Poster Presentation

2nd International Symposium on Solid Phase Synthesis & Related Technologies - August, 1991

High Speed Continuous Flow Peptide Synthesis using PEG-PS™ Support at Standard Scales

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High Speed Continuous Flow Peptide Synthesis using PEG-PS™ Support at Standard Scales

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The Millipore PEG-PS, as well as other polyethylene glycol (PEG) grafted supports[1] have been shown to provide excellent results in a wide variety of peptide synthesis applications. Due to the rigidity imparted to the polystyrene bead by the addition of the PEG linker, the support is ideal for use in continuous flow synthesis systems at high flow rates (20-30 ml/min). The high speed of reagent delivery imparted by these flow rates allows reactions to proceed more quickly than at lower flow rates. Using standard sized (100-200 mesh beads) PEG-PS PAL support loaded at 0.22 meq/gm and high flow rates, peptides were synthesized on a Millipore model 9050 peptide synthesizer at standard 0.2mmol scale with a four-fold amino acid excess. Cycle times averaging less than 13min (including wait periods for liquid handler) were employed.

Well characterized polyethylene glycol molecules have been incorporated into the linkers on standard sized 1% crosslinked polystyrene beads [2] imparting superior properties of solvation [3] and rigidity to the support. In order for this matrix to be truly valuable, it must be able to function at a variety of scales including the 0.2mmol scale which is standard on the model 9050 peptide synthesizer. The following experiments indicate that the support is not only suitable for use at the standard scale, but that its inherent mechanical stability can be exploited to reduce cycle times.

EXPERIMENTAL

A Millipore model 9050 peptide synthesizer was used with modifications to existing protocols as outlined in *Fig 1.* PEG-PS PAL support (0.9 gm) was used in a standard 1cm column with a 1cm head space allowed for expansion of the

support. A flow rate of 20 ml/min was used for all wash and recycle steps. All activation of Fmoc amino acids was by PFP ester except as noted when HBTU activation was employed. The amino acids were dissolved using a bubbling pattern that was modified to yield six cycles of 30 sec bubble/4 sec settling. This cycle was sufficient to dissolve all amino acids used in these syntheses satisfactorily. Caution must be used when dissolving GLN residues, because both the PFP ester and (TMOB) protected free acid derivative, do not dissolve well using this protocol. Use of the (TRT) protected free acid is the best choice for the addition of GLN. Peptides were cleaved as indicated below and precipitated into anhydrous ethyl ether. Crude precipitates were then solubilized in water and subjected to further analysis.

Analytical HPLC was performed on a system consisting of: a Waters model 712 WISP sample injector, a model 600 pump, and a model 990 photodiode array detector, all controlled by Powerline™ software. The column used was a 3.9 X 150mm Delta Pak™ C18, 5µm, 300Å. The A buffer was water with 0.1% TFA and the B buffer was acetonitrile with 0.65% TFA. Flow rates were 0.75 ml/min and gradients were as indicated below.

Small scale prep chromatography was performed on a system consisting of a Waters Delta Prep™ 4000 pump, and a model 486 tunable wavelength detector equipped with a preparative flow cell. Segmented column technology was employed by the use of the three 8 X 100 mm Radial-Pak™ cartridge columns containing Delta Pak C18,15µm, 300Å packing, in a radial compression module. Buffers and gradients were the same as above and a flow rate of 4 ml/min was used. Fractions were collected by hand, dried down, solubilized in water and subjected to further analysis.

Amino acid analysis was performed using standard Pico Tag® methods[4] employing a Waters 3.9 X 150mm Pico Tag column, two model 510 pumps, and a model 440 detector. The sample was hydrolyzed for 22.5hrs at 110°C in a solution of 6N HCl and 0.1% phenol.

Peptide sequencing was performed on a Millipore model 6600 Prosequencer™ using adsorptive protocols on an Immobilon N™ membrane. Successive 269nm traces are aligned and then subtracted to yield the illustrated dynasub chromatograms.

