Biocatalysts for the Synthesis and Modification of Biopolymers

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5.1. INTRODUCTION

Biopolymers are polymers that are biosynthesized from monomeric building blocks. Although biopolymers have been used for many years by the food, pharmaceutical, and specialty chemical industries, they have recently taken on increasing industrial importance often for novel applications. The heightened interest in these materials has primarily been the result of new biotechnological methods which have given industry access to large quantities of highly-pure biopolymers of various structures representing both natural and nonnatural products.

Three major classes of biopolymers have commanded the most attention from the scientific community; these are proteins, polysaccharides, and nucleic acids. Proteins can be conveniently broken into two classes—those with catalytic activity and those without. Enzymes have catalytic activity and thus are not consumed in the chemical reaction which they facilitate, while many other bioactive proteins (i.e., antibodies, growth factors, polypeptide hormones, etc.) participate stoichiometrically in a reaction. Other proteins, such as collagen, may play structural roles, supporting tissues in higher organisms. Polysaccharides are similar to proteins in their...
multiple roles as both bioactive (catalytic and noncatalytic) and structural biopolymers. Nucleic acids, although primarily acting as informational molecules, also have demonstrated catalytic or pseudocatalytic activities.

Biopolymers can be prepared and modified in several ways. The first and oldest method is by extraction from plant and animal tissues followed by fractionation or chemical modification. Although extractive techniques are still widely used to prepare biopolymers, this method has certain shortcomings. Extraction generally only affords the natural product, and chemical modification of a biopolymer resulting from extraction procedures is usually uncontrolled and often undesirable.

Fermentation is an ancient method used to prepare natural products including biopolymers. When it is coupled with modern methods such as recombinant genetics and tissue culture technology, biopolymers having very specific structural characteristics can often be produced. For the preparation of proteins, genetic engineering and site-directed mutagenesis have become the methods of choice to prepare both structurally modified proteins and protein-based natural products. Chemical methods have also been widely used to synthesize both natural and nonnatural biopolymers. Chemical synthesis of proteins and nucleic acids has been accomplished using solid-phase supports utilizing automated synthesizers. Polysaccharide synthesis is somewhat less developed, in part due to the high level of chirality present in these molecules. Recent advances in the chemical synthesis of oligosaccharides and polysaccharides suggest that someday automated solid-phase synthesizers will be available even for the complex-carbohydrate-based biopolymers.

Enzymatic synthesis as an additional method for the preparation and modification of biopolymers has recently received a great deal of attention. Increased interest in the use of enzymes as tools to synthesize and modify biopolymers has resulted from several recent scientific developments. First, enzymes of high purity have become increasingly available due to recent progress in biotechnology. These enzymes can now be modified using site-directed mutagenesis to impart different specificity or increased stability. Designer enzymes represent a set of precision tools useful in the construction and modification of biopolymers. Second, advances in the use of enzymes as reagents have resulted from the discoveries that enzymes can catalyze nonnatural reactions, that enzymes can work well in organic solvents, and that many enzymes can be immobilized and used efficiently in bioreactors. Third, the scientific community has begun to recognize that enzymes often represent the best way to prepare or modify biopolymers both from the standpoint of their high regio- and stereo-selectivity and that of their ability to be used under very mild reaction conditions.

This chapter will focus on the use of enzymes to prepare and modify
biopolymers including proteins, nucleic acids, and polysaccharides. The preponderance of biopolymers and modified biopolymers, having high commercial value, are being investigated for their therapeutic potential. Therefore, the major emphasis of this chapter will be on biopolymers which have potential importance to the pharmaceutical industry.

5.2. PROTEINS AND POLYPEPTIDES

5.2.1. Biosynthesis

Proteins are prepared biosynthetically in ribosomes by the addition of amino acids to the carboxyl terminal of the growing peptide chain. The activating enzymes are aminoacyl-tRNA synthetases. These enzymes construct a protein having a sequence directed by a mRNA template. This class of enzymes requires a cofactor such as a nucleoside triphosphate like ATP, to form a peptide linkage between the amino acid residues of the growing peptide chain. Following their template-directed synthesis, proteins are often further processed by cleavage at certain sites catalyzed by specific proteolytic enzymes. The reader is referred to more detailed reviews in this field. The enzymes that are responsible for the biosynthesis of proteins have not been useful for in vitro synthesis because of problems associated with cofactor regeneration, enzyme availability, cost, and stability. Therefore, alternative chemical methods for the synthesis of proteins and polypeptides have been developed.

