Synthesis Methodology

Peptides are used to prepare epitope-specific antibodies, map antibody epitopes and enzyme binding sites and to design novel enzymes, drugs and vaccines. While peptide synthesis used to be labor-intensive and produce low yields, improved methods of production and peptide chemistry have made peptide synthesis more available for general research applications

Introduction to Peptide Synthesis

Peptide synthesis is characterized as the formation of a peptide bond between two amino acids. While the definition of a peptide is not definitive, it usually refers to flexible (little secondary structure) chains of up to 30-50 amino acids. The ability to form peptide bonds to link amino acids together is over 100 years old, although the first peptides to be synthesized, including oxytocin and insulin, did not occur for another 50-60 years, demonstrating the difficult task of chemically synthesizing chains of amino acids. In the last 50 years, advances in protein synthesis chemistry and methods have developed to the point where peptide synthesis today is a common approach in even high-throughput biological research and product and drug development.

The benefit of peptide synthesis strategies today is that besides having the ability to make peptides that are found in biological specimens, creativity and imagination can be tapped to generate unique peptides to optimize a desired biological response or other result. This page highlights the important aspects of peptide synthesis, the most common methods of synthesis and purification and the strengths and limitations of the respective strategies.

Applications for Synthetic Peptides

The invention of peptide synthesis in the fifties and sixties spurred the development of different application areas in which synthetic peptides are now use, including the development of epitope-specific antibodies against pathogenic proteins, the study of protein functions and the identification and characterization of proteins. Furthermore, synthetic peptides are used to study

enzyme-substrate interactions within important enzyme classes such as kinases and proteases, which play a crucial role in cell signaling.

In cell biology, receptor binding or the substrate recognition specificity of newly discovered enzymes can often be studied using sets of homologous synthetic peptides. Synthetic peptides can resemble naturally occurring peptides and act as drugs against cancer and other major diseases. Finally, synthetic peptides are used as standards and reagents in mass spectrometry (MS)-based applications. Synthetic peptides play a central role in MS-based discovery, characterization and quantitation of proteins, especially those that serve as early biomarkers for diseases.

Process of Synthesizing Peptides

Peptide synthesis most often occurs by coupling the carboxyl group of the incoming amino acid to the N-terminus of the growing peptide chain. This C-to-N synthesis is opposite from protein biosynthesis, during which the N-terminus of the incoming amino acid is linked to the C-terminus of the protein chain (N-to-C). Due to the complex nature of in vitro protein synthesis, the addition of amino acids to the growing peptide chain occurs in a precise, step-wise and cyclic manner. And while the common methods of peptide synthesis have some critical differences, they all follow the same step-wise method to add amino acids one-at-a-time to the growing peptide chain.

Peptide Deprotection

Because amino acids have multiple reactive groups, peptide synthesis must be carefully performed to avoid side reactions that can reduce the length and cause branching of the peptide chain. To facilitate peptide formation with minimal side reactions, chemical groups have been developed that bind to the amino acid reactive groups and block, or protect, the functional group from nonspecific reaction.

Purified, individual amino acids used to synthesize peptides are reacted with these protecting groups prior to synthesis, and then specific protecting groups

are removed from the newly added amino acid (a step called deprotection) just after coupling to allow the next incoming amino acid to bind to the growing peptide chain in the proper orientation. Once peptide synthesis is completed, all remaining protecting groups are removed from the nascent peptides. Three types of protecting groups are generally used.

The amino acid N-termini are protected by groups that are termed "temporary" protecting groups, because they are relatively easily removed to allow peptide bond formation. Two common N-terminal protecting groups are tert-butoxycarbonyl (Boc) and 9-fluorenylmethoxycarbonyl (Fmoc), and each group has distinct characteristics that determine their use. Boc requires a moderately strong acid such as trifluoracetic acid (TFA) to be removed from the newly added amino acid, while Fmoc is a base-labile protecting group that is removed with a mild base such as piperidine.

