Module No. 31: Peptide Synthesis: Definition, Methodology & applications

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   - 2.2. Activation C-terminal carboxylic acid
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Peptides are present in living cell as enzyme, hormone, antibiotic, and receptor and have diverse biochemical activities. It is very important and interesting to develop the synthetic peptide in the laboratory. The main objectives of peptide synthesis are:

1. To study the relationship between structure function relationship of biologically active protein and peptides and establish their molecular mechanisms.
2. To synthesize peptides those are of clinically importance such as antibiotic, vaccines and other drugs.
3. To develop new peptide-based kits for immunogen.

1. Introduction

Emil Fischer defined in 1901 [1] the peptide bond as the amide-like linkage between amino acids and chosen the term peptide which was called peptones in Fisher’s day are the mixtures of peptides formed on the proteases on proteins. Peptide has various crucial roles in biological system. It governs the structural and functional properties of bioactive proteins which is important objective in biological and medical research.

Amino acids and peptides

Peptides and proteins are prime important in the regulation and maintenance of all biological processes.

The variation in properties of peptide and proteins depends on the nature of the side chain of amino acids:

i) Some amino acids partially transfer the protein and give rise to hydrogen bond interaction like M…..H…..X

ii) Some completely transfer the proton and form an ion and they are acidic in nature

iii) Some amino acids can accept proton easily due to the presence of free amino group which are basic in nature

-NH₂ + H⁺ ----NH₃

Under physiological condition in neutral solution, amino acid acts as a double ion or Zwitterion of the form
NH₃⁺---------CHR----COO⁻

**Hydrophobic amino acids** – side chains are non-polar. They are water hating group and usually located interior of the protein. Hydrophobic interaction is the important aspects for the stability of the protein.

Eg: Phenylalanine (F), Glycine (G), Alanine (A), Isoleucine (I), Leucine (L), Valine (V)

**Basic amino acids** – positively charged at physiological pH. They are usually involved in electrostatic interaction with negatively charged group of amino acids. They are usually occur in enzyme active sites as it can functions as very efficient general acid-base catalyst.

Eg: Arginine (R), Lysine (K), Histidine (H)

**Acidic amino acids** – They are negatively charged in physiological pH. They are most often found on the surface of the protein where they can interact with favorable solvent molecules. They take part in electrostatic interaction with positively charged amino acids.

They take part in catalytic role in active sites of the enzyme and well known for their metal-ion binding capabilities.

Eg: Aspartic acid (D), Glutamic acid (E)

**Properties of different amino acids**

**Glycine (G)** – It is very flexible due to the absence of side chain. It frequently found in the turn region of the protein where the backbone has to make sharp turn.

**Alanine (A), Valine (V), Leucine (L), Isoleucine (I)** Phenylalanine (F) – They do not have any reactive group on their side chain, so they do not interact with water but interact with each other and non-polar atoms which is the main factor in stabilizing the folded confirmation of protein.

**Serine (S), Threonine (T)** – ‘S’ has OH group and ‘T’ has CH₂-OH group which are not very chemically reactive.

**Aspartic acid (D), Glutamic acid (E)** - Difference between D and E is only one methylene group. This slight difference in the length of the side chain causes to have different tendencies of their chemical interaction with peptide backbone. They have different effects of the conformation and chemical reactivity of the peptide backbone. They are found on the surface of the protein where they can easily interact with solvent molecules and take part in electrostatic interactions with positively charged basic amino acids.

**Tyrosine (Y), Tryptophan (W)** – These residues are nearly found buried in the hydrophobic interior of the proteins as they are predominantly non-polar in nature. They allow hydrogen
bonding interaction to be made with other residues or even solvent molecules due to the presence of OH and NH group in their side chain.

**Cysteine (C), Methionine (M)** – C and M are non-polar have a unique property of being able to form a covalent cross-link with another C residue elsewhere in the protein, thus forming a disulphide bridge involve –S-S– bond between two C residues. C frequently occur at metal binding sites as their sulphur atoms can form native covalent bond with central metal ions.

**Histidine (H), Lysine (K), Arginine (R)** – These basic amino acids occur very frequently in enzyme active sites as it can function as a very efficient acid-base catalyst and also acts as a metal ion ligand in numerous protein families. Leucine (L), Histidine (H) and Arginine (R) are strongly basic and are usually involved in electrostatic interactions with negatively charged group such as Aspartic acid (D), Glutamic acid (E). They have important roles in anion binding proteins as they can interact electrostatically with ligand.