5.2.2. Chemical Synthesis

Various chemical methods have been developed by which single amino acids, dipeptides, or polypeptides are covalently coupled, affording a target polypeptide sequence. Most of the reported methods deal with the production of polypeptides that have properties similar or identical to natural products.

Although chemical synthesis offers different ways for the preparation of desired peptides in reasonable quantities, problems such as racemization and side reactions still exist. The development of solid-phase synthesis and its constant improvement have resulted in convenient methods for obtaining pure peptides, particularly since the introduction of automated synthesis. Solid-phase synthesis is based on the addition of a single amino acid residue at a time to a growing chain by coupling the α-COOH group of one amino acid derivative with the α-NH₂ group of another, as shown in Figure 1. Peptide synthesis utilizes a variety of approaches to active
functional groups including azide coupling,25 carbodiimide coupling,26 and mixed anhydride coupling.27 Enzyme-catalyzed coupling has also been used in the formation of polypeptide bonds.28 and is discussed in detail later.

Amino group protection of the reactant with the available carboxyl group, and carboxyl group protection of the reactant with the available amino group, are blocking steps essential for the prevention of side reactions. There are a number of amine protective groups including urethane derivatives29 and trifluoroacetyl derivatives that can be removed under very mild conditions.30 Carboxyl groups are generally protected by esterification.31 Other functional groups, such as the nitrogen of the histidine side chain, are protected as an imidazole derivative32 and tyrosine and serine hydroxyl groups are protected by etherification.33

There are two general strategies of protein synthesis both of which use these protection and coupling procedures. The stepwise chemical approach starts at a terminal group and proceeds by the addition of single amino acids in the desired sequence. The peptide condensation approach requires the assembly of separate portions of the peptide chain often prepared by stepwise chemical synthesis with appropriate blocking and activating groups in place. These peptides are then coupled to form a larger peptide of the desired sequence.

Liquid-phase peptide synthesis, carried out in a homogeneous solution,34 requires multiple synthetic (blocking and de-blocking) steps. Purification by crystallization, extraction, or chromatography is required after each synthetic step. Consequently, the yield is considerably lower than solid-phase synthesis. Thus large-scale peptide synthesis is typically performed using the solid-phase peptide synthesis method introduced by Bruce Merrifield35 and subsequently modified.36 Its practical application of insoluble, polymer-bound reagents has enhanced its utility particularly with the advent of automated solid-phase peptide systems. These automated systems can produce large quantities of synthetic peptides in a relatively short time.

The synthetic scheme used in automated, modified Merrifield synthesis begins with the attachment of the C-terminal residue of the peptide to be synthesized to an insoluble solid support. The peptide chain is then synthesized from the amino terminus of the growing peptide chain. When the desired peptide chain has been completed, it is cleaved from the support (Figure 2).
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\[
R^-NH-R^\text{'}CH-COOH \rightarrow \text{polymerr resin} \\
R^-NH-R^\text{'}CH-COO \rightarrow \text{polymerr resin} \\
-R^\text{'} \downarrow \text{deblocking step} \\
+ R^-NH-R^\text{'}CH-CO-X \downarrow \text{coupling step} \\
R^-NH-R^\text{'}CH-CO-NH-R^\text{'}CH-COO \rightarrow \text{polymerr resin} \\
\downarrow \text{repeat} \\
R^-\text{peptide} \rightarrow \text{polymerr resin} \\
\downarrow \text{cleave, deblock} \\
\text{peptide} \rightarrow \text{polymerr resin}
\]

Figure 2. Automated solid-phase synthesis of peptides. The C-terminus of a protected amino acid is coupled to a solid, polymer resin support. A peptide of the desired amino acid sequence is then synthesized on the polymer resin by adding amino-blocked carboxy-activated (X) amino acids. The synthesized peptide is then released from the support by treatment with mild acid. R\text{'}, is the amino-protecting group, while R\text{'} and R\text{''} designate the first two amino-acid residues added to the growing peptide chain.