Boc chemistry was first described in the 1950s and requires acidic conditions for deprotection, while Fmoc, which was not reported for another twenty years, is cleaved under mild, basic conditions. Because of the mild deprotection conditions, Fmoc chemistry is more commonly used in commercial settings because of the higher quality and greater yield, while Boc is preferred for complex peptide synthesis or when non-natural peptides or analogs that are base-sensitive are required. The use of a C-terminal protecting group depends on the type of peptide synthesis used; while liquid-phase peptide synthesis requires protection of the C-terminus of the first amino acid (C-terminal amino acid), solid-phase peptide synthesis does not, because a solid support (resin) acts as the protecting group for the only C-terminal amino acid that requires protection. Amino acid side chains represent a broad range of functional groups and are therefore a site of considerable side chain reactivity during peptide synthesis. Because of this, many different protecting groups are required, although they are usually based on the benzyl (Bzl) or tert-butyl (tBu) group. The specific protecting groups used during the synthesis of a given peptide vary depending on the peptide sequence and the type of N-terminal protection used (see next paragraph). Side chain protecting groups are known

as permanent protecting groups, because they can withstand the multiple cycles of chemical treatment during the synthesis phase and are only removed during treatment with strong acids after synthesis is complete.

Amino acid functional group protection. Prior to peptide synthesis, the N-termini and amino acid side chains are "protected" with chemical groups that block nonspecific reaction during synthesis. The C-terminus of the C-terminal amino acid of the peptide is also protected to facilitate peptide extension in the correct orientation. Because multiple protecting groups are normally used in peptide synthesis, it is evident that these groups must be compatible to allow deprotection of distinct protecting groups while not affecting other protecting groups. Protecting schemes are therefore established to match protecting groups so that deprotection of one protecting group does not affect the binding of the other groups. Because N-terminal deprotection occurs continuously during peptide synthesis, protecting schemes have been established in which the different types of side chain protecting groups (Bzl or tBu) are matched to either Boc or Fmoc, respectively, for optimized deprotection. These protecting schemes also incoroprate each of the steps of syntheis and cleavage, as described in the table and in later sections of this page.

Common Protecting Scheme-specific Solvents

Protecting Scheme	Deprotection	Coupling	Cleavage	Wash
Boc/Bzl	TFA	Coupling	HF, HBr, TFMSA	DMF
Fmoc/tBut	Piperidine	agent in DMF	TFA	

The act of removing protecting groups, especially under acidic conditions, results in the production of cationic species that can alkylate the functional groups on the peptide chain. Therefore, scavengers such as water, anisol or thiol derivatives can be added in excess during the deprotection step to react with any of these free reactive species.

Amino Acid Coupling

Synthetic peptide coupling requires the activation of the C-terminal carboxylic acid on the incoming amino acid using carbodiimides such as dicyclohexylcarbodiimide (DCC) or diisopropylcarbodiimide (DIC). These coupling reagents react with the carboxyl group to form a highly reactive O-acylisourea intermediate that is quickly displaced by nucleophilic attack from the deprotected primary amino group on the N-terminus of the growing peptide chain to form the nascent peptide bond.

Carbodiimides form such a reactive intermediate that racemization of the amino acid can occur. Therefore, reagents that react with the O-acylisourea intermediate are often added, including 1-hydroxybenzotriazole (HOBt), which forms a less-reactive intermediate that reduces the risk of racemization. Additionally, side reactions caused by carbodiimides have led to the examination of other coupling agents, including benzotriazol-1-yl-oxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP) and 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), which both require activating bases to mediate amino acid coupling.

The N-terminal protecting group on the C-terminal amino acid of the peptide to be synthesized is first deprotected. After removing the unbound protecting groups, the next amino acid is activated at the C-terminal end by a coupling agent (e.g., DCC;), which facilitates peptide bond formation between the deprotected N-terminus of the first amino acid and the activated C-terminus of the incoming amino acid. The new N-terminus of the growing peptide is then deprotected and coupled to the next amino acid. This cycle of deprotection and coupling is repeated until the full-length peptide is formed.