**Asparagine (N), Glutamine (Q)** – They have amide group in their side chains which are usually hydrogen bonded whenever they occur in the interior of the protein

**Proline (P)** – Proline is the most rigid of the twenty amino acids since it’s side chain is covalently linked with main chain nitrogen.

**Peptides**

The peptide bond is formed between amino group of one amino acid with carbonyl group of next amino acid by the formation of amide bond and one molecule of water.

The peptide bond is planer and short bond length between C' – N and C' – O.

\[
C' – N = 1.33 \text{ Å (normally it is 1.47 Å)}
\]

\[
C' – O = 1.24 \text{ Å (normally it is 1.43 Å)}
\]

This shortening of the bond length is due to the delocalization of double bond of the carbonyl group into the C' – N bond.

\[
\begin{align*}
\text{O} \\
\text{C} - \text{C'} - \text{N} - \text{C} & \leftrightarrow \text{C} - \text{C'} - \text{N}' - \text{C} \\
\text{H} & \text{H}
\end{align*}
\]

Because of resonance the six atoms shown above tend to lie on a same plane. C and N have trigonal planar sp³ orbital.
The shortness of the bond and partial double bond character gives more strength and rotation around the bond is restricted and gives rise to a planer structure. So the peptide bond; NH – CO is planar and always in trans configuration. Cα – N and Cα – C’ are always in trans to each other with respect to peptide bond C’ – N.

Rotation around C’ – N is restricted and the flexibility of polypeptide chain is only due to the rotation around C’ - Cα and Cα – N.

**Why peptide:**

i) Restriction of conformational flexibility will lead to higher degree of predictability

ii) Highly active; small amount is required for biological function

iii) Highly specific and have therefore relatively low systematic toxicity. Do not accumulate in the body for short half life

iv) Easily acceptable by body, with very low side effect.

**2. Synthesis of peptide**

The peptide synthesis is occur by coupling between two amino acids and formation of peptide bond between carboxyl group one amino acid to amino group of another amino acid.

Synthesis of peptide coupling between two amino acids required the following steps:

i) Protection of N-terminal group of one amino acid and C-terminal of other amino acid by protected group.

ii) Activation of C—terminal of carboxy group of amino acid which is protect by N-terminal protected group.

iii) Coupling reaction: Mixing two amino acids to form an amide bond and release water molecule.

**2.1. N-terminal protected group of amino acid**
i) Boc- Tertiary butyl carbonyl chloride is added dropwise during one hour to a well stirred solution of amino acid in NaOH and t-BuOH [2].

\[
\text{(CH}_3\text{)}_3\text{COCCl} + \text{H}_2\text{N}\text{-R} \xrightarrow{1. \text{aq NaOH}} \text{(CH}_3\text{)}_3\text{COCONH-R} \quad \text{or} \quad \text{Boc-N-R}
\]

tert-butoxycarbonyl chloride

De-protection:
Boc group is de-protected by tri-fluoro acetic acid (TFA)

ii) Z- Benzyloxycarbonyl chloride is added to amino acid in NaOH and water

\[
\text{CH}_2\text{OCCl} + \text{H}_2\text{N}\text{-R} \xrightarrow{1. \text{aq NaOH}} \text{CH}_2\text{OCNONH-R} \quad \text{or} \quad \text{Z-N-R}
\]

benzyloxycarbonyl chloride

Deprotection:
Deprotected by catalytic hydrogenation.

iii) Fmoc - Fluorenylmethyloxycarbonyl chloride is added to amino acid [3].

De-protected by piperidine

Protected carboxyl group: by methyl or ethyl group
Methyl or ethyl group is attached to carboxyl group of amino acid by methylation and ethylation with methyl or ethyl alcohol.

\[
R' - \text{OH} \quad \text{HOC} - R \quad \quad \text{H}^+ / \text{heat} \quad \quad \text{R'O} \quad \text{C} - R
\]

\[R' = \text{Me-}, \text{Et-} \text{or} \text{Bn}\]

Deprotection: it can deprotect by hydrolysis with NaOH.

2.2. Activation C-terminal carboxylic acid

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).

2.3. Coupling between two amino acids: The coupling of two amino acids form an amide bonds

Precautionary reagents: the activating group of DCCI forms 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.

3. Methods of peptide synthesis

There are two methods for synthesis: solid and solution phase

3.1. Solid phase synthesis: Solid phase synthesis was first introduced in 1963 by Merrifield [4]. It involves the successive addition of amino acids to form a linear peptide chain. C-terminus of the first amino acid is covalently bound to a solid support called resin and the chain of amino acid binds from the N-terminal of the amino acid. Four steps chemical reactions are repeated for each amino acids that is added to the peptide chain: de-protection, activation, coupling and cleavage of resin.
i) Protected amino acid is removed to make the alpha-amino group on the end of the peptide chain accessible.

ii) Activation converts the next amino acid to be added to an active ester.

iii) Activation converts the next amino acid to be added to an active ester. During coupling the active ester forms an amide bond with the de-protected alpha-amino group on the end of the peptide chain. After coupling new cycle of synthesis begins with the next de-protection.

iv) Cleavage of resin uses different reagents depending upon the resin used for solid support. The side chain protecting groups also remove during cleavage.

Types of resin:

i). Wang resin [5]:

![Wang resin]

Cleavage – Wang resin is cleaved by trifluoroacetic acid (TFA)

ii) Pam resin:

![Pam resin]

Cleavage – Pam resin is cleaved by Trifluoromethane sulphonic acid (TFMSA)

iii) Merrifield resin:

![Merrifield resin]
Cleavage- Merrifield resin is cleaved by stable dimethyl ether–poly(hydrogen fluoride) (DMEPF).

**Precautionary reagents**: Some amino acids like C,M,W,Y,T have a potentially reactive side chain for the formation of carbonium ion called carbocation during cleavage with acid. So to prevent carbocation various scavengers are used like anisole and thioanisole. Water is also added with scavenger to suppress alkylation.

**Kaiser’s Test:**

The completion of coupling is tested manually by Kaiser’s test for the presence of free amino group (Kaiser et al 1970)[6]. Three solutions are required for this test

Solution A: Ninhydrin in ethanol

Solution B: Phenol in ethanol

Solution C: Potassium cyanide in pyridine.

Add two drops from each solvent to few beads of resin, heat in 100°C water bath. Free amino group gives blue colour beads which show the incomplete coupling and white colour beads show the absence of free amino group.

**3.2. Solution phase synthesis**: Solution phase synthesis is a classical method which was first introduced by scientist for peptide synthesis. In this method, C-terminal of one amino acid is protected by ethyl of methyl ester and couple with another N-terminal protected amino acid. After each coupling peptide is isolated and purified and proceed for next coupling.

**4. Different Mechanism of amino acid coupling:**

i) Mixed anhydride coupling

ii) DCCI/HOBt coupling

iii) Salt coupling

iv) Acyl halide and pseudo halide coupling

**5. Difference between solid and solution phase peptide synthesis**

<table>
<thead>
<tr>
<th>Solid Phase</th>
<th>Solution phase</th>
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<tbody>
<tr>
<td>Solid polymer support at C-terminal end is required</td>
<td>No solid support is required. C terminal is protected with methyl or ethyl group</td>
</tr>
<tr>
<td>The synthesis is always proceed via N-terminal</td>
<td>Synthesis is possible from both N and C</td>
</tr>
<tr>
<td>One pot reaction</td>
<td>Isolation and purification is required after every step of coupling.</td>
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<tr>
<td>Very rapid method</td>
<td>Very slow and labour-intensive as product has to isolate after every coupling manually.</td>
</tr>
<tr>
<td>Yield is very high</td>
<td>Yield is low as it looses at every step of washing after coupling.</td>
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### 6. Solid Phase Peptide synthesis by automatic peptide synthesizer:

The machine is fully automated microprocessor controlled instrument for peptide synthesis. It has a software consist of menu includes deprotection, washing, activation and coupling.

#### 6.1 The parts of the machines:

i) Three reaction vessels

ii) Four solvent bottles: 2 reaction solvent bottles

   - 1 De-protector bottle
   - 1 Activator bottle

iii) One rack with the capacity of 45 amino acid vials

iv) LCD with front panel control

v) Connector to connect with nitrogen cylinder to run the machine

#### 6.2 Software loaded in the machine: There six programs in the machine to run each steps of the synthesis as well as manual run. The programs are built for washing, de-protection, activation, coupling and repetition of coupling.
The cleavage of resin after peptide synthesis is not included in the machine, so peptide resin manually cleaved by using the reagent required for it.

7. **Application of synthetic peptide**

i) To study enzyme substrate kinetic reaction

ii) To determine the structure of naturally occurring peptide since they are resemble.

iii) To mass spectrometry for characterization and quantification of proteins

iv) To determine the structure-activity relationship of biologically active proteins and peptide

v) Use as a drug

vi) Use as diagnostic reagent

8. **Summary**

i) Easy to synthesize

ii) Less immunogenic

iii) Easily absorbed through metabolism as any other biomolecules

iv) Can mimic protein-protein interaction

v) High therapeutic index with low toxicity

vi) Chemical and biological diversity