Thousands of synthetic peptides having biological activity have been prepared using chemical methods. One of many applications is the chemical synthesis of peptides resembling specific regions of viral coats as immunogens or for blocking viral infection.\textsuperscript{37,38} For example, an 18-residue synthetic peptide corresponding to the region of the envelope glycoprotein of HIV (responsible for acquired immune-deficiency syndrome) has been chemically synthesized and found to elicit antibodies that recognize intact precursor envelope glycoprotein.\textsuperscript{39}

5.2.3. Enzymatic Synthesis and Modification

Protease-catalyzed enzymatic synthesis of bioactive proteins and polypeptides is an area of great interest and activity.\textsuperscript{40-43} These proteolytic enzymes are used in organic solvents or in the presence of molecular traps to form peptide bonds.\textsuperscript{44} Peptide synthesis using reverse enzymatic catalysis decreases the requirements for protective blocking groups and often results in enhanced stereo- and regioselectivity, representing the main advantage over chemical methods of peptide synthesis.

Semisynthetic techniques, which combine chemical and enzymatic methods, have been widely used in the preparation and coupling of bioactive peptides.\textsuperscript{45} For example, a peptide isolated from naturally occurring
protein can be coupled to a synthetic peptide to form the same protein or an entirely new protein. This approach was applied successfully to the preparation of ribonuclease-S[1–20],46 cytochrome C,47 human insulin (HUMULIN),48 and somatotropin49 and other proteins.50

Aspartame is a low-calorie dipeptide sweetener, commercially important as a sugar substitute. Enzymatic synthesis of aspartame is based on the coupling of N-carbobenzoxy-L-aspartic acid and D, L-phenylalanine methyl ester. The reaction is catalyzed by thermolysin, a metalloprotease, in either aqueous or organic solvents.51

The two main strategies used in peptide synthesis by enzymatic condensation are thermodynamic control and kinetic control.

5.2.3.1 Thermodynamically Controlled Synthesis

Thermodynamically controlled synthesis involves the direct reversal of protease-catalyzed peptide hydrolysis (Figure 3). The equilibrium of reactions (a) and (b) in Figure 3 favors hydrolysis (a) in an isolated or undisturbed system under physiologic conditions. However, if a substrate of reaction (b), R′CO2H and/or R′NH2, is used at a high concentration, reaction (b) can be driven to produce the condensation product.52 The formation reaction can also be driven by continuously removing the product R′CONHR2 through precipitation53 or adsorption to a solid phase, or by using a molecular trap (such as an antibody which binds product but not starting materials). The use of a molecular trap couples the peptide formation reaction to a second, thermodynamically highly favorable, ligand-binding reaction. This approach was used for reversible modification of the active site of soybean trypsin inhibitor.54 The use of an organic cosolvent which can accommodate both the substrates and the enzyme but precipitates the peptide product will drive the reaction towards product formation.55 The equilibrium of a reversible reaction (Figure 3, reactions (a) and (b)) can be altered by using an organic cosolvent such as glycerol, dimethylformamide, ethylene glycol, or dimethylsulfoxide. Water-organic

\[ \text{a. } R′\text{CONHR}_2 + H_2O \rightarrow R′\text{CO}_2 + ^+\text{HNR}_2 \text{ (hydrolysis)} \]
\[ \text{b. } R′\text{CO}_2H + ^+\text{HNR}_2 \rightarrow R′\text{CONHR}_2 + H_2O \text{ (formation)} \]
\[ \text{c. } R′\text{CO}_2H + ^+\text{HNR}_2 \leftrightharpoons R′\text{CO}_2 + ^+\text{H}_2\text{NR}_2 \]

