HPLC Purification of Peptides

J. Frederick

Abstract

Peptide purification counts after the synthesis, or other forms of making of it. For drug applied peptide like cell-penetrating peptides are supposed to ba paid speical attention as impurities may bring unwanted effects, as well as APIs peptides like pt-141.

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Protocol

Everything that goes into the HPLC must be filtered first, through a 0.45 mm or 0.2 mm filter and special glassware to remove particles that can get caught up on the column and interfere with absorption and separation.

Step 1.

This includes your buffers and your sample. Omission of this step can result in damage to the instrument.

Sample preparation

Step 2.

The crude peptide, prepared by manual or automated synthesis, will be supplied as a lyophilized (dried by freezing in a high vacuum) substance. For b-peptides, the sample is dissolved in 50% H20/50% CH3CN (or a range of others; solvent selection depends on solubility of the sample). Filter your sample.

Buffer Preparation

Step 3.

Buffer A and Buffer B are prepared according to the following recipes:
Schepartz Lab Protocols
Buffer A Buffer B
80 mL CH3CN 3200 mL CH3CN
3920 mL H20 800 mL H20
2.4 mL TFA* 2.0 mL TFA*
Safety precaution: Trifluoroacetic acid (TFA) is highly corrosive and causes severe burns when inhaled or upon contact with skin. This chemical should only be handled in the fume hood while wearing safety goggles, gloves, and protective clothing.

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Filter your buffers, using the designated glassware and following the specific instructions provided by your TA. This can be done prior to use and buffers stored at room temperature until you are ready to use the HPLC.

HPLC Operation

Step 4.

Your TA will provide specific instructions pertaining to the use of the HPLC. Typically, a run starts by attaching your buffers and washing the column (100% Buffer B for 5-10 minutes). Next allow the column to re-equilibrate to conditions that will start your run. For a run with a gradient of 20% Buffer B to 100% Buffer B, this means allowing about 5-10 minutes for the starting conditions for injection to be achieved (that is, to get the entire column in 20% Buffer A). Once a specific separation method is specified, you may review the parameters such as pump flow gradient, run time, and the PDA setup (acquisition). On some instruments, you will need to specify the lamp used for detection. Your TA will supply the details for the instrument you are using. When making an injection, choose the amount based on the type of column you are using and the approximate amount of your sample. For a- and b-peptides, the following general guidelines apply: Column scale Amount peptide per injection Analytical Up to 0.01 mg Semi-preparative Up to 0.05 - 0.1 mg Preparative Up to 0.1 – 0.5 mg Use either a glass syringe or a disposable plastic syringe fitted with a luer lock needle (only use flat-tipped needles). Before drawing up your sample, wash out the syringe several times with Buffer B. Draw your sample into the syringe, then carefully remove ALL bubbles from the sample by inverting the syringe, tapping gently, and expelling air until liquid just appears at the needle tip. Load your sample as instructed by your TA. You will want to adjust the view on the PC screen for convenient monitoring of the run, which means selecting the appropriate wavelength(s). For a- and b-peptides, 214 nm (the absorption frequency of peptide bonds) and 280 nm (the absorption of tyrosine and tryptophan) are recommended. Notice the retention times listed (in minutes) at the bottom of the graphs as well as in the status bar at the top of the screen (this may vary depending on the software used; your TA will clarify this). You will need to record the retention times as you collect peaks so you can correlate your fractions with peaks on the chromatogram. For the first injection of a peptide you've never purified before, you will need to carefully analyze the output. To do this, label a set of 15-20 tubes (15-ml conical vials usually work; you may want to do this ahead of time and loosen the caps so they are Schepartz Lab Protocols ready for collecting peaks as they come off the column. Once you have collected all the relevant peaks from the first injection, you will analyze them by mass spectrometry and determine which fraction or fractions contain your peptide by looking for its molecular weight (calculated in advance). Matching these fractions to their corresponding peaks will give you the retention time for your molecule. At this point, further injections will be simplified as you can accurately predict the retention time of your sample, and you'll know where to expect the peak containing your molecule. The first peaks that come off the column (after 3-4 minutes dead time for the semi-prep column, 5 minutes dead time for a prep column) represent a variety of leftovers from the synthesis (usually incomplete removal of reagents during wash steps). Once you get beyond this point you should collect every peak as it comes off the column, noting the retention time (for example: 11.23-11.5) for each numbered tube. Try to separate shoulders from main peaks, and isolate peaks that appear within multiple peaks. Keep in mind that the method you choose will impact the appearance of the chromatogram, and hence your ability to collect a pure, isolated fraction. For example, a longer method will give better resolution, but broader peaks. You will get better at this technique with practice, and bear in mind that it usually takes at least two passes through a column to purify a crude peptide synthesis.

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