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RESIN-COUPLED CYCLIC PEPTIDES AS PROTEINASE INHIBITORS

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Many screening methodologies applicable to combinatorial peptide libraries require that the peptide is presented in a biologically active form while remaining covalently-coupled to a synthetic support. Here we describe methodology appropriate to the generation of disulfide cross-linked cyclic peptides that allow high efficiency on-resin cyclisation. This is applied to the generation of a resin-bound cyclic proteinase inhibitor peptide, which is shown to retain enzyme binding activity.

Recent advances in the generation of combinatorial peptide libraries have revealed their potential as effective tools in the drug discovery process [1 , 2]. To date, almost all such work has used linear peptides as the basis for the library, and structural constraint by cyclisation has only been moderately explored [3 , 4]. In principle, cyclic peptides offer several advantages over their linear counterparts, as they have a more defined conformation which can result in enhanced binding affinities [5]. Generation of cyclic structures has often been employed as an optimisation procedure in traditional methodologies [6 , 7 , 8], but an alternative approach would be to create a combinatorial cyclic library directly. One of the most elegant combinatorial approaches is the 'one bead, one peptide' method [9], where a solid phase immobilised library is produced that includes all possible sequence combinations, but where individual beads of synthesis resin have unique sequences. Screening of such libraries for a desired activity (typically by protein binding) can then identify active sequences. In order to create a cyclic combinatorial library, it is first essential to establish that the cyclisation reactions can be performed at high efficiencies whilst the peptides remain immobilised. In this paper we show that it is possible to create cyclic disulfide-linked peptides in high yields. Furthermore, we illustrate that such material is suitable for direct screening by constructing an immobilised cyclic peptide and demonstrating that the resin-bound peptide retains enzyme-binding capability.

Whilst peptide cyclisation is usually performed in solution at low concentrations to minimise potential aggregation, dimerisation and oligomerisation, 'on-resin' cyclisation takes advantage of the pseudo-dilution phenomenon [10] as well as the removal of reagents by simple filtration. In addition to on-resin disulfide bond formation [11 , 12 , 13 , 14], procedures have also been described for the formation of side chain to side chain amide bonds [15 , 16 , 17], and side chain to amino terminus bonds [18 , 19 , 20]. In all instances, however, the objective has been purely preparative, and the degree of dimerisation can still remain high. To be useful in the construction of a peptide combinatorial library, the cyclisation procedure must ideally proceed in high yield, as no subsequent purification is possible.

In choosing a cyclisation protocol for solid phase peptide libraries it is important to use conditions that involve minimal side reactions and are compatible with all protected amino acids. In this paper we have performed the cyclisation via a disulfide bond. The commonly used methods of air oxidation and thiol-disulphide interchange using oxidised and reduced glutathione require relatively long reaction times to produce thermodynamically-controlled products. More rapid procedures give kinetically-controlled products and typically employ stronger oxidants such as $K_3Fe(CN)_6$ and I_2. However care is required for the latter procedure due to the susceptibility to modification of nucleophilic amino acids including methionine, tyrosine, tryptophan and histidine [21]. An attractive mild procedure is to use DMSO as an oxidising agent, which has recently been described for solution phase cyclisation [22 , 23]. When used in aqueous buffered media in the pH range 3-8 this is found to give no modification of methionine and tryptophan [22], and we have found it to give very high yields of monomeric cyclic peptides in solution [24].

In order to study the feasibility of using resin-bound cyclic peptides, we have examined a model peptide consisting of the anti-tryptic loop region of the Bowman-Birk proteinase inhibitor D4 from *Macrotyloma maxillare* [25] where the specificity has been re-directed to chymotrypsin by a lysine to phenylalanine substitution [24]. To monitor the purity of the product we included the acid insensitive linker 4-hydroxy-methylbenzoic acid (4HMBA) prior to coupling. This allows the final cyclic product to be cleaved off the resin and subjected to analysis.

Construction of the Immobilised Cyclic Peptide TentaGel™-S-NH_2 was employed as the solid phase resin (Rapp Polymere, Tubingen, Germany). This resin consists of polyoxyethylene grafted onto polystyrene giving good swelling properties in aqueous media. In addition this resin has a low loading capacity, a property that has been shown to reduce the level of dimerisation during on-resin cyclisation [14]. The sequence of the peptide used is given below:

