DNA Modifying Enzymes used in Recombinant DNA technology

Introduction

Molecular cloning has become a routine laboratory technique and it is important to understand the function and specificities of the different enzymes used to generate and manipulate nucleic acids. To obtain or to insert specific properties and characteristics, nucleic acids are extensively manipulated by number of enzymes including polymerases, ligases, nucleases, phosphatases, and methylases. Such manipulations include propagation, ligation, digestion, or addition of modifying groups such as phosphate or methyl groups. Nowadays numerous sources of enzymes are available, and selecting the appropriate enzyme for a specific task may seem difficult to the beginner. DNA recombinant technology requires a whole toolkit for modifying/manipulating DNA. These tools invariably include a variety of enzymes called as DNA modifying enzymes. Enzymes that modify DNA are useful became they allow the investigator to manipulate DNA defined ways

These include– 1. Nucleases **a)Endo nucleases cut DNA fragments in the middle of the molecule. 2) Exo nucleases degrade DNA from the ends.**2. Polymerases **elongate DNA molecules by adding free nucleotides to the 3'ends (usually according to an opposite template strand)**3. Ligases **join loose ends of DNA together.**

Let's discuss the description of the main enzymes of each group, their properties and mechanism of action.

Nucleases

A nuclease is an enzyme capable of cleaving the phosphodiester bonds between the nucleotide subunits of nucleic acids. These enzymes play crucial roles in various

DNA repair processes, which involve DNA replication, base excision repair, nucleotide excision repair, mismatch repair, and double strand break repair. In recent years new nucleases involved in various DNA repair have been discovered. Deoxyribonucleases cleave DNA whereas ribonucleases cleave RNA.

Range of nucleases has found applications in recombinant DNA technology. Some nucleases have a broad range of activities but most are either exonucleases, removing

nucleotides from the ends of DNA and/or RNA molecules, or endonucleases, making cuts at internal phosphodiester bonds. Some nucleases are specific for DNA and some for RNA; some work only on double-stranded DNA and others only on single-stranded DNA. The most widely used nucleases are DNase I and RNase A, both of which are purified from bovine pancreas. Based on their mode of action, they are classified as: -

1) Exo- nucleases 2) Endo- nucleases

A. Exonucleases They are active at the end of nucleic acid molecules, removes the terminal nucleotide of the DNA molecule by breaking the phosphodiester bond.

Different types of exonucleases can be categorized on the basis of number of strands they degrade in a double stranded DNA molecule.

Bal31: Bal31 is isolated from a marine bacterium Alteromonas espejiana. It is a Ca2+ dependent enzyme that degrades the nucleotides from both the strands of ds DNA molecule. The longer the DNA is incubated with Bal31, the shorter the DNA molecule becomes. It also acts as single stranded endonucleases that cleave DNA at nicks, gaps and single stranded regions.

Exonuclease III: An enzyme isolated from E. coli called exonuclease III digests only one strand of the dsDNA molecule. It removes the nucleotide from the 3'

terminus of the strand, thus leaving protruding 5' overhangs. Exonuclease III is used for generating single stranded templates.

Similar to exonucleases, endonucleases can also be categorized based on whether they act on single or double stranded DNA.

B. Endonucleases

S1 nuclease: It is an endonuclease that is isolated from the fungus Aspergillus oryzae. It is a heat stable enzyme that functions at high ionic strength, low pH and in the presence of Zn2+ ions. It cleaves only single stranded DNA. Also, it is able to cleave the single stranded nicks in ds DNA molecules.

DNase I: Another type of endonuclease called as DNase I that is isolated from cow's pancreas is a non–specific enzymes. It is able to cleave both single and double stranded DNAs. It can cleave any of the internal phosphodiester bonds, thus prolonged digestion of DNA with DNase I results in its complete chewing leaving only a mixture of mononucleotides.

Restriction endonucleases: Restriction endonuclease is an enzyme that binds to a DNA molecule at a specific sequence and makes a double-stranded cut at or near that sequence. Because of the sequence specificity, it is easy to predict the correct positions of cuts within a DNA molecule, assuming that the DNA sequence is known, enabling defined segments to be excised from a larger molecule.

This ability underlies gene cloning and all other aspects of recombinant DNA technology in which DNA fragments of known sequence are required. Hundreds of restriction endonucleases have been isolated from bacteria, and some of them are commercially available. The progress and growth of biotechnology is unbelievable without the availability of restriction enzymes.

The cleavage of DNA by these enzymes is very specific at 'particular sites'. Specific order of nucleotide sequences are recognized by restriction enzymes on the DNA that are then cleaved. Two kinds of ends (blunt or staggered) may be formed due to digestion of DNA by different kinds of restriction enzymes. This specific sequence

which is recognized by restriction endonuclease enzyme is known as recognition sequence. Majority of restriction endonucleases (particularly type II) cut DNA at defined sites within recognition sequence. The cut DNA fragments by restriction endonucleases may have mostly sticky ends (cohesive ends) or blunt ends. DNA fragments with sticky ends are particularly useful for recombinant DNA experiments. This is because the single-stranded sticky DNA ends can easily pair with any other DNA fragment having complementary sticky ends.

Ribonucleases: Ribonuclease A is an endoribonuclease that cleaves single-stranded RNA at the 3' end of pyrimidine residues. It degrades the RNA into 3'phosphorylated mononucleotides and oligonucleotides. Some of the major use of RNase A are: • Eliminating or reducing RNA contamination in preparations of plasmid DNA. • Mapping mutations in

DNA or RNA by mismatch cleavage. • RNase will cleave the RNA in RNA: DNA hybrids at sites of single base mismatches, and the cleavage products can be analysed.

