Purification of a Lipid Peptide: Method Development for Hydrophobic Peptides

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Introduction

A current strategy for production of adjuvant-independent synthetic peptide vaccines involves combining three entities in a hybrid molecule:

1) a CTL (cytotoxic T lymphocyte) epitope peptide derived from the sequence of the target protein antigen,

2) the non-natural helper T lymphocyte epitope peptide known a PADRE (pan-DR helper epitope) [Ref. 1],

3) lipid moieties attached to the N-terminal lysine residue of a shor linker sequence, demonstrated to increase immunogenicity and allow production of immune response after injection without adjuvant.

Such hybrid lipid-peptides can be very hydrophobic molecules and thus present a challenge for purification.

This report details steps in method development for process purification of a synthetic peptide vaccine candidate for producing cellular immune response against human cytomegalovirus (HCMV), of particular importance as a critical determinant of patient recovery after allogeneic bone marrow transplantation therapy. The product structure consists of a 25-amino-acid peptide including a dipalmitoylated terminal lysine residue, PADRE, and a nine-amino-acid CTL epitope ($pp65_{495\text{-}503}$) from the HCMV 65 kilodalton lower matrix phosphoprotein. It contains a number of

hydrophobic amino acids.

Initial sample dissolution studies provide valuable information leading to identification of appropriate mobile phases for gradient

Dissolution and Preparative Reversed-Phase Chromatography

Direct dissolution of hydrophobic peptides in aqueous solvent mixtures can be slow. Frequently a peptide will appear to be insoluble when it is actually the kinetics of dissolution that are causing difficulty. The following procedure with a selection of likely solvents and trial samples of peptide will usually succeed.

1) Add pure solvent, i.e., the organic component, first. This brings hydrophobic parts of the peptide into the liquid phase first.

2) Next add concentrated buffer components. This sets the pH, which adjusts charges on the ionic amino acids.

3) Finally, add the aqueous portion of the mixture. In many cases the peptide will not dissolve until this final step, but a peptide that appeared insoluble will dissolve rapidly when these steps are followed.

In the present study, approximately one milligram of peptide was added to each of six 6 x 50 mm test tubes, and the dissolution sequences in Table I were attempted.

Based on the results of Table I, 10 mg of the peptide was solubilized by adding 3.6 mL of *n*-propanol, then 200 µL of glacial acetic acid, and finally 0.6 mL of 5% acetic acid in water. The peptide does not dissolve until the 5% acetic acid is added. The resulting solution contains 2.27 mg/mL peptide in 86% *n*-propanol/5% acetic acid. When an aliquot was diluted 8/1 with 0.5% acetic acid, the peptide remained in solution.

Table I

Step 1. Strong Component (Organic)	Step 2. Buffer	Step 3. Weak Component (Aqueous)	Success?
$CHCl_3$	TEAA pH6	acetone	NO
$CHCl_3$ + *n*-propanol	TEAA	water	NO
THF + *n*-propanol	TEAA	water	NO
100µL *n*-propanol + 100µL THF	100µL HOAc	100µL water	YES
200µL *n*-propanol	100µL HOAc	100µL water	YES
200µL *n*-propanol	10µL HOAC	100µL 5%HOAc in water	YES

Initial attempts at chromatography using a gradient from 5% to 85% *n*-propanol in 0.5% HOAc on a C4 column (Vydac 214TP54) resulted in no elution of peptide (measured as A280). The same procedure on a diphenyl column (Vydac 219TP54) produced the chromatogram of Figure 1.

Based on these results, a much larger sample load of peptide was applied to the diphenyl column equilibrated at 29% *n*-propanol in 0.5% HOAc. This produced the chromatogram of Figure 2, the starting point for a preparative method.

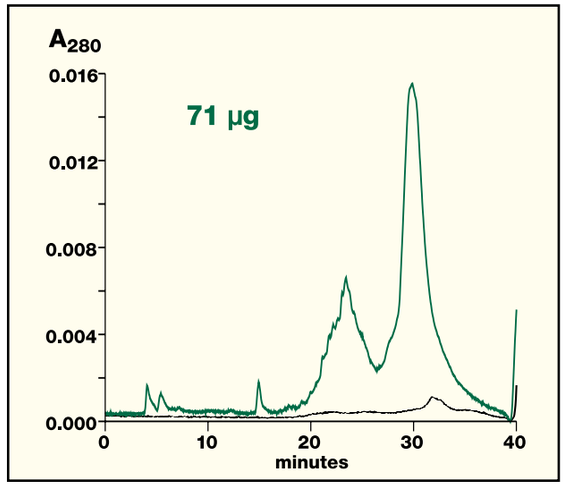

Figure 1. Initial trial chromatogram. Column: Vydac 219TP54 phenyl reversed-phase, 4.6mmID x 250mmL. Sample: 250 µL of 8:1 diluted peptide solution = 71 µg. Flow rate: 0.75 mL/min. Mobile phase: A = 5% *n*-propanol/0.5% HOAc. B = 85% *n*-propanol/0.5% HOAc. Gradient: 0% to 100% B in 30 minutes. The black trace is a post-run blank.

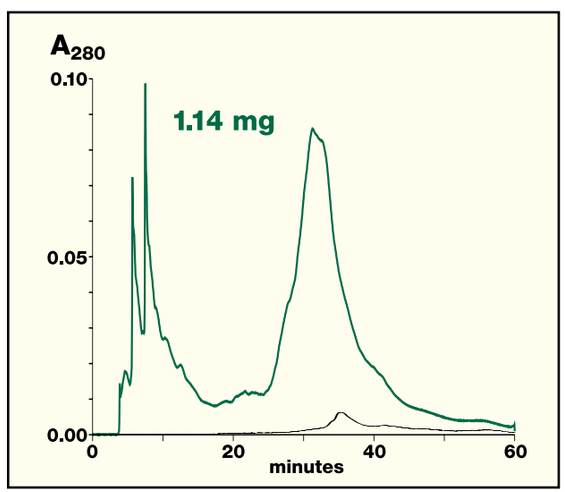

Figure 2. Initial preparative chromatogram of lipid peptide. Column: Same as Figure 5. Sample: 1.5 mL of 3:1 diluted peptide solution = 1.14 mg. Three 500 µL injections at 1.5 minute intervals. Flow rate: 0.75 mL/min. Mobile phase: A = 29% *n*-propanol/0.5% HOAc. B = 61% *n*-propanol/0.5% HOAc. Gradient: Hold 100% A for 6 minutes. Then, 0% to 100% B in 30 minutes. Black trace is post-run blank.

Analytical Reversed-Phase Chromatography

To monitor success of purification, a high-resolution analytical method is also needed. The major concern for preparative chromatography is to maintain solubility and avoid irreversible aggregation of high concentrations of sample on the chromatographic column. Under analytical conditions, however, solubility and aggregation should be less problematic. Lower sample loads allow more latitude in choice of solvents. The primary objective: to maximize resolution. In addition, using different chromatographic conditions, for example TFA as the modifier vs. acetic acid used for preparative runs, can provide greater assurance of detecting coeluting impurities.

Higher viscosity and slower diffusion generally make gradients of solvents such as *n*-propanol less suitable for analytical chromatography than methanol or acetonitrile. In this case, first attempts at analytical chromatography were made substituting acetonitrile in the eluting solvent for reversed phase on the Vydac 219TP diphenyl column. Figure 3 shows analytical chromatograms with gradients of acetonitrile alone as well as 50% blends of acetonitrile with isopropanol and *n*-propanol. The surprising result is that the peptide appeared to elute more rapidly with acetonitrile alone than in combination with the solvent that was known to favor dissolution. At first glance, a gradient of acetonitrile in 0.1% TFA appeared to be suitable for analytical chromatography.

This proved not to be so, however, as revealed by post-run blank chromatograms, an important check on chromatography of any difficult-to-dissolve material. When chromatography was done with acetonitrile alone (Figure 4) the column showed significant "memory", in the form of ghost peaks appearing in the eluate with no sample applied.

With a 50:50 acetonitrile:*n*-propanol blend as the eluting solvent, the post-run blank was clean (Figure 5), demonstrating the importance of the stronger solvent in this case for preventing aggregation even at analytical concentrations. Use of *n*-propanol as the blended solvent appears to have a slight edge in resolution over isopropanol for this particular peptide.

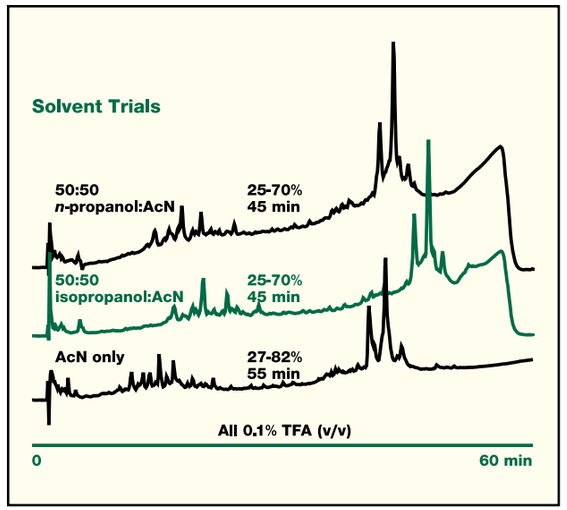

Figure 3. Trial chromatograms for analytical reversed-phase separation with different eluting solvents. In each case, gradients were run with solvents in the proportions indicated, 0.1% TFA (v/v), and water as the aqueous mobile-phase component. Column: Vydac 219TP54 (diphenyl, 4.6mmID x 250mmL). Flow rate: 1.5 mL/min. Temperature: 45°C.

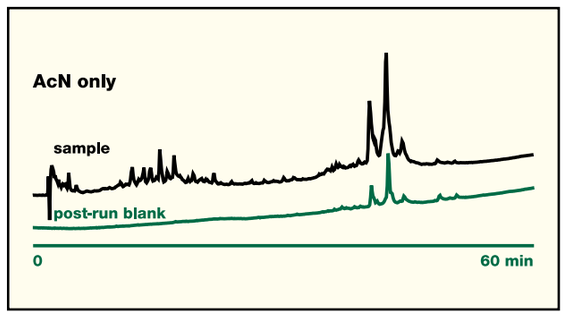

Figure 4. Examination of post-run blank for chromatogram with acetonitrile only. Conditions as in Figure 3.

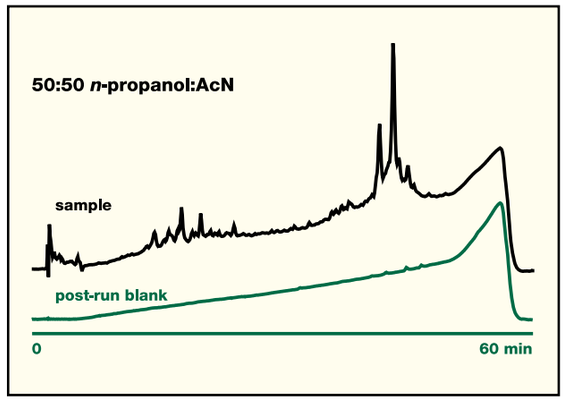

Figure 5. Examination of post-run blank for chromatogram run with 50:50 acetonitrile:*n*-propanol. Conditions as in Figure 3.

Summary

Hydrophobic peptides are difficult to purify because of solubility problems with typical ACN/H_2O/TFA mobile phases. Despite problems in their preparation and purification, hydrophobic peptides have taken on increased importance in the field of vaccination for stimulation of cellular immune responses. Previous work has established that introduction of lipid moieties at the amino terminus of MHC Class I CTL epitope vaccine peptides endows them with adjuvant-independent properties for immunization in both the murine and human setting. Such lipopeptides have been shown to be efficacious in clinical studies.

We have previously demonstrated [Ref. 2] that a CTL epitope from cytomegalovirus derived from the protein pp65 will be recognized by the Class I HLA A*0201 molecule. The pp65 CTL epitope was synthesized attached to a helper T lymphocyte epitope, referred to as PADRE, and dipalmitoylated at the amino terminus. The two palmitic acid side chains are attached to an N-terminal lysine, and the 25-amino-acid peptide contains a number of hydrophobic amino acids.

Initial sample dissolution studies provide valuable information that leads to the proper mobile phases for gradient elution. The order in which sample, solvents and buffers are added is very important because the rate of dissolution of peptides can be slow and can lead to inadvertently abandoning solvent mixtures that actually do work if the proper order of addition is followed.

After the mobile phase was developed, a C4 column was initially tried, but the results were equivocal. Injecting the sample with a coil of tubing and no column gave the expected area, proving the peptide had been tightly retained and did not elute from the C4.

Next, a less retentive diphenyl column (Vydac 219TP54) was used. The peptide eluted from this column with a good yield and very little memory, providing a starting point for development of the final process chromatography protocol.

Analytical reversed-phase separation was attempted with a mobile phase containing only acetonitrile, but a check of post-run blanks revealed significant memory effects on the column. Use of equal parts *n*-propanol and acetonitrile as the organic component of the mobile phase produced reliable elution and provided a basis for analytical chromatography.

References

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2. **Development of a candidate HLA A*0201 restricted peptide-based vaccine against human cytomegalovirus infection.** D.J. Diamond, J. York, J-Y Sun, C.L. Wright, and S.J. Forman. (1997) *Blood* **90**(5):1751-1767.

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