Ser-Cys-Thr-Phe-Ser-Ile-Pro-Pro-Gln-Cys-Tyr-Gly-Gly-Gly-Gly-4HMBA-Gly-Resin

The first eleven residues, when cyclised via the two Cys residues, form the chymotrypsin inhibiting portion; the additional C-terminal glycine residues were added to act as a spacer between the active peptide and the resin.
Synthesis was carried out semi-automatically on an ABI 431A peptide synthesiser at 0.1 mM scale as described previously [24]. Tentagel-S-NH_2 (0.23 meq g^{-1}) was first loaded with Fmoc-glycine as the symmetric anhydride using DCC activation (catalysed using DMAP). Following Fmoc deprotection (20% piperidine in NMP), 4-hydroxy-methylbenzoic acid as linkage agent was similarly coupled by DCC activation. For the remainder of the synthesis, coupling was achieved by activating a 4 fold molar excess of each amino acid with equimolar amounts of HBTU and HOBT. Side chain deprotection was performed using 95% TFA with 2.5% ethanedithiol as a scavenger. Cyclisation was then performed by gentle shaking of the resin-peptide overnight in 20% DMSO, 5% acetic acid which had been adjusted to pH 6 with aqueous ammonia. Finally the cyclic peptide was cleaved from the resin as the methyl ester using 10% triethylamine in methanol [26].

Analysis of the Cyclic Peptide Analysis by HPLC and FAB-MS confirmed a high yield of the cyclic peptide, with no dimerisation or polymerisation detected. The yields of cyclic material were found to be comparable to or greater than those obtained in solution, which suggests that the resin-bound peptide acts effectively as an infinitely dilute solution. However, we observed that when using deprotection/cleavage times appropriate for this peptide when using standard resins with TFA-cleavable linkers the product was incompletely deprotected, with approximately 50% of the material retaining one t-butyl side chain protecting group (Figure 1a). By extending the deprotection time from 1.5 hours to 3.5 hours, the desired cyclic peptide was obtained in yields of 70-90% monomeric form by analytical HPLC (Figure 1b). A potential drawback of library syntheses is the assumption that all steps in the synthesis have been carried out with complete efficiency. In the case of this model cyclic peptide, it is clear that the final product can be obtained with good purity, which implies that libraries based on this sequence are likely to be synthesised successfully.
The overall recovered yield of desired cyclic monomeric product after cleavage was of 38%. Although we have used this protocol to allow the development of immobilised peptides for library studies, the overall efficiency of the process suggests that the protocol may also be useful for the preparation of larger quantities of cyclic peptide, avoiding more traditional cyclisation protocols that require handling large volumes of diluted material.

Activity of the Immobilised Cyclic Peptide The library screening protocol of Lam and co-workers [9] uses direct binding to distinguish active from inactive library sequences. In order to use such methods in any library based on the cyclic sequence described above, it is essential that the immobilised peptide retains binding activity. To test this, the resin-bound peptide was tested for the ability to bind to the target enzyme, chymotrypsin. The cyclic peptide-resin conjugate was packed into a column, and a chymotrypsin solution was applied. It was found that chymotrypsin was specifically retained by the column, and could be eluted by 10 mM HCl (Figure 2).

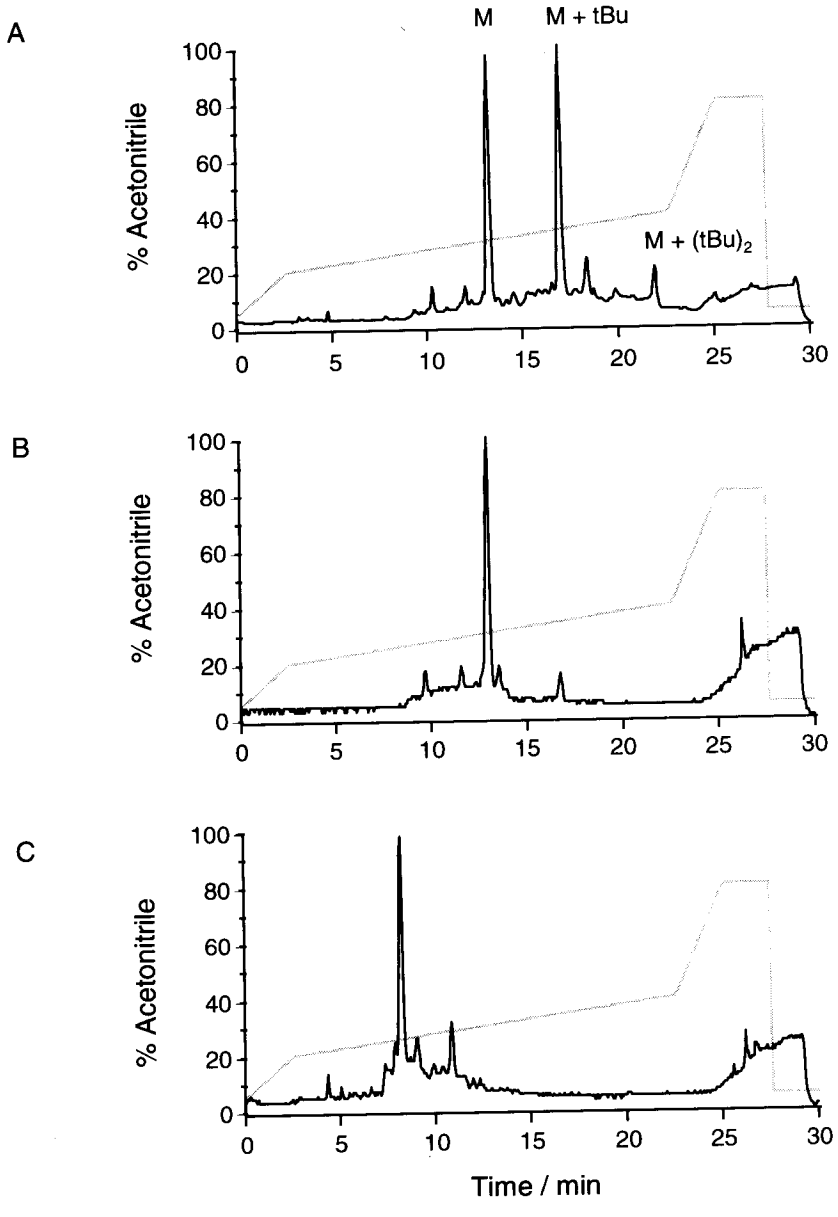

Figure 1 Analytical HPLC traces (Waters-Millipore Nova-Pak C_{18} reversed-phase column, 8 x 100 mm) of the crude cleavage products after on-resin cyclisation following A) standard deprotection time of 1.5 hours (as would be appropriate for this peptide in solution); B) extended deprotection time of 3 hours. Trace C shows the corresponding linear peptide. In trace A the elution positions of the fully deprotected peptide (M) and the peptide with one and two residual t-Butyl protecting groups are shown. In each case the black line represents the absorbance at 223 nm, and the grey line the percentage of acetonitrile. The mobile phase included 0.1% TFA.

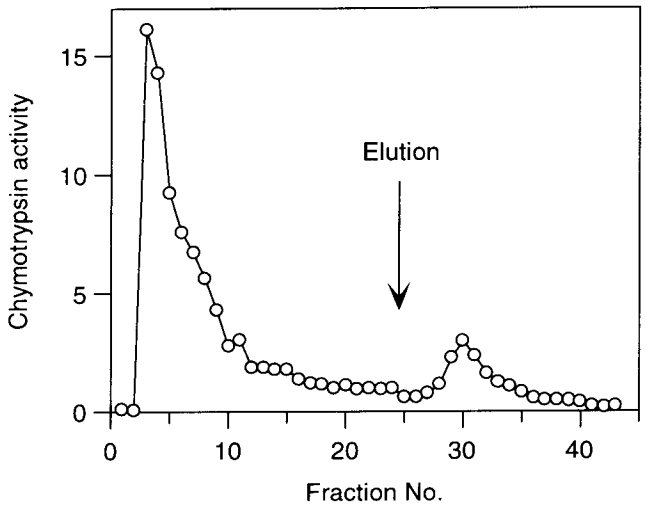

Figure 2 Binding of chymotrypsin to the cyclic peptide immobilised on resin. The peptide-resin was packed into a column (10 x 4 mm) and equilibrated with 50 mm tris-HCl, 100 mm NaCl, pH 8.0. Chymotrypsin (20 mg in 500 mL) was applied, and the column washed with buffer at 1 mL min^{-1} until the absorbance returned to baseline. Bound chymotrypsin was eluted with 10 mM HCl. The activity of chymotrypsin in the fractions (0.5 mL) was determined monitoring the hydrolysis of the chromogenic substrate succinyl-Ala-Ala-Pro-Phe-p-nitroanilide at 405 nm in 50 mM tris-HCl pH 8.0, and is expressed as A_{405} min^{-1} x 10^{-3}.

This shows that the resin-coupled cyclic peptide is active at binding chymotrypsin. The properties of the Rapp resin are not optimal for use as an affinity matrix as it has a size exclusion above M_r ~ 3,000 [27], which explains the relatively poor capacity for chymotrypsin binding. Nevertheless, the retention of activity indicates that it should be possible to use enzyme binding as a means of screening.

Summary Cyclic peptides have great potential for use in combinatorial libraries. We have demonstrated that it is possible to incorporate a disulfide cross-link with high efficiency into a resin-bound peptide using DMSO oxidation. This procedure is compatible with all protected amino acids, and so is applicable to libraries of peptides. However, we observe that it is essential to use extended deprotection times when using resin-bound material. We have used this protocol to generate a resin-bound cyclic peptide that is known to interact with the protein chymotrypsin, and have shown that the immobilised peptide retains enzyme binding capability. We are currently investigating the possibility of constructing libraries based on this cyclic peptide sequence in order to screen for proteinase inhibitors.

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