DNA Polymerases

DNA polymerase is an enzyme that synthesizes DNA and one that copies an existing DNA or RNA molecule is called a template-dependent DNA polymerase. For initiating DNA synthesis the enzyme requires: - 1) Primer a short double-stranded region provides 3′end on to which the enzyme will add new nucleotides. The new polynucleotide synthesized in the 5′→3′ direction. 2) A pool of all the four dNTPs, used to synthesize the new DNA strand. 3) In addition, some cofactors like Mg2+ ions may be required in a buffer solution with correct pH for optimum activity.

DNA polymerases can have either 3′→5′ exonuclease or 5′→3′ exonucleases activity. There are different types of DNA polymerases used in recombinant DNA technology. We will study the following types in detail. A) E. coli DNA polymerase I B) Klenow Fragment C) Thermostable DNA polymerase D) Reverse Transcriptase

A) E. coli DNA polymerase I DNA polymerase I was discovered by Arthur Kornberg in 1960. It has both the 3′→5′ and 5′→3′ exonuclease activities, which limits its usefulness in DNA manipulation. It catalyses the template-directed polymerization of nucleotides into duplex DNA in a 5′→3′ direction. DNA Polymerase I possesses a 3′→5′ exonuclease activity or "proofreading" function, which lowers the error rate during DNA replication, and also contains a 5′→3′exonuclease activity, which enables the enzyme to replace nucleotides in the growing strand of DNA by nick translation. DNA polymerase I can also excise mismatched regions in DNA.

B) The Klenow fragment The Klenow fragment is a proteolytic product of E. coli DNA polymerase I that retains polymerization and 3’ to 5’ exonuclease activity, but lack 5’ to 3’ exonuclease activity.

This Klenow fragment can synthesize the new DNA strand complementary to the template but cannot degrade the existing strand. Klenow polymerase is now rarely used in sequencing and has its major application in DNA labelling.

C) Thermostable DNA polymerase The E. coli DNA polymerase I enzyme has an optimum reaction temperature of 37 ℃ and cannot function at high temperatures. Thermostable DNA polymerases are a class of enzymes which remain functional at high temperatures. Suitable enzymes can be obtained from bacteria such as Thermus aquaticus, which live in hot springs at temperatures up to 95 ℃, and whose DNA polymerase I enzyme has an optimum working temperature of 72 ℃. Thermostable DNA polymerase is the major component of PCR which requires one that is able to function at temperatures much higher than 37 ℃.

D) Reverse transcriptase One additional type of DNA polymerase important in molecular biology research is reverse transcriptase, which is an RNA-dependent DNA polymerase.

Reverse transcriptase (RT) is found in RNA viruses also called as retroviruses. RT uses mRNA template instead of DNA for synthesizing new DNA strand. The complementary DNA strand formed on the mRNA template is called the complementary DNA (cDNA). Formation of a double stranded cDNA from the mRNA molecule using RT finds applications in genetic engineering.

Ligases

Ligases are enzymes that join the nucleic acid molecules together. These nucleic acids can either be DNA or RNA. DNA ligases connect DNA fragments by catalyzing the formation of a phosphodiester bond between a 3'-OH and a 5'phosphate group at a single-strand break in double-stranded DNA.

In nature DNA ligases are essential for joining Okazaki fragments during replication, and in the last step of DNA repair process. Basically there are two types of ligases a) DNA ligases b) RNA ligases

Because it can join two pieces of DNA, DNA ligase became a key enzyme in genetic engineering. If restriction-digested fragments of DNA are placed together under appropriate conditions, the DNA fragments from two sources can anneal to form recombinant molecules by hydrogen bonding between the complementary base pairs of the sticky ends. DNA ligases are used in molecular biology to join DNA fragments generated by restriction enzymes, add

linkers or adaptors to DNA, or repair nicks. Ligases join the DNA known as ‘insert’, with the vector molecule and the reaction is known as ligation.

The discovery of DNA ligases was equally important as that of restriction endonucleases in the development of recombinant DNA technology. In recombinant DNA technology the most commonly used ligases is T4 DNA obtained from bacteriophage T4. It requires ATP as cofactor and Mg2+ions for its activity and is able to perform both blunt and sticky end ligations. The greater efficiency of sticky-end ligation has stimulated the development of methods for converting blunt ends into sticky ends. In one method, short double stranded molecules called linkers or adaptors are attached to the blunt ends. Linkers and adaptors contain a recognition sequence for a restriction endonuclease and so produce a sticky end after treatment with the appropriate enzyme. Apart from the above enzymes there are some more enzymes which are included in the category of DNA end modifying enzymes. These are alkaline phosphatase, polynucleotide kinase and terminal transferase. Alkaline phosphatase removes the terminal phosphate group while polynucleotide kinase is involved in the addition of phosphate groups. Alkaline phosphatase plays

an important role in cloning experiments and for preparing radio labelled DNA probes. Terminal transferase (also called terminal deoxynucleotidyl transferase) repeatedly adds nucleotides to any available 3′-terminal ends, the most suitable being the protruding 3′-ends. This enzyme is particularly useful to add homo-polymer tails prior to the construction of recombinant DNA molecules.

Conclusion The term genetic engineer may be appropriate for an individual who is involved in genetic manipulations. The genetic engineer’s toolkit or molecular tools are the enzymes most commonly used in recombinant DNA experiments. The basis of recombinant DNA technology is the ability to manipulate DNA molecules in the test tube. This, in turn, depends on the availability of purified enzymes whose activities are known and can be controlled, and which can therefore be used to make specified changes to the DNA molecules that are being manipulated. Enzymes like DNA polymerases, ligases, restriction endonucleases and other end modifying enzymes are the basic tools for DNA modifications in the field of genetic engineering.