Figure 3. Reactions important in thermodynamically controlled peptide formation and hydrolysis. These are: (a) peptide hydrolysis; (b) peptide formation; and (c) deprotonation and protonation of carboxyl and amino groups. In peptide hydrolysis (a), the carboxyl and amino groups in the products are charged as is typically favored under physiologic conditions. In peptide formation (b), the carboxyl and amino groups in the reactants are uncharged. R1 and R2 designate different amino acids.
solvent systems increase the pKa value of the carboxyl component. This favors the protonated form $R'\text{CO}_2\text{H}$ over $R'\text{CO}_2\text{H}^-$ by shifting the equilibrium (reaction (c) in Figure 3) to the left. The increased concentration of the protonated form of the acid, a substrate in the reaction (b) (Figure 3) results in increased peptide formation. For example, the synthesis of Cbz—Try—Gly—NH$_2$ from Cbz—Try—OH and Gly—NH$_2$ was maximum in 60% trimethylene glycol. The increased peptide formation in the presence of an organic cosolvent is attributable to the suppression of the transfer of the acidic proton from the carboxyl group to the amino group. This method has been used to prepare human insulin from porcine insulin$^{43}$ by condensation of desoctapeptide insulin with synthetic tripeptide using trypsin in 50% dimethyl formamide.$^{51}$ Human insulin is also prepared by tryptic transpeptidation of porcine insulin and threonine derivatives using cosolvents.$^{58}$

5.2.3.2. Kinetically Controlled Synthesis

Enzymatic synthesis of proteins and peptides can also be achieved by taking into account the kinetic considerations governing the reaction shown in Figure 4.

Kinetically controlled synthesis involves the enzymatic coupling of an $N$-blocked amino acid ester (acyl donor) with an amino acid or amino acid ester (acyl acceptor).$^{56,60}$ This approach is limited to enzymes forming an acyl-enzyme intermediate such as lipases and pig liver esterases$^{61,62}$ and having no amidase activity. Proteases which form acyl-enzyme intermediates can also be used; however, cleavage of peptide bonds in the resulting products can be caused by their amidase activity. Sometimes proteases can be modified to remove their amidase activity while retaining their esterase activity.$^{61}$ For example, the active-site serine of subtilisin could be modified, reducing its amidase activity while maintaining its weak esterase activities.$^{63}$

A number of biologically important peptides containing unusual

\[
R'\text{CO}_2\text{H} + E \rightleftharpoons R'\text{CO}_2\text{R}' + E \rightleftharpoons R'\text{COE} \rightleftharpoons R'\text{CONHR}'
\]

\[
\downarrow \text{H}_2\text{O}
\]

$R'\text{CO}_2\text{H} + E$

Figure 4. Kinetically controlled synthesis of peptides. $R'\text{CO}_2\text{R}'$ is an $N$-blocked amino acid ester representing an acyl donor and $R'\text{NH}_2$ is an amino acid or an amino acid ester representing an acyl acceptor. E is an esterase, lipase, or modified protease having no amidase activity capable of forming an acyl-enzyme intermediate $R'\text{COE}$. 

acyl acceptor

acyl donor
D-amino acids have been prepared. Proteases will often not accept peptide linkages composed of D-amino acids as substrates. Thus protease-catalyzed bond formation involving these D-amino acids is not possible, nor is recombinant technology useful in preparing peptides or proteins from such unusual amino acids. However, esterase-catalyzed, kinetically controlled peptide synthesis has been used successfully to prepare peptides containing D-amino acids.62,64

5.3. POLYSACCHARIDES AND OLIGOSACCHARIDES

5.3.1. Structure

Oligosaccharides and polysaccharides are comprised of monosaccharide residues in either the furanose (5-membered ring) or the pyranose (6-membered ring) form, attached through glycosidic linkages.4 The difference between an oligosaccharide and a polysaccharide is primarily one of size. Typically, oligomers having less than ten sugar residues are called oligosaccharides while those with more than twenty residues are called polysaccharides. Glycosidic linkages occur between the 1-position (anomeric position) of one saccharide unit and a nonanomeric hydroxyl group on the second saccharide unit. A typical disaccharide unit composed of two simple hexopyranose saccharide units can be linked between the 1 → 2, 1 → 3, 1 → 4, or 1 → 6 positions in either of two linkage configurations α or β owing to the chirality of the anomeric center. Thus there are sixteen possible disaccharides comprised of any two simple hexopyranose saccharide residues. If the chirality, ring size, and substitution by nonsugar moieties (i.e., acetate, amino groups, phosphate, sulfate, etc.) are considered, thousands of different disaccharide structures can be drawn. While many oligosaccharides and polysaccharides are linear, like proteins and nucleic acids, branching is often observed, increasing the number of possible structural isomers. Glycoconjugates are carbohydrates attached to small molecules (aglycones) such as steroids, and lipids or larger molecules such as peptides and proteins. Glycoproteins (and glycopeptides) are biopolymers in which an oligosaccharide is attached to a structurally dominant protein component. Alternatively, in proteoglycans (and peptidoglycans) a structurally dominant polysaccharide is attached to a smaller protein or peptide core. In this section pure carbohydrate containing biopolymers as well as biopolymeric glycoconjugates are discussed.

