chemical coupling to form N→C terminal amides. These methods, however, are inefficient for cyclization of large peptides (>10 residues) due to the large entropic barriers for such reactions and competing intermolecular oligomerization.[8] Here we report the synthesis of several head to tail cyclic peptide amides 12–25 residues in length via enzymatic cyclization of linear peptide esters.

A mutant of subtilisin BPN (S221C/P225A) called subtiligase has been shown to efficiently ligate peptides in aqueous solution.[9] Subtiligase has also been used for the semisynthesis and total synthesis of proteins via sequential enzymatic ligation of peptide fragments.[10,11] Here we extend subtiligase technology to the enzymatic cyclization of linear peptide esters. Peptide substrates were synthesized as described[12] using standard solid phase methods.[13] The linear peptide substrates were synthesized as C-terminal glycolate phenylalanylamide esters to allow efficient acylation by subtiligase at pH 8 (Scheme 1). The resulting acyl-enzyme intermediates can then undergo hydrolysis, intermolecular dimerization, or intramolecular aminolysis by the N-terminal α amine resulting in head to tail cyclization (Scheme 1). Linear peptide substrates were cyclized as described,[14] and the yields for cyclization of several peptide esters ranged from 30% to 88% as determined by analytical HPLC[15] (Table 1). The efficiency of cyclization appears to depend primarily on peptide sequence and length. In general subtiligase prefers large hydrophobic residues at P1 and nonpolar residues at P1′ (Scheme 1).[9–11] A variety of sequences are accepted by subtiligase at the remaining positions making this method a general one for the synthesis of cyclic peptides.[9–11] To date we have never detected or isolated products resulting from amide formation between side chains even though the peptide side chains are fully deprotected.[15]

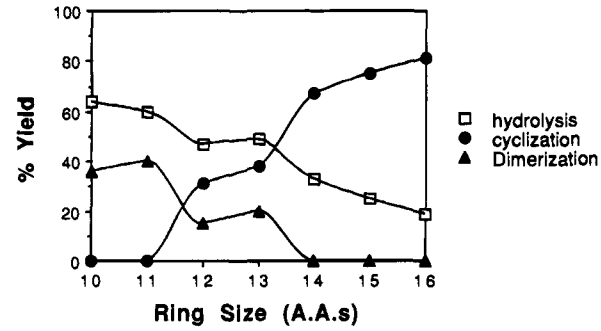

Figure 1. Cyclization yield vs peptide length.

In order to test the length dependence of subtiligase for cyclization, we constructed seven peptides (10–16 residues long) with N and C terminal sequences known to be efficient substrates for subtiligase (Figure 1). Because subtiligase binds peptides in an extended conformation and has a binding site which accommodates four residues to the amino terminal side of the ligation junction (P_1-P_4 subsites) and three residues to the carboxy terminal side (P_1'–P_3' subsites),[16] we expected that the enzyme would cyclize peptides about twice this length, 13–14 residues long. To test this we varied the peptide lengths using glycine spacers and determined the cyclization efficiency by analytical reverse phase HPLC.[15] We found that subtiligase has a minimum length requirement of 12 residues. Peptides shorter than 12 residues hydrolyze or dimerize but do not cyclize

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(12) Glycolate activated peptides were synthesized as follows. Phenylalanyl-*p*-methylbenzhydrylamine (MBHA) resin (0.63 mequiv/gm, Advanced ChemTech) was stirred with bromoacetic acid (5 equiv) and diisopropylcarbodiimide (5 equiv) for 1 h at 25 °C in dimethylacetamide (DMA) to afford the bromoacetyl derivative. The resin was washed extensively with DMA, and individual butyloxycarbonyl (BOC)-protected amino acids (3 equiv, Bachem) were esterified by stirring with sodium bicarbonate (6 equiv) in dimethylformamide (DMF) for 24 h at 50 °C to yield the corresponding glycolate-phenylalanyl-amide-resin. The amino acetylated resin was washed with DMF and dichloromethane (CH_2Cl_2) and could be stored at room temperature for several months. The resin **3** was loaded into an automated peptide synthesizer (Applied Biosystems 430A), and the peptides were elongated using standard solid phase procedures. The *N*-α-BOC group was removed with a solution of 45% trifluoroacetic acid in CH_2Cl_2. Subsequent BOC-protected amino acids (5 equiv) were preactivated using (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP, 4 equiv) and *N*-methylmorpholine (NMM, 10 equiv) in DMA and coupled for 1–2 h. Cleavage and deprotection of the peptide via treatment with anhydrous HF (5% anisole/5% ethyl methyl sulfide) at 0 °C for 1 h afforded glycolate-phe-amide activated peptide, which was purified by reverse-phase C18 HPLC (CH_3CN/H_2O gradient, 0.1% TFA). The identity of all substrates was confirmed by mass spectrometry.

