Taq DNA Polymerase is isolated from the gram-negative, rod-shaped thermophilic bacterium, Thermus aquaticus. It consists of a single polypeptide chain having the molecular weight of 95,000 daltons. It has a thermo-stable enzyme that can withstand temperature up to 95°C or more. The polymerization activity, however, is maximum at around 75°C. This enzyme is resistant to pH 9. It adds complementary nucleotides onto the template DNA strand to form complementary DNA strand. This enzyme has an unusual property that, it adds an extra adenine base to the 3' end of the growing strand.

USES

Taq DNA polymerase is used to make numerous identical copies of desired DNA segment by polymerase chain reaction.

2.2.2: Sequencing techniques

A. DNA sequencing.

Determination of the order of the nucleotides of a DNA is called DNA sequencing.

It is very important for the genetic analysis of DNAs.The nucleotide sequence data is very useful to have a thorough understanding of traits coded by that DNA. Knowledge of nucleotide sequence data of the DNA is a pre-requisite for taking attempts to manipulate the DNA in the desired way.

There are two techniques for DNA sequencing;

a. Maxam-Gilbert method

b. Sanger's method

a. Maxam and Gilbert Method

Maxam and Gilbert (1977) developed a chemical degradation method for sequencing the nucleotides of DNA.

In this method, the DNA is cut into small pieces with the help of a restriction enzyme. Each piece should not be higher than 300bps size. They are separated by electrophoresis on a polyacrylamide gel. Individual DNA bands are separated. Each band contains many identical copies of a DNA. Thus DNA is isolated from each and every band separately. DNA isolated from a band is denatured using alkali treatment and separated by electrophoresis to separate identical strands of the DNA. As a result of electrophoresis, two DNA bands are formed on the gel. The DNA strands in one band are complementary to DNA in the other band. These two strands are sequenced by using the Maxam-Gilbert method separately.

The first strand will give the order of nucleotides in one strand and the second strand will give the order of nucleotides in its complementary strand. The errors in the sequence data may be corrected by matching the nucleotide sequence of these two DNA strands based on the rules of complementarity between the bases in DNA. The ssDNA fragment is labeled with the p32-phosphate group and subjected to the DNA sequencing procedure.

The labeled DNA in the solution is divided into four equal parts each of which is then subjected to a specific chemical treatment to cut the DNA at specific bases.

The four chemical treatments are as follows:

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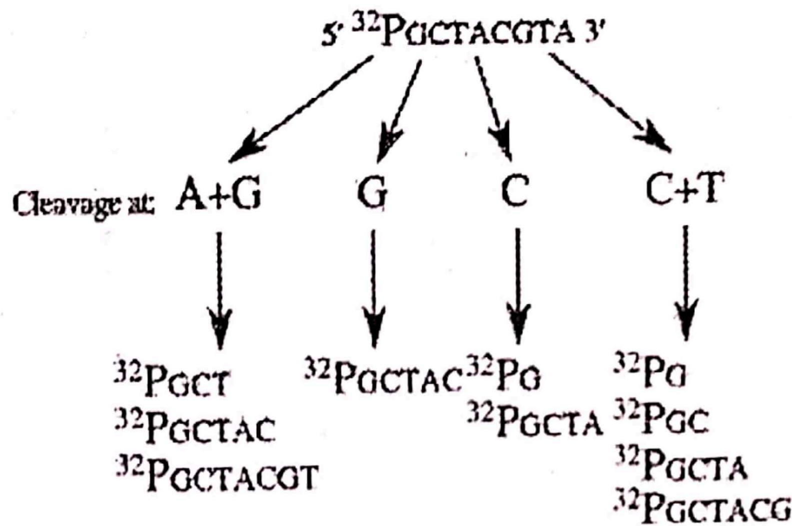
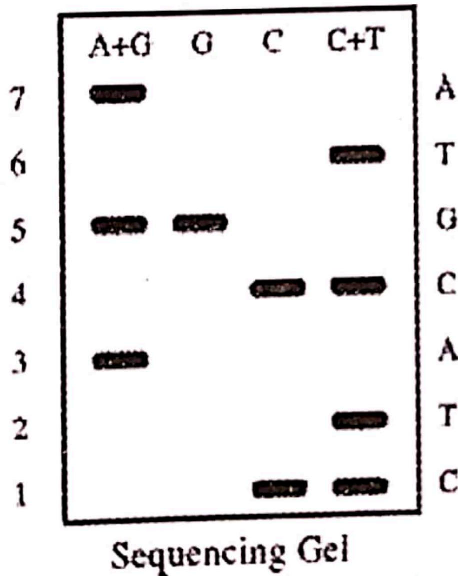
DNA in one tube is treated with dimethyl sulfate (DMS) and piperidine to cut the DNA at guanine bases. The DMS methylates guanine bases at their N7 position and the piperidine removes the methylated guanine and sugar residue attached to it.
DNA in the second tube is treated with DMS, acid, and piperidine to cut the DNA at adenine bases. Here, DMS methylates N3 position of adenine, the acid removes the methylated base and piperidine removes the sugar attached to it.
DNA in the third tube is treated with hydrazine and piperidine to cut the DNA at cytosine or thymine. DNA in the fourth tube is treated with hydrazine, NaCl, and piperidine to break the DNA at cytosine residues. NaCl enhances the frequency of cutting at cytosine residues.