Mass Spectroscopy was performed using standard matrix assisted laser desorption time of flight (maLD-TOF) protocols[7]. An insulin internal standard was used with a sinapinic acid matrix to generate the illustrated spectra.

DMF wash	6 sec
PIP wash	90 sec
DMF wash	120 sec
Acylation	240 sec
loop wash	50 sec
DCM wash	25 sec
loop wash	20 sec
total	9:11 mins

Fig. 1 High Speed synthesis cycle

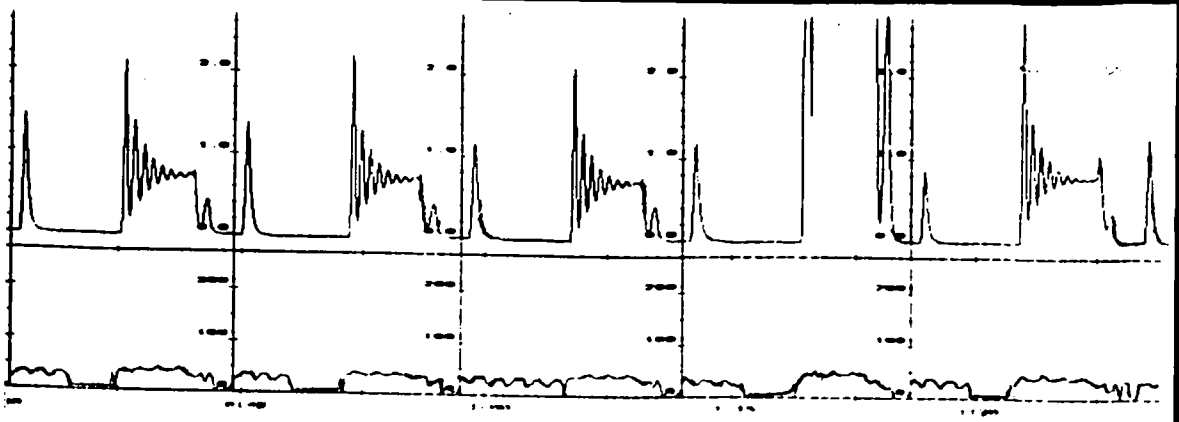
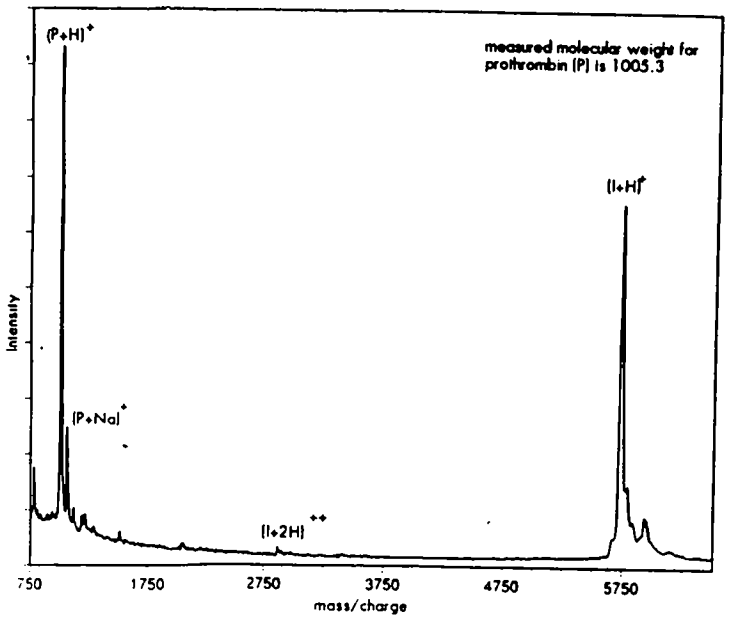


Fig. 2 Online cycle monitoring of synthesis cycles #5-1



ig. 3 maLD-TOF spectrum of synthetic Prothrombin fragment mass axis calibrated using an internal insulin standard