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(14) Cyclizations were carried out at 25 °C in 100 mM tricine, pH 8. Typically 5–10 mg of the glycolate activated peptide containing a free N-terminus was dissolved in 1–2 mL of buffer (2–5 mM peptide), and a 10× stock solution of subtiligase (1 mg/mL in 100 mM tricine, pH 8) was added to bring the final enzyme concentration to ~5 μM. The reactions were monitored by analytical reverse-phase C18 HPLC (CH_3CN/H_2O gradient with 0.1% TFA) and were usually complete in 1–2 h as judged by complete disappearance of starting material. The products of 2% yield or greater were then isolated by preparative C18 HPLC (CH_3CN/H_2O gradient), and fractions containing pure material were lyophilized. The identity and purity of all isolated products were confirmed by mass spectrometry on a Sciex electrospray ionization mass spectrometer. Cyclic products were identified as those products having a mass of 18 mass units less than their linear counterparts due to a loss of water upon cyclization.

(15) The reported percent yields represent the integrated areas at 214 nm of the product peaks of 2% or greater after the complete disappearance of starting glycolate peptides. The extinction coefficients of starting peptide, hydrolyzed products, and cyclic products were identical, and no corrections were made. The dimerization products had extinction coefficients twice that of the corresponding monomers, and the areas were corrected accordingly. No products other than those of hydrolysis, dimerization, or cyclization were detected.

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Table 1. Peptide Carboxy Esters Cyclized by Subtiligase

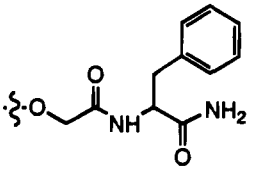
peptide no., sequence	size	% yield[b]
1, H_2N-KETAAAKFERQHMDSSTSAA-CO-R[a]	20	56
2, H_2N-SSSNYCNQMMKSRNLTKDRCKPVNTFVHESL-CO-R	31	85
3, H_2N-ADVQAVCSQKNV-CO-R	12	36
4, H_2N-ACKNGQTNCYQSY-CO-R	13	68
5, H_2N-STMSITDCRETGSSKYPNCAY-CO-R	21	75

[a] R = glycolate-phenylalanine-amide (glc-F-NH_2). [b] Yields determined by HPLC after complete disappearance of starting material.

Scheme 1. Cyclization of Peptide Esters by Subtiligase

$$H_2N\text{-}P_1'P_2'P_3'(AA)_n\text{-}P_3\text{-}P_2P_1\text{-}COR \xrightarrow[\text{pH 8}]{\text{Subtiligase}} HN\text{-}P_1'P_2'P_3'(AA)_n\text{-}P_3\text{-}P_2P_1\text{-}C{=}O$$

R = (glycolate phenylalanylamide) =



presumably because the enzyme cannot accommodate both ends in a productive binding geometry. As the length of the peptides increases, the cyclization efficiency also increases while dimerization and hydrolysis decrease (Table 1). Presumably the longer peptides are more flexible and are better able to adopt a productive binding conformation. No dimerization was observed for peptides greater than 14 residues in length, indicating that intramolecular cyclization is much faster than the corresponding intermolecular dimerization.

We believe this technology to be a general method for making large cyclic peptide and protein amides. To date the properties of such long cyclic peptides and proteins have not been described because such peptides have been very difficult to synthesize. Cyclic proteins may prove to possess enhanced stability and activity in vivo,[2] and we are currently investigating these properties. In general subtiligase has a broad sequence specificity and should be useful for making a wide range of cyclic peptides. Cyclization efficiency appears to depend primarily on peptide sequence and not concentration. No side chain protection is necessary because the subtiligase enzyme ensures head to tail ligation and will not accept the ϵ-amino group of lysine as a substrate.[9-11] In addition, both Boc and Fmoc peptide synthesis strategies can be used to construct the peptide substrates. Finally subtiligase mutants with altered sequence specificities and stabilities have been produced that broaden the range of possible substrates.[9-11]

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