Sequencing Gel

Figure 2.17 : Maxam-Gilbert method of DNA sequencing

These four chemically treated DNA solutions are separately loaded in the wells of polyacrylamide gel and allowed to run across the gel.

7M urea is added to the gel to prevent the formation of secondary structures that affect the mobility of the DNA fragments in the gel.
Temperature raised by the current is also helpful to prevent the formation of the secondary structures.

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As a result, four parallel series of bands are formed on the gel.

The image of the radiolabelled DNA bands is captured on an X-ray film by means of autoradiography. The autoradiogram thus obtained shows four series of bands which indicate the relative position of respective nucleotides in the DNA.

The base sequence is read from bottom to top. The uppermost band resolves the 3' nucleotide and the lowermost one resolves the 5' nucleotide.

Each and every band identifies a particular nucleotide and its position in the DNA. The proceeding base of any base is one base shorter than the previous one. It may occur in the same lane or in any of the other lanes. Therefore, the base sequence is read in a zig-zag pattern. It gives the nucleotide sequence of a ssDNA fragment.

The complementary strand of the ssDNA is then subjected to Maxam-Gilbert sequencing to detect its base sequence. The error in the first sequence is corrected based on complementary matching with the nucleotide sequence of the complementary strand. The missed base may be added to the correct location of the first strand. This is a precaution to avoid errors in DNA sequencing.

In the way, all fragments of the cell DNA are sequenced and the sequences of these fragments are properly arranged to get the complete base sequence of the DNA. They arranged in the correct order based on the principle of overlapping fragments.

At least a few bases, at the end of one DNA fragment, are homologous to some bases at one end of another fragment. Such two DNA fragments are brought together as the adjacent fragments. In this way, all these DNA fragments are arranged in a proper order to get the complete sequence of the cell DNA.

b. Sanger's Method (Dideoxynucleotide Chain termination) method of DNA Sequencing.
Frederick Sanger: (13 August 1918 –19 November 2013) was a British biochemist who twice won the Nobel Prize in Chemistry. Sanger et al; (1977) developed a DNA sequencing method that makes use of enzymes to synthesize new DNA chains on the target site and dideoxy reagent to stop the DNA synthesis at random sites. This method is therefore called the dideoxy method or chain termination method. It is a suitable method for the large-scale sequencing of DNA.

Preparation of DNA :

Sample DNA is a single-stranded DNA with 150-350 bp size. It is prepared in the following way:
The DNA to be sequenced is cut with the restriction enzyme to generate DNA fragments of variable lengths.

These DNA fragments are separated by electrophoresis.

These DNA fragments are cloned in the replicative form (RF) of M13 phage.

The recombinant RFs are introduced into E.coli cells by transformation. Thus as many bacterial clones, as the number of types of DNA fragments, are produced.

Recombinant phages are isolated from each and every clone.M13 phage has the circular single-stranded genome and the genome is a positive strand. So, all the phage particles contain a particular ssDNA.

The ssDNA is isolated from the phage and used for sanger's method of DNA sequencing.

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DNA sequencing procedure

Sanger's DNA sequencing procedure involves the following essential steps:

The circular single-stranded DNA containing DNA to be sequenced is taken in four tubes. The tubes are labeled as A, C, G, and T.

A primer is added to all these tubes. The 5' end of the primer is labeled with radioactive p32. It provides a free 3'-OH group for the synthesis of a daughter strand.

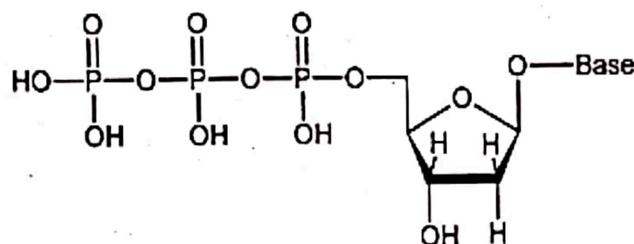
Klenow enzyme and four kinds of radiolabelled nucleotides such as dATP, dGTP, dTTP and dCTP are added to the reaction solution. A small amount of (1%)-

dideoxy adenine triphosphate is added to the A tube.

dideoxycytidine triphosphate is added to the C tube.

dideoxyguanosine triphosphate is added to the G tube.

dideoxythymidine triphosphate is added to the T tube.



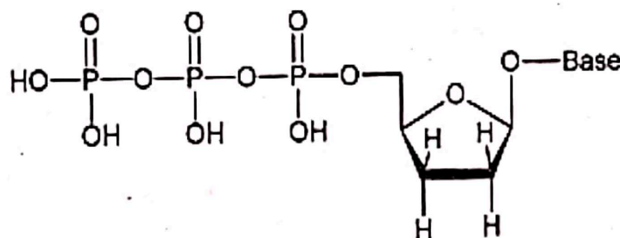


Figure 2.18 : A-Deoxyribonucleotide, B-Dideoxyribonucleotide

The Klenow enzyme adds complementary nucleotides one by one to 3'-OH group of the primer so that the new strand elongates in the 5'-3' direction on the DNA to be sequenced.

When a dideoxynucleotide is incorporated into the daughter strand, the DNA synthesis stops at that site because it has no free OH-group at the 3' end.

The dideoxynucleotides are incorporated at random. Hence the chain synthesis is stopped at any length. It generated fragments of DNA with a variable length. All these fragments have a particular dideoxynucleotide lacking free OH group at the 3' end.

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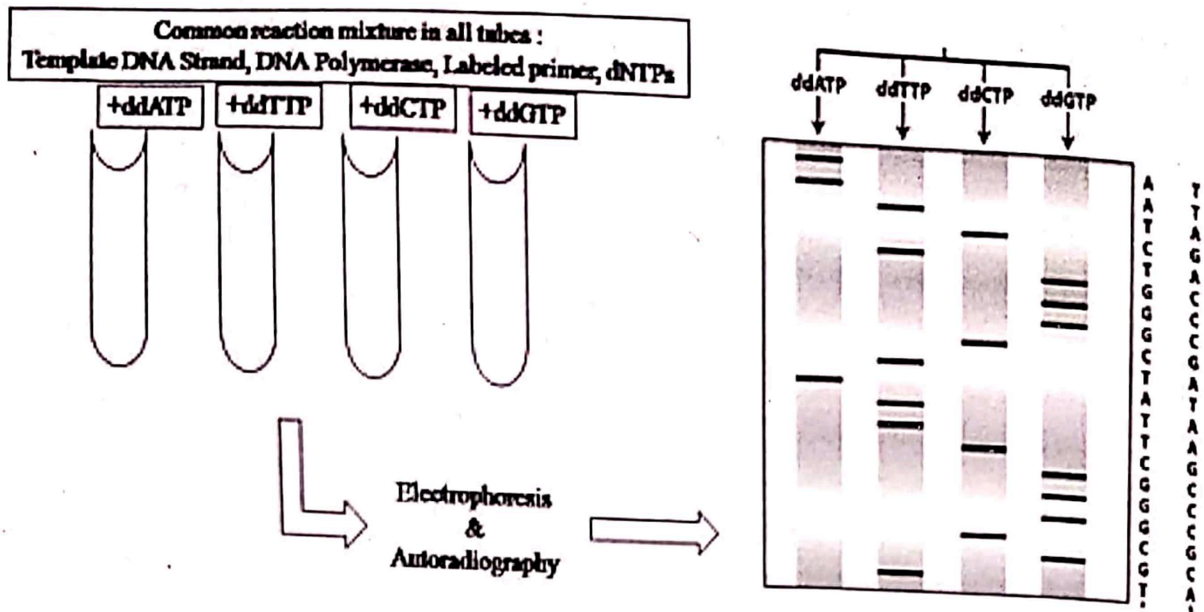

Figure 2.19 : Steps involved in Sanger's method of DNA sequencing

After the completion of the chain synthesis, the reaction tubes are heated to 70°C for separation of daughter strands from the templates.

The contents of these four tubes are loaded into separate wells and electrophoresed on a polyacrylamide gel.

Four series of bands are formed on the gel during electrophoresis.

By means of autoradiography, the images are captured on an X-ray film.

The DNA bands are read from the bottom to top of the gel in a zig-zag manner in relation to the position of the bands. The smallest fragments will move to the bottom of the gel and the subsequent fragments will be one nucleotide longer than the previous one. So each band in the gel indicates the position of one nucleotide.

It gives the complete base sequence of the DNA fragment.

In this way, all the DNA fragments are sequenced simultaneously and the nucleotide sequences are aligned in the correct order based on the overlapping sequences. It gives the complete base sequence of the cell DNA.

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B. Protein Sequencing

a. Sangers method:

Frederick Sanger (13 August 1918 – 19 November 2013) was a British biochemist who won the Nobel Prize in Chemistry twice, one of which was for the development of Sanger's reagent. Sanger's reagent is 1-fluoro-2,4-dinitrobenzene, a trisubstituted, highly activated benzene ring towards nucleophilic aromatic substitution because all three groups are electron-withdrawing (fluoride is a mildly activating group and the nitro groups are strongly activating).

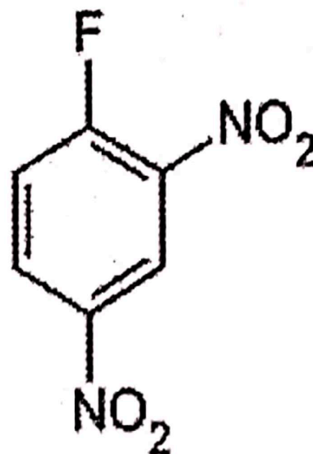

Figure 2.20 : Sanger's reagent :1-fluoro-2,4-dinitrobenzene

All of the above means that the most reactive site on Sanger's reagent is the carbon bonded to the fluorine atom and that carbon is strongly electropositive. Hence this molecule reacts with the terminal amino acid of a given protein and forms a complex which can be cleaved and detected chromatographically. Cyclic repetition of the process allows us to identify amino acid sequence part of the polypeptide chain.
The mechanism obeys the same principles as any nucleophilic aromatic substitution reaction.

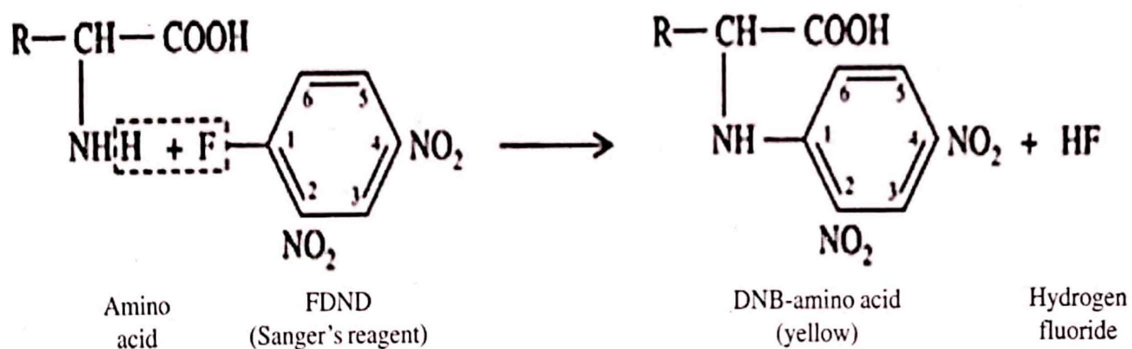

Figure 2.21 :Sanger's protein sequencing reaction

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B. Protein Sequencing by Edman Degradation

The sequence of amino acids in a protein or peptide can be identified by Edman degradation, which was developed by Pehr Edman. In this phenylisothiocyanate molecule is employed to label and cleave the peptide from N-terminal without disrupting the peptide bonds between other amino acid residues. The Edman degradation reaction was automated in 1967 by Edman and Beggs. Nowadays, the automated Edman degradation (the protein sequenator) is used widely, and it can sequence peptides up to 50 amino acids.

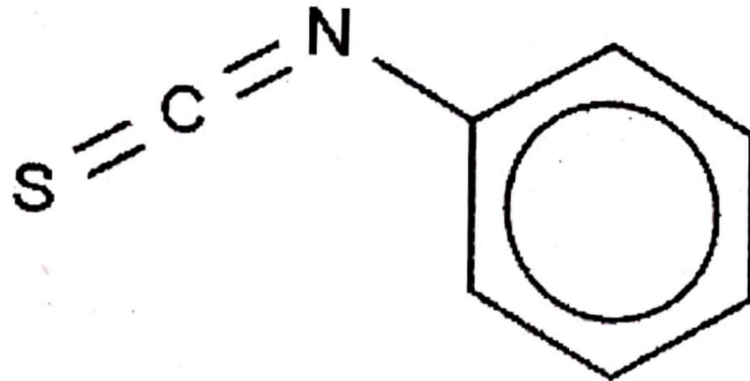

Figure 2.22 : Phenylisothiocyanate

Cyclic degradation of peptides based on the reaction of phenylisothiocyanate with the free amino group of the N-terminal residue such that amino acids are removed one at a time and identified as their phenylthiohydantoin derivatives. An uncharged peptide is reacted with phenylisothiocyanate (PITC) at the amino terminus under mildly alkaline conditions to give a phenylthiocarbamoyl derivative (PTC-peptide). Then, under acidic conditions, the thiocarbonyl sulfur of the derivative attacks the carbonyl carbon of the N-terminal amino acid. The first amino acid is cleaved as anilinothiazolinone derivative (ATZ-amino acid) and the remainder of the peptide can be isolated and subjected to the next degradation cycle. Once formed, this thiazolone derivative is more stable than phenylthiocarbamyl derivative. The ATZ amino acid is then removed by extraction with ethyl acetate and converted to a phenylthiohydantoin derivative (PTH-amino acid). And then chromatography can be used to identify the PTH amino acid residue generated by each cycle.

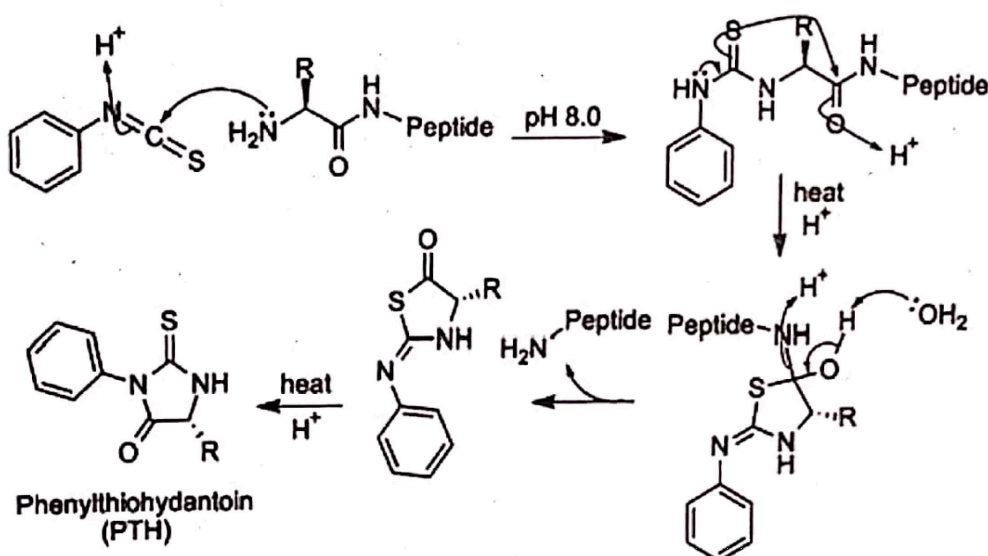

Figure 2.23 : Edman Degradation reaction steps

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