This high level of structural diversity in complex carbohydrates suggests the utility of enzyme-assisted synthesis and structural modification with its expected high level of regio- and stereoselectivity. The large-scale production of recombinant proteins for pharmaceutical applications has
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demonstrated the need for developing methods for the enzyme-assisted synthesis and enzymatic modification of the carbohydrate portion of glycoproteins. Many recombinant proteins either totally lack attached carbohydrate or are glycosylated differently from the natural products which they are intended to supplant. Carbohydrate “remodeling” has been proposed and uses purified enzymes to modify these glycoproteins to enhance their activities or alter their in vitro half-lives. Before such modification of synthesis can be undertaken, the structure of the glycoprotein to be remodeled and the desired carbohydrate structure (often that of the natural product) must first be determined. The determination of the carbohydrate structure of glycoproteins—molecules that are very complex, structurally heterogeneous, and available in very small quantities—often requires an involved, integrated approach using chemical, enzymatic, and spectroscopic methods.

5.3.2. Biosynthesis

Polysaccharide and oligosaccharide biosynthesis differs from nucleic acid and protein biosynthesis in that it is not template directed. However, this does not mean that it is any less complex or that information cannot be stored (coded) within the resulting carbohydrate biopolymer. The biosynthesis of polysaccharides and oligosaccharides is a highly regulated, multienzyme process which often takes place in specialized cellular com-

![Figure 5. Carbohydrate synthesis by stepwise addition of nucleotide diphosphate sugar. UDP is uridine diphosphate, Ac is acetate, and R represents one or more sugar residues.](image-url)
parts. Once the glycosidic linkages are formed, further enzymatic modification, such as acetylation, sulfation, phosphorylation, etc., takes place. The final polysaccharide or oligosaccharide component that "decorates" a glycoprotein is often more structurally complex than the protein portion of the molecule.

There are three major types of glycosylation reaction. The first type

\[
\text{Sugar nucleotide} + \text{HO-PP-Phosphate} \rightarrow \text{Carrier molecule}
\]

\[
\text{Carrier molecule} + \text{NH-Asn-Polypeptide} \rightarrow \text{NH-Asn-Polypeptide + PP-Phosphate}
\]

Figure 6. Carbohydrate synthesis by transfer of a glycosyl from a sugar nucleotide to a carrier molecule. UDP is uridine diphosphate, PP-phosphate is polyphosphoryl phosphate, and Asn is asparagine.
(Figure 5) is the formation of carbohydrate chains usually on core protein by stepwise addition of nucleotide diphosphate sugar to an acceptor with the release of nucleotide diphosphate. This irreversible transfer reaction resulting in glycosylated acceptor is catalyzed by glycosyl transferase. This enzyme is specific for both the acceptor and the donor nucleotide sugar.

The second glycosylation process (Figure 6) involves the transfer of glycosyl residue from a sugar nucleotide to a carrier molecule such as

![Diagram of glycosylation process]

Figure 7. Transglycosylation of preassembled oligosaccharides leading to branching. R, R', and R'' represent one or more sugar residues.
polypropenyl phosphate or diphosphate with the release of inorganic phosphate. Core oligosaccharides are then assembled on polypropenyl diphosphate carriers. Glycosyl transfer from carrier to protein is followed by glycosidase-catalyzed remodeling of oligosaccharide, eventually leading to the formation of the final carbohydrate chain.

The last type of glycosylation (Figure 7) is the transglycosylation of preassembled oligosaccharide fragments, which often leads to polysaccharide branching. This type of assembly is usually applied to the preparation of storage polysaccharides, resulting in the formation of branched structures from linear structures. This mechanism does not involve phosphate-activated species; rather, the enzyme is glycosylated as an acyl-enzyme-activated intermediate.69

5.3.3. Chemical Synthesis

The chemical synthesis of oligosaccharides requires careful planning due to the large number of possible arrangements of monosaccharide residues. The pivotal step in the chemical synthesis of oligosaccharide is the stereospecific and regiospecific formation of glycosidic linkages between polyfunctional sugar components. This coupling process generally involves the reaction of an activated glycosyl donor68,69 such as a glycosyl halide with a suitably protected sugar acceptor (Figure 8). The coupling step itself must be controlled to give a product with the required configuration (either α or β). All steps, i.e., activation, formation of glycosidic linkage, protection, deprotection, and other modifications must have high regio- and stereoselectivity to prevent the formation of complex product mixtures.

![Figure 8](image_url)

Figure 8. Chemical synthesis of a disaccharide using a glycosyl halide and appropriately protected sugars. X represents a halide, Ac is acetate, and Bzl is benzyl.
Multiple synthetic steps, required for appropriate blocking and deblocking reactions, lower the yields, as do chromatographic and other purification steps.

5.3.4. Enzymatic Synthesis

Enzymatic synthesis of oligo- and polysaccharides is less laborious than chemical synthesis. With the increased availability of enzymes acting on sugars and the development of new approaches for enzymatic synthesis, enzymatic methods are becoming a routine practice in polysaccharide synthesis. The advantages of enzymatic synthesis over chemical synthesis are a decrease in the number of required synthetic steps and higher regio- and stereo-selectivity. The chemical synthesis of a simple disaccharide may take as many as six synthetic steps due to required protection and deprotection, while the same enzymatic synthesis can often be performed in a single step. As discussed earlier, although regioselectivity can be controlled in chemical synthesis by the appropriate use of well-placed protective groups, stereoselectivity is often more difficult to control. Enzymes are chiral catalysts and thus have very high levels of stereoselectivity. Thus, by decreasing the number of steps while increasing the regio- and stereoselectivity, higher yields and greater product purity are obtained.

The main two types of enzymes which are used for the preparation of complex oligo- and polysaccharides are the glycosyltransferases (EC 2.4.-.-.) and the glycosidases (EC 3.2.-.-.).

5.3.4.1. Glycosyltransferase-Catalyzed Synthesis

The glycosyltransferases are a group of enzymes that catalyze the specific glycosidic synthesis by the transfer of a monosaccharide from glycosylnucleotide donor substrate to an acceptor saccharide substrate (Figure 5). This transfer process is both stereo- and regiospecific. Glycosyltransferases are present at very low concentrations on the intracellular membrane and are difficult to purify; thus only a small number are commercially available. In addition, these enzymes require expensive activated nucleotide sugars as substrates, and are highly specific enzymes catalyzing only certain reactions, which limits their general utility.

A few glycosyltransferases that are used in oligosaccharide synthesis have been isolated and purified. A pure glycosyltransferase typically catalyzes the synthesis of only one linkage and the product can be purified with relative ease. Some examples include the enzyme-catalyzed synthesis of N-acetyllactosamine using galactosyltransferase. UDP galactosyl-
transferase has also been utilized in the coupling reaction to form di- and trisaccharides (Figure 9).

Solid-phase synthesis of oligosaccharides using glycosyltransferases represents an important advance. An interesting compound, the trisaccharide Neu-5-Ac-α(2 → 3)Gal-β(1 → 3)Glc-NAc, has been synthesized by the use of the immobilized enzyme, sialyltransferase. This sequence is of particular interest because it is frequently bound to the terminus of glycoproteins.

Figure 9. Enzymatic synthesis of a disaccharide and a trisaccharide using glycosyltransferases and nucleotide sugars. UDP is uridine diphosphate, GDP is guanosine diphosphate, and Ac is acