

Enzymes used in Genetic Engineering

The ability to manipulate DNA *in vitro* depends entirely on the availability of purified enzymes that can cleave, modify and join the DNA molecule in specific ways.

At present, no chemical method can achieve the ability to manipulate the DNA *in vitro* in a predictable way. Only enzymes are able to carry out the function of manipulating the DNA. Each enzyme has a vital role to play in the process of genetic engineering. The various enzymes used in genetic engineering are as follows:

- ❖ Nucleases
- ❖ Restriction enzymes
- ❖ DNA ligase
- ❖ Kinase
- ❖ Phosphatase
- ❖ Reverse transcriptase
- ❖ Terminal Deoxynucleotide Transferase
- ❖ RNaseP

The functions of the various enzymes used in genetic engineering are depicted below:

Nucleases - Nucleases are a group of enzymes which cleave or cut the genetic material (DNA or RNA).

DNase and RNase

Nucleases are further classified into two types based upon the substrate on which they act. Nucleases which act on or cut the DNA are classified as DNases, whereas those which act on the RNA are called as RNases.

DNases are further classified into two types based upon the position where they act. DNases that act on the ends or terminal regions of DNA are called as exonucleases and those that act at a non-specific region in the centre of the DNA are called as endonucleases.

Exonucleases require a DNA strand with at least two 5 and 3 ends. They cannot act on DNA which is circular. Endonucleases can act on circular DNA and do not require any

free DNA ends (*i.e.*, 5 or 3 end). Exonucleases release nucleotides (Nucleic acid + sugar + phosphate), whereas endonucleases release short segments of DNA.

Restriction Enzymes

DNases which act on specific positions or sequences on the DNA are called as restriction endonucleases. The sequences which are recognized by the restriction endonucleases or restriction enzymes (RE) are called as recognition sequences or restriction sites. These sequences are palindromic sequences. Different restriction enzymes present in different bacteria can recognize different or same restriction sites. But they will cut at two different points within the restriction site. Such restriction enzymes are called as isoschizomers. Interestingly no two restriction enzymes from a single bacterium will cut at the same restriction site.

Mode of action

The restriction enzyme binds to the recognition site and checks for the methylation (presence of methyl group on the DNA at a specific nucleotide). If there is methylation in the recognition sequence, then, it just falls off the DNA and does not cut. If only one strand in the DNA molecule is methylated in the recognition sequence and the other strand is not methylated, then RE (only type I and type III) will methylate the other strand at the required position. The methyl group is taken by the RE from S-adenosyl methionine by using modification site present in the restriction enzymes.

However, type II restriction enzymes take the help of another enzyme called methylase, and methylate the DNA. Then RE cleaves the DNA. If there is no methylation on both the strands of DNA, then RE cleaves the DNA.

It is only by this methylation mechanism that, RE, although present in bacteria, does not cleave the bacterial DNA but cleaves the foreign DNA. But there are some restriction enzymes which function exactly in reverse mode. They cut the DNA if it is a methylate.

Star activity

Sometimes restriction enzymes recognize and cleave the DNA strand at the recognition site with asymmetrical palindromic sequence, for example Bam HI cuts at the sequence GA TCC, but under extreme conditions such as low ionic strength it will cleave in any of the following sequence NGA TCC, GPOA TCC, GGNTCC. Such an activity of the RE is

called star activity.

Nomenclature of Restriction Enzymes

As a large number of restriction enzymes have been discovered, a uniform nomenclature system is adopted to avoid confusion. This nomenclature was first proposed by Smith and Nattens in 1973.

Every restriction enzyme would have a specific name which would identify it uniquely. The first three letters, in italics, indicate the biological source of the enzymes, the first letter being the initial of the genus and the second and third being the first two letters of the species name. Thus restriction enzymes from *Escherichia coli* are called *Eco*; *Haemophilus influenzae* becomes *Hin*; *Diplococcus pneumoniae* *Dpn* and so on. Then comes a letter that identifies the strain of bacteria; *Eco R* for strain R. Finally there is a roman numeral for the particular enzyme if there are more than one in the strain in question; *Eco RI* for the first enzyme from *E. coli* R, *Eco RII* for the second.

Types

The restriction endonucleases can be divided into three groups as type I, II and III. Types I and III have an ATP dependent restriction activity and a modification activity resident in the same multimeric protein. Both these types recognize unmethylated recognition sequences in DNA. Type I enzymes cleave the DNA at random site, whereas Type III cleave at a specific site. Type II restriction modification system possess separate enzymes for endonuclease and methylase activity and are the most widely used for genetic manipulation.

Type I Restriction Enzymes

These restriction enzymes recognize the recognition site, but cleave the DNA somewhere between 400 base pairs (bp) to 10,000 bp or 10 kbp right or left. The cleavage site is not specific. These enzymes are made up of three peptides with multiple functions. These enzymes require Mg⁺⁺, ATP and S adenosyl methionine for cleavage or for enzymatic hydrolysis of DNA. These enzymes are studied for general interest rather than as useful tools for genetic engineering.

Type II Restriction Enzymes

Restriction enzymes of this type recognize the restriction site and cleave the DNA within

the recognition site or sequence. These enzymes require Mg^{++} as cofactor for cleavage activity and can generate 5'-PO₄ or 3'-OH. Enzymes of this type are highly important because of their specificity. Type II restriction enzymes are further divided into two types based upon their mode of cutting.

Type II Restriction Enzymes - Blunt End Cutters

Blunt end cutters Type II restriction enzymes of this class cut the DNA strands at same points on both the strands of DNA within the recognition sequence. The DNA strands generated are completely base paired. Such fragments are called as blunt ended or flush ended fragments.

Type II Restriction Enzymes - Cohesive End Cutters

Cohesive end cutter Type II restriction enzymes of this class cut the DNA stands at different points on both the strands of DNA within the recognition sequence. They generate a short single-stranded sequence at the end. This short single strand sequence is called as sticky or cohesive end. This cohesive end may contain 5'-PO₄ or 3'-OH, based upon the terminal molecule (5'-PO₄ or 3'-OH). These enzymes are further classified as 5' end cutter (if 5'-PO₄ is present) or 3'-end cutter (if 3'-OH is present).

Type III Restriction Enzymes

Type III Restriction enzymes of this type recognize the recognition site, but cut the DNA 1 kbp away from the restriction site. These enzymes are made up of two peptides or subunits. These enzymes require ATP, Mg^{++} and S-adenosyl methionine for action.

The properties of three types of restriction endonucleases and a list of enzymes are given below.

Property	Type I	Type II	Type III
Structure	Enzyme complex of 500-600 k dal composed of three separate subunits	Normally homodimers of 20-70 k dal	Heterodimers with subunits of 70 and 100 k dal
Composition	Multienzyme complex with R (endonuclease), M (methylase) and S (specificity) subunits	Separate enzymes; endonuclease is a homodimer, methylase a monomer	M subunit provides specificity on its own; functions as methylase; as heterodimer with R subunit; functions as methylase-endonuclease

Cofactors	Mg ²⁺ , ATP, S-adenosylmethionine (SAM) (needed for cleavage as well as methylation)	Mg ²⁺ , SAM (for methylation only)	Mg ²⁺ , ATP (for cleavage), SAM (needed for methylation: stimulate cleavage)
Recognition sites	Asymmetric, bipartite, may be degenerate; 13-15 base pairs containing interruption of 6 to 8 base pairs	Asymmetric, may be bipartite, may be degenerate; 4 to 8 base pairs normally 180° rotational symmetry	Asymmetric, uninterrupted, 5-6 nucleotide long with no rotational symmetry
Cleavage	Non-specific, variable distance (100-1000 nucleotides) from recognition site	Precise cleavage within recognition site at defined distance	Precise cleavage at a fixed distance; 25-27 nucleotides from recognition site
Example	<i>EcoK</i>	<i>EcoRI</i>	<i>EcoP1</i>

Some restriction endonucleases and their recognition sites

Enzyme	Recognition site	Enzyme	Recognition site
4-base cutters		6-base cutters	
<i>Mbol</i> , <i>Dpnl</i> , <i>Sau3AI</i>	/GATC	<i>BglI</i>	A/GATCT
<i>Mspl</i> , <i>HpaII</i>	C/CGG	<i>ClaI</i>	AT/CGAT
<i>AluI</i>	AG/CT	<i>PvuII</i>	CAG/CTG
<i>HaeIII</i>	GG/CC	<i>PvuI</i>	CGAT/CG
<i>Tal</i>	AC/GT	8-base cutters	
		<i>NotI</i>	GC/GGCCCC
		<i>SbfI</i>	CCTGCA/GG

DNA Ligase

Recombinant DNA experiments require the joining of two different DNA segments or fragments *in vitro*. The cohesive ends generated by some RE will anneal themselves by forming hydrogen bonds. But the segments annealed thus are weak and do not withstand experimental conditions. To get a stable joining, the DNA should be joined by using an enzyme called ligase. DNA ligase joins the DNA molecule covalently by catalysing the formation of phosphodiester bonds between adjacent nucleotides.

DNA ligase isolated from *E. coli* and T 4 bacteriophage is widely used. These ligases more or less catalyse the reaction in the same way and differ only in requirements of cofactor. T4 ligase requires ATP as cofactor and *E. coli* ligase requires NADP as

cofactor. The cofactor is first split (ATP - AMP + 2Pi) and then AMP binds to the enzyme to form the enzyme-AMP complex. This complex then binds to the nick or breaks (with 5' -PO₄ and 3' -OH) and makes a covalent bond in the phosphodiester chain. The ligase reaction is carried out at 4°C for better results.

Kinase

Kinase is the group of enzymes, which adds a free pyrophosphate (PO₄) to a wide variety of substrates like proteins, DNA and RNA. It uses ATP as cofactor and adds a phosphate by breaking the ATP into ADP and pyrophosphate. It is widely used in molecular biology and genetic engineering to add radiolabelled phosphates.

Alkaline phosphatases

Phosphatases are a group of enzymes which remove a phosphate from a variety of substrates like DNA, RNA and proteins. Phosphatases which act in basic buffers with pH 8 or 9 are called as alkaline phosphatases. Most commonly bacterial alkaline phosphatases (BAP), calf intestine alkaline phosphatases (CIAP) and shrimp alkaline phosphatases are used in molecular cloning experiments. The PO₄ from the substrate is removed by forming phosphorylated serine intermediate. Alkaline phosphatase is metalloenzymes and has Zn⁺⁺ ions in them.

BAP (bacterial alkaline phosphatase) is a dimer containing six Zn⁺⁺ ions, two of which are essential for enzymatic activity. BAP is very stable and is not inactivated by heat and detergent.

Calf intestine alkaline phosphatase (CIAP) is also a dimer. It requires Zn⁺⁺ and Mg⁺⁺ ions for action. CIAP is inactivated by heating at 70°C for twenty minutes or in the presence of 10 mM EGTA. Alkaline phosphatases are used to remove the PO₄ from the DNA or as reporter enzymes.

Reverse Transcriptase

This enzyme uses an RNA molecule as template and synthesizes a DNA strand complementary to the RNA molecule. These enzymes are used to synthesize the DNA from RNA. These enzymes are present in most of the RNA tumour viruses and retroviruses. Reverse transcriptase enzyme is also called as RNA dependent DNA polymerase. Reverse transcriptase enzyme, after synthesizing the complementary

strand at the 3' end of the DNA strand, adds a small extra nucleotide stretch without complementary sequence. This short stretch is called as R-loop.

Terminal Deoxynucleotide transferase

Terminal deoxynucleotide transferase is a polymerase which adds nucleotides at 3' -OH end (like Klenow fragment) but does not require any complementary sequence and does not copy any DNA sequence (unlike Klenow fragment). Terminal deoxynucleotide transferase (TDNT) adds nucleotide whatever comes into its active site and it does not show any preference for any nucleotide.

RNase P

It specifically cleaves at the 5' end of RNA. It is a complex enzyme consisting of small protein (20 kilodaltons) and a 377 -nucleotide RNA molecule. It has been observed that the RNA molecule possesses at least part of the enzymatic activity of the complex. Hence, it is an example of ribozyme.

Klenow fragment

E. coli DNA polymerase I consists of a single polypeptide chain. Pol I carry out three enzymatic reactions that are performed by three distinct functional domains. Two fragments are obtained when DNA pol I is treated with trypsin/subtilisin in mild conditions. The larger fragment is called as Klenow fragment. This fragment is 602 amino acids in length. The function of the Klenow fragment is to add nucleotides to the 3' end and 3' -5' exonuclease activity.

Klenow fragment adds nucleotides by using complementary strand as reference. It cannot extend the DNA without the presence of the complementary strand. If any nucleotide is added by mistake and the base pair is wrong (i.e., if A is paired to G instead of T) then by using 3' -5' exonuclease activity present in Klenow fragment, this mispaired base pair is removed. In general the Klenow fragment has 5' -3' polymerase and exonuclease activity.

Questions

1. The enzymes used in rDNA technology includes with star activity is/are
a). Nucleases
b). Restriction enzymes
c). DNA ligase
d). All the above
2. The enzymes used in rDNA technology includes with star activity is/are
a). Kinase
b). Phosphatase
c). Reverse transcriptase
d). All the above
3. The enzymes used in rDNA technology includes with star activity is/are
a). Terminal Deoxynucleotide Transferase
b). RNaseP
c). Reverse transcriptase
d). All the above
4. The group of enzymes used in rDNA technology which cleave or cut the genetic material
a). **Nucleases**
b). RNaseP
c). Reverse transcriptase
d). All the above
5. Nucleases act on
a). DNA
b). RNA
c). Both a and b
d). None of the above
6. Nucleases act on
a). DNA
b). RNA
c). Both a and b
d). None of the above
7. The nomenclature of restriction enzymes was proposed by
a). Smith
b). Nattens
c). Both a and b
d). None of the above
8. The restriction enzymes are grouped into types
a). **3**
b). 5
c). 2
d). None of the above
9. The restriction enzymes used widely in rDNA technology is
a). Type I
b). **Type II**
c). Type III
d). None of the above
10. The restriction enzyme with star activity is/are
a). *EcoRI*
b). *BamHI*

c). *SalI*

d). All the above

11. DNA ligase joins the DNA molecule covalently by catalysing the formation of bonds between adjacent nucleotides.

a). **Phosphodiester**

c). Both a and b

b). Phosphotriester

d). None of the above

12. DNA ligase isolated is from

a). *E. coli*

c). **Both a and b**

b). T 4 bacteriophage

d). None of the above

13. DNA ligase isolated from and is widely used.

a). *E. coli*

c). **Both a and b**

b). T 4 bacteriophage

d). None of the above

14. Reverse transcriptase isolated from and is widely used.

a). *E. coli*

c). **Both a and b**

b). T 4 bacteriophage

d). None of the above

15. RNase P specifically cleaves at the of RNA.

a). 5' end

c). **Both a and b**

b). 3' end

d). None of the above