Peptide Cleavage

After successive cycles of amino acid deprotection and coupling, all remaining protecting groups must be removed from the nascent peptide. These groups are cleaved by acidolysis, and the chemical used for cleavage depends on the

protection scheme used; strong acids such as hydrogen fluoride (HF), hydrogen bromide (HBr) or trifluoromethane sulfonic acid (TFMSA) are used to cleave Boc and Bzl groups, while a relatively milder acid such as TFA is used to cleave Fmoc and tBut groups. When properly executed, cleavage results in the removal of the N-terminal protecting group of the last amino acid added, the C-terminal protecting group (either chemical or resin) from the first amino acid and any side-chain protecting groups. As with deprotection, scavengers are also included during this step to react with free protecting groups. Because of the importance of cleavage in proper peptide synthesis, this step should be optimized to avoid acid-catalyzed side reactions.

Peptide Synthesis Strategies

Liquid-phase peptide synthesis is the classical method that scientists used when first discovering how to generate peptides in vitro and is still commonly used for large-scale synthesis. This method is slow and labor-intensive, though, because the product has to be manually removed from the reaction solution after each step. Additionally, this approach requires another chemical group to protect the C-terminus of the first amino acid. A benefit of liquid-phase synthesis, though, is that because the product is purified after each step, side reactions are easily detected. Additionally, convergent synthesis can be performed, in which separate peptides are synthesized and then coupled together to create larger peptides.

By far, though, solid-phase peptide synthesis is the most common method of peptide synthesis today. Instead of C-terminal protection with a chemical group, the C-terminus of the first amino acid is coupled to an activated solid support, such as polystyrene or polyacrylamide. This type of approach has a two-fold function: the resin acts as the C-terminal protecting group and provides a rapid method to separate the growing peptide product from the different reaction mixtures during synthesis. As with many different biological manufacturing processes, peptide synthesizers have been developed for automation and high-throughput peptide production.

Peptide Purification

Although peptide synthesis strategies have been optimized and can be mass-produced, the process to generate peptides is by no means perfect. Events such as incomplete deprotection or reaction with free protecting groups can cause truncated or deletion sequences, isomers or other side products. These events can occur at any step during peptide synthesis, and therefore the longer the peptide sequence, the greater probability that something will negatively affect the synthesis of the target peptide. Thus, peptide yield is inversely correlated with peptide length.

Purification strategies are usually based on a combination of separation methods that exploit the physiochemical characteristics of peptides, including size, charge and hydrophobicity. Purification techniques include:

- size-exclusion chromatography
- ion exchange chromatography (IEC)
- partition chromatography
- high-performance liquid chromatography (HPLC)

Reverse-phase chromatography (RPC) is the most versatile and most widely used method of peptide purification. With traditional methods of HPLC, the stationary phase captures polar, hydrophilic molecules that are then differentially eluted by increasing the concentration of polar solvents in the mobile phase. In RPC, as the name implies, hydrophobic molecules from aqueous solutions are instead captured by the stationary phase using hydrophobic C4, C8 or C18 n-alkyl hydrocarbon ligands, and their retention time is a function of the hydrophobicity of the molecule and that of the mobile phase.

For peptide purification, RPC separates the target peptides from impurities from the synthesis steps, such as isomers, deletion sequences, peptide products from side reactions with free coupling and protecting groups or peptides that have undergone side-chain reactions.

Peptide purity is measured as a percentage of the target peptide to impurities that absorb at the peptide bond absorption wavelength (210-220nm), and varying levels of purity are commercially available based on the application in which the peptides will be used:

- >95% – Quantitative studies such as NMR, receptor-ligand binding studies, ELISA and RIA, monoclonal antibody production, in vivo studies
- >80% – High-throughput screening, non-quantitative blocking in immunohistochemical (IHC) and Western blot analyses, non-quantitative enzyme-substrate studies, antibody affinity purification and plate coating for cell attachment
- >70% – ELISA standards, ELISPOT assays and polyclonal antibody production