The Most Efficient Method of Synthesizing and Purifying Peptides

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ABSTRACT

The most expensive and time-consuming step in the production of purified peptides is the purification. Measures to increase the purity and yield of crude peptides not only enhance efficiency by optimizing the utilization of materials during the synthesis, but also reduce the difficulty of purification. We have demonstrated the efficient and cost-effective synthesis of peptides requires the use of high quality amino acids, resins, efficient washing protocols, and accurate and precise delivery of reagents. Utilization of PEG modified resins such as OctaGel™, pseudoproline dipeptides, reaction heating and other methods of disrupting peptide inter- and intra-chain hydrogen bonding are highly effective in the synthesis of hydrophobic peptides. We have derived an equation for complete and efficient washing of resin for the removal of piperidine and other reagents. The optimization of HPLC purification is dependent on choice of the correct column with highest number of plates, and the optimal gradient which provides the final step in the efficient production of peptides.

HIGH QUALITY AMINO ACIDS AND RESINS

The use of high purity amino acids is essential in the synthesis of long peptides. Table 1 compares the theoretical purity of peptide prepared utilizing 99.7% pure, 99% pure and 98% pure amino acids. As the table shows, the purity of a 10 residue peptide prepared with 99.7% pure amino acids would have a maximum expected purity of approximately 97% while a peptide prepared with 98% pure amino acids would only be approximately 81% pure. For a 15 residue peptide prepared with 99.7% pure amino acids the maximum theoretical purity is greater than 95%, but is less than 74% if 98% pure amino acids are used if all reaction goes 100% in each step.

Maximum Theoretical Purity of Synthesized Peptide

Residue	99.7%	99%	98%
1	99.7%	99%	98%
2	99.4%	98.01%	96.04%
4	98.81%	96.06%	92.24%
6	98.21%	94.15%	88.58%
8	97.63%	92.27%	85.08%
10	97.04%	90.44%	81.71%
15	95.59%	86.01%	73.86%

Table 1 – Use of 99.7% Pure vs. 99% Pure vs. 98% Pure Amino Acids

The selection of the type of resin is also another very important parameter for producing high quality peptides. Resins should be highly uniform in bead size to assure uniform reaction rates. Broken beads and very small beads may partially clog filters or frits within a peptide synthesizer resulting in extended synthesis times or impurities due to an increased amount of solvent trapped in the resin.
Resin with hydrophilic properties such as PEG based resin can make higher quality peptides.

Resins should have reactive sites on the surface of the beads for the highest quality peptides. Peptides formed at sites in pores within the beads are more likely to be truncated or contain deletions because the peptide chains become increasingly hindered and inaccessible within the resin pores as the peptide chain increases in length. Polyethylene glycol (PEG) attached to the surface of the resin reduces inter- and intra-chain hydrogen bonding of the peptide chains, leaving the peptide N-terminals more accessible for further reaction. Resins including each of these three factors produce optimal crude peptides.

PEG Resins (OctaGel™)

The use of resins containing polyethyleneglycol (PEG) often improves the yield and purity of hydrophobic peptides.[1] The peptide chains hydrogen bond to the PEG (Figure 1A) instead of hydrogen bonding inter- and intra-molecularly, leaving the N-terminal more accessible for reaction. As a result, peptides prepared on PEG-containing resins have fewer impurities arising from deletions or chain termination.[1,2]

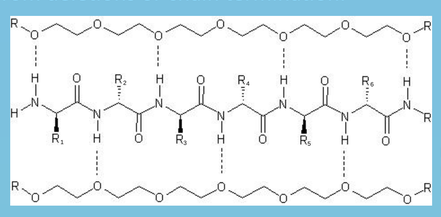

Fig. 1A – Peptide – PEG Hydrogen Bonding on OctaGel™

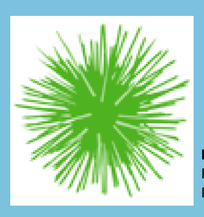

Fig. 1B – OctaGel™ Resin Bead with PEG on the Surface

OctaGel™ is a highly uniform resin with PEG chains bonded perpendicular to the surface of the resin beads (Figure 1B). In addition, sites within the resin pores are capped with PEG, assuring that the only reactive sites are on the bead surface. OctaGel™ resins, even without heating, produce crude peptides in higher yield and greater purity than other standard resins. Delayed gradient heating with OctaGel™resins results in crude peptides with the highest purity and yield. For example Figure 2A shows the ACP (65-74) peptide synthesized with gradient heating and Figure 2B without heat on OctaGel™.