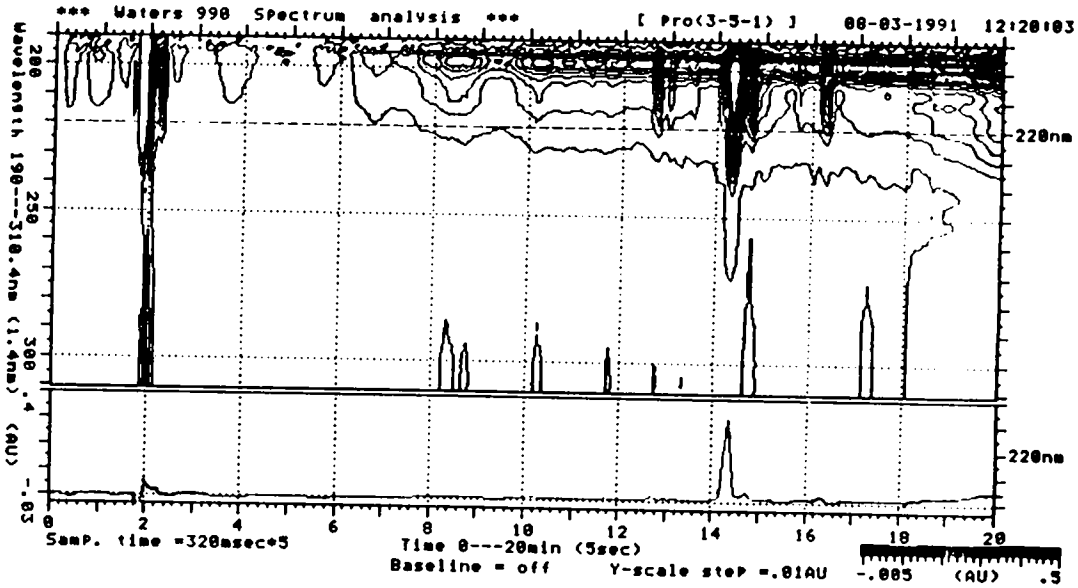


Fig. 4 Contour plot of analytical HPLC separation of crude prothrombin fragment preparation.

Peptide #1 (prothrombin fragment) NH_2-ANKGFLEEV-$CONH_2$ the standard peptide used to test Millipore synthesizers in the field, was synthesized in 1hr 47min *Fig. 2.* The peptide resin was cleaved for 1hr using 95% TFA and 5% triethylsilane. HPLC was performed using a linear gradient of 7-47% B buffer over 25min *Fig. 4.* ASN (TMOB) was added using HBTU activation. amino acid and mass spectroscopy analyses were performed on crude precipitated preparations. The expected weight of 1005 was verified *Fig. 3* and AAA indicated the proper ratios of amino acids.

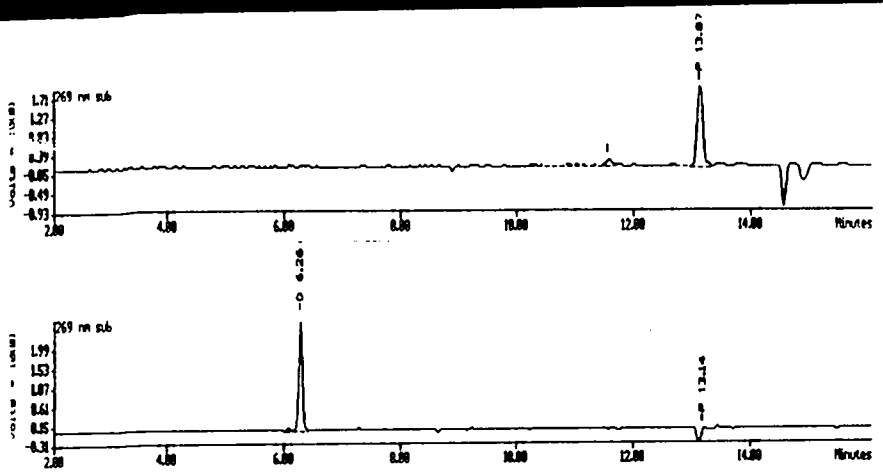


Fig. 5 Dynasub chromatograms of sequencing cycles #11 & 14

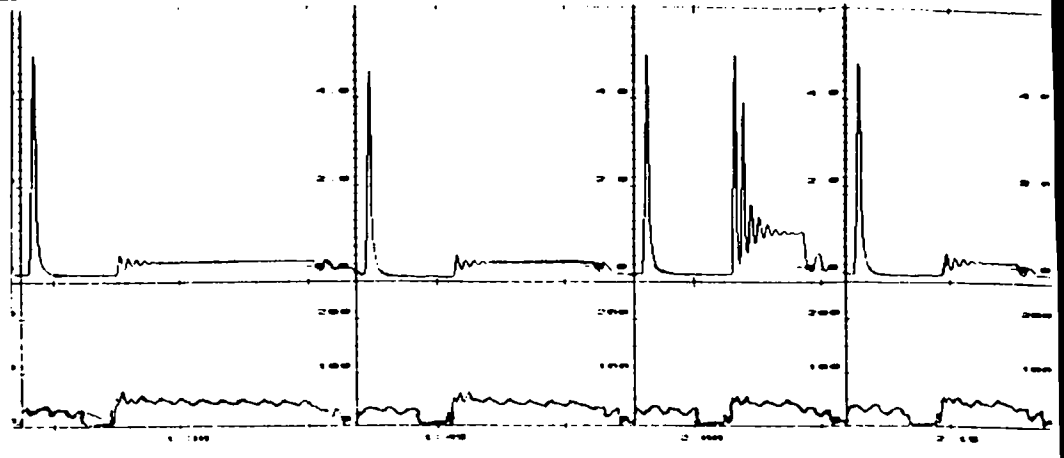
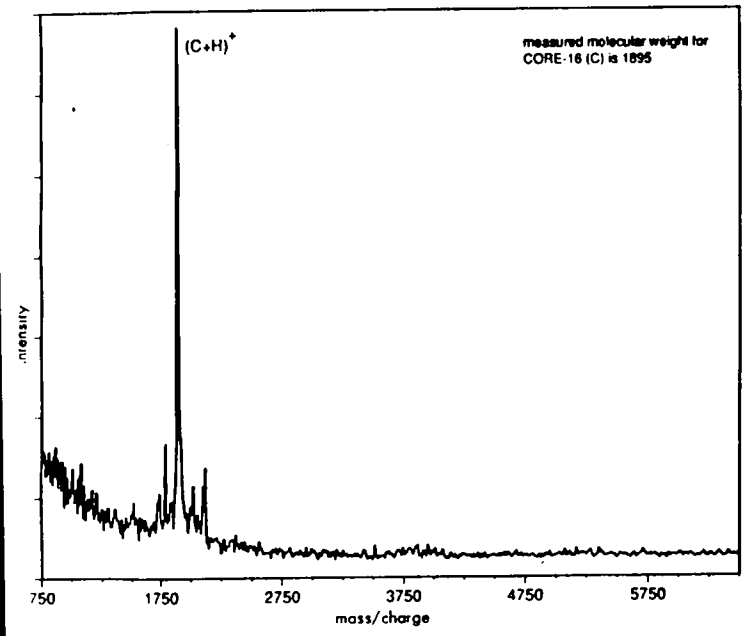


Fig. 6 Online cycle monitoring of synthesis cycles #10-7



ig. 7 maLD-TOF spectrum of synthetic Core-16 peptide mass axis calibrated using an external standard

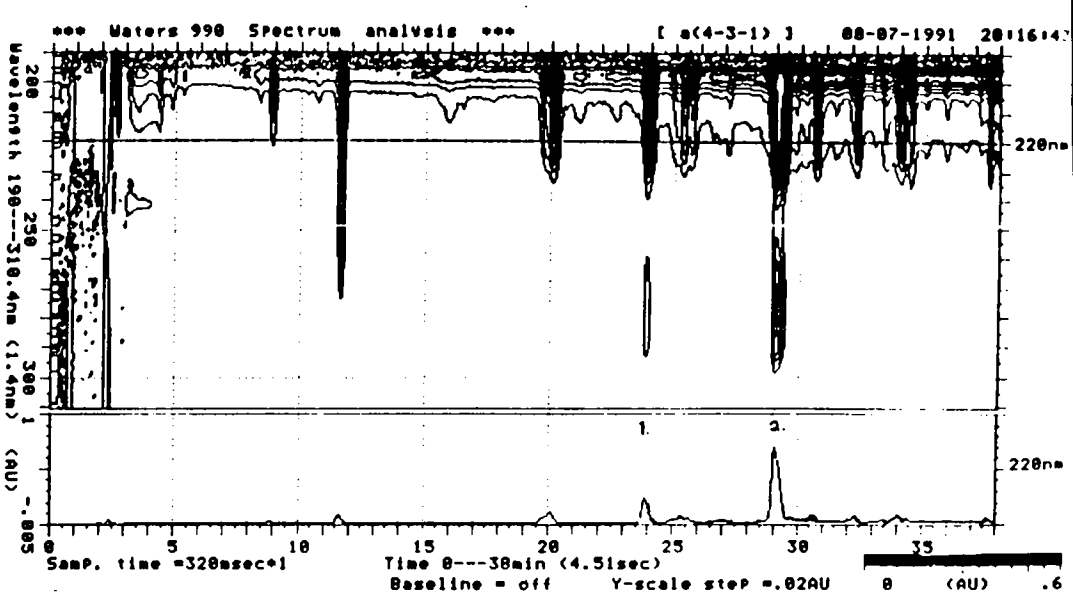


Fig. 8 Contour plot of analytical HPLC separation of crude Core-16 fragment preparation.

Peptide # 2 NH_2-VKKRCSMWIIPTDDEA-$CONH_2$ is the peptide that was used in a survey of core facilities to test synthesis capability(5). The survey was administered by the Association of Biomolecular Resource Facilities. The synthesis was completed in 3hr 45min *Fig. 5*. The peptide resin was cleaved for 1hr using reagent K (6). ARG(PMC) was added using HBTU activation. Both ILE residues were coupled for 10mins. HPLC was performed using a linear gradient of 13-31% B buffer over 35mins *Fig. 8*. Peaks labeled #1 and #2 were collected and subjected to further analysis. Peak #1 was shown to be a VAL deletion and peak #2 was confirmed by mass spectroscopy (mw theoretical = 1891 observed = 1895) *Fig. 7* and sequencing *Fig. 6* as the desired product.

DISCUSSION

Both of the peptides were synthesized in a significantly shortened time period and yielded products of good quality as verified by AAA, mass spectroscopy, and sequencing. At 20 ml/min the matrix showed considerable compression of bed length (approximately 10%) versus that observed when the flow was halted. This compression did not appear to have affected the efficiency of the synthesis in any way. The largest deletion peak observed in the peptide #2 preparation a des VAL moiety, is not surprising and would likely be reduced if acylation time was extended. Even if coupling times were extended to 10 mins the resulting cycle times would still lend themselves to routine one day peptide syntheses. The inherent ability of these standard sized PEG-PS beads to stand up to the elevated pressures of high flow rate protocols is the key to the success of these rapid cycles. When attempting these high flow rate protocols it is imperative that all instrument parameters are precisely calibrated, especially those of pick-up volume and loop excess which are resident in the .DAT files of the 9050 software. During the course of this work the optimal linear velocity was not determined, but it is probable that the lowering of the linear velocity experienced by a doubling of column diameter, should not have a deleterious effect on synthesis results.

REFFERENCES

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