Crude ACP (65-74) Prepared on OctaGel™
Fig. 2A – OctaGel™ Resin Bead with

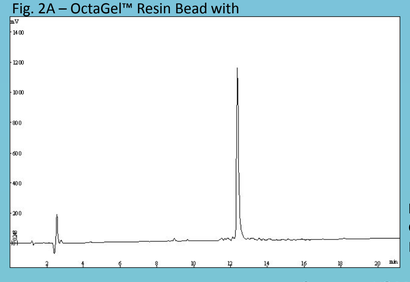

Fig. 2A – Gradient Heating to 75 °C

Crude ACP (65-74) Prepared on OctaGel™
PEG on the Surface

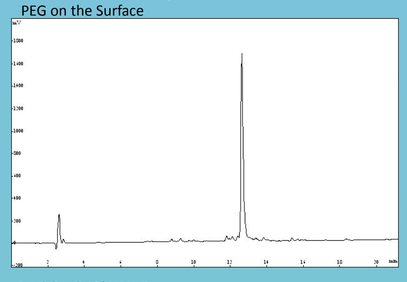

Fig. 2B – Room Temperature

Sequence: VQAAIDYING HPLC Conditions: 10-50%B, 20 min; A-H2O + 0.1% TFA; B-Acetonitrile + 0.1% TFA; Detection- 220 nm; Column-Spirit C18 Peptide, 5µ, 150 x4.6 mm

Resin Wash

Washing resin after each step is another factor for producing high quality peptides.
Washing is a dilution factor; protocols should be optimized to efficiently remove excess reagents and impurities without using excessive amounts of solvent. Efficient washing is critical after Fmoc deprotection because piperidine is used in great excess and will react with activated amino acid in the following coupling if it is not removed. We have determined that each gram of dry resin retains approximately 3ml of solvent when swollen. When using a 20% piperidine/DMF solution for Fmoc removal, this corresponds to 0.6 ml (517 mg, 6.07 mmol) of piperidine per gram of resin remaining. Each wash of the resin corresponds to a serial dilution of the piperidine. Table 2 shows the theoretical amount of piperidine left in 1 gram of resin after each 7 ml wash.

The following formulas can be used to calculate minimum wash volume needed to reduce the remaining piperidine to a desired level or the mmoles of piperidine per gram of resin remaining after a number of washes.

$$V_{wash} = [10.123(3R)^{n+1}\, C_{init}/Mmol]^{1/n} - 3R$$
$$Mmol = 10.123(3R)^{n+1}\, C_{init}/(V_{wash} + 3R)^{n}$$

Where V_{wash} = the volume of the DMF wash
$Mmol$ = the millimoles of piperidine after n washes
C_{init} = the initial concentration of piperidine in ml per ml DMF
R = grams of resin
n = the number of washes (e.g. 2-7)
10.123 = conversion factor to convert milliliters of piperidine to mmoles of piperidine which is derived below
d_{pip} = density of piperidine = 0.862 g/ml
MW_{pip} = molecular weight of piperidine = 85.15 g/mole
$1000d_{pip}/MW_{pip} = 1000(0.862)/85.15 = 10.123$ mmol/ml

Amount of Piperidine Retained in 1 Gram of Resin after n Washes

Wash (7 ml)	Concentration of Piperidine	Volume Piperidine	Milligrams Piperidine	Mmoles Piperidine
0	20%	0.6 mL	517	6.07
1	6%	0.18 mL	155	1.822
2	1.8%	0.054 mL	46.5	0.55
3	0.54%	0.016 mL	14	0.164
4	0.16%	0.0048 mL	4.2	0.049
5	0.048%	0.00146 mL	1.26	0.015

Table 2 – Residual Piperidine after Washes

Peptide Synthesis

Peptide synthesizers must be able to deliver reagents accurately with high precision repeatedly, especially in research scale syntheses. Inaccurate, imprecise delivery will result in an excess of amino acid or reagents present in the reaction mixture which may produce side products that reduce the purity and yield of the crude peptide. An excess of base during coupling will increase racemization and introduce diasteromeric peptide impurities. Excess HBTU, HATU, or HCTU can cause termination of peptide chains, producing short chain contaminants.

One method of measuring the amounts of solution delivered in a peptide instrument is to use time-flow based calibration. The flow rate depends on the solvent, the temperature, the viscosity of the amino acid or reagent solution and the concentration of the solution. This method lacks the precision needed to deliver small volumes repeatedly.

Syringe pumps provide high accuracy and precision in delivering very small volumes. Syringe pumps are utilized on Apex 396, where delivery volumes are generally less than 1 mL. Syringe pumps are not as practical for delivering volumes greater than several milliliters. The Focus XC measures the level (Figure 3) of solution in a flow-cell calibrated vessel to consistently measure and deliver accurately and precisely any amount of any amino acid or reagent with varying viscosity.

"Difficult" Hydrophobic Peptides

Peptides with large hydrophobic regions are often difficult to synthesize. Inter-chain hydrogen bonding in the hydrophobic regions causes poor solvation of the peptide chains and slowed reaction rates. Measures to disrupt this hydrogen bonding often significantly improve the purity and yield of the crude peptide.

Heating disrupts the hydrogen bonding between peptide chains leading to increased reaction rates in addition to rate acceleration normally observed due to increased temperature. Microwave heating directly increases the vibration of molecules within the solution resulting in rapid temperature increase. The rapid temperature increase can lead to overshooting the desired temperature. Microwave heating (Figure 4) accelerates peptide synthesis and improves the purity of crude peptides. Rapid conventional heating (Figure 4), although slower within a minute of microwave heating, produces nearly identical results as microwave heating in improving the purity and yield of crude "difficult" peptides.[6] Heating, either conventionally or by microwaves, also accelerates the racemization of the easily-racemized cysteine and histidine residues.

Fig. 3 –AAPPTec Focus XC 6RV

Temperature Profiles of Microwave, Conventional, Gradient and Delayed Gradient Heating for the Coupling Reaction

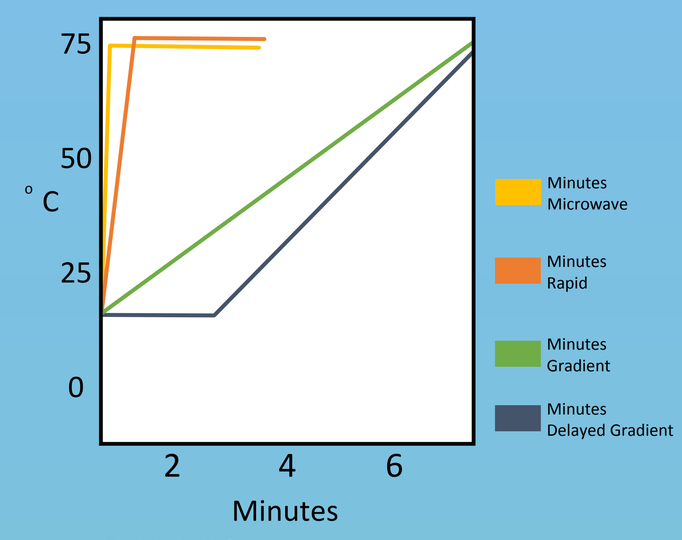

Fig. 4 –Heating Profile

Slower heating on a temperature gradient (Figure 4) also improved the yield and purity of crude peptides but with less racemization of cysteine. Peptide chains on the surface of the resin beads are exposed to the reaction mixture and react rapidly at room temperature or with mild heating while racemization is slow. Usually 80-90% of coupling to reactive sites occurs within the first 2 to 4 minutes of reaction time at room temperature. Heating is only required to accelerate the reactions at the remaining sites. Rapid heating and microwaves result in approximately 4% racemization. In gradient heating, racemization affects only the 10-20% of peptide chains that had not already reacted at lower temperature. This results in diastereomeric impurities reduced by 80-90%.

With microwave and conventional heating, the accelerated racemization of cysteine is competitive with the coupling rate leading to increased formation of diastereomeric impurities. In gradient heating, the majority of the peptide chains react at lower temperatures where the racemization reactions are slow and less diasteriomeric impurities are formed. Advancing on this finding, we allowed the coupling to proceed at room temperature for 2 minutes before starting the temperature gradient (Figure 4). With this delayed gradient procedure, the yield and purity of the crude peptides was maximized and the racemization of cysteine residues was reduced.

Pseudoproline Dipeptides and Dmb Amino Acids

Incorporating pseudoproline dipeptides and/or Dmb protected amino acids at strategic points in the synthesis of hydrophobic peptides can significantly improve the yield and purity of long or difficult peptides.[7] The pseudoproline introduces a bend in the peptide backbone which disrupts hydrogen bonding and promotes the solvation of the peptide chain. A pseudoproline disrupts inter- and intra-chain hydrogen bonding for the next 5 to 6 amino acids. This makes the N-terminal of the peptide chain more accessible for reaction resulting in fewer deletion and chain termination peptides in the crude product and increased yield. Pseudoprolines and Dmb protected amino acids also prevent succinimide formation at Asp residues which results in the formation of β-Asp containing impurities.

Purification

Efficient purification of crude peptides depends upon the optimization of the HPLC separation. First, a column with high selectivity and a high number of theoretical plates should be chosen. This provides the highest loading capacity. This will reduce the amount of solvent required to purify the peptide. In addition, the volume containing the desired peptide will be minimized which will reduce the lyophilization time. Optimum resolution is achieved when the peak of the desired product has a retention of 4 to 5 column volumes. The HPLC gradient should be adjusted until the desired peak elutes within this range. Shallow gradients produce greater resolution than steeper gradients. If the crude peptide contains close-eluting impurities, extremely shallow gradients, as low as 0.1% per minute, may be utilized. The optimized gradient does not need to be linear. Impurities eluting much earlier or much later than the desired peak can be eluted with a steep gradient, then a much shallower gradient used to obtain maximum resolution of the desired peak.

CONCLUSIONS

These factors are important for the efficient synthesis and purification of peptides.

1. Utilization of high quality amino acids.
2. Utilization of high quality resins such as OctaGel™.
3. Efficient washing of resin for the removal of piperidine and excess reagents.
4. Accurate and precise measuring and delivery of amino acid and reagent solutions.
5. Utilize delayed gradient heating.
6. Utilize pseudoproline dipeptides and Dmb-protected amino acids in "difficult" hydrophobic regions.
7. Use of a column with high selectivity and a high number of theoretical plates for purification.
8. Optimization of the purification gradient.

These factors will maximize peptide production while reducing time and overall cost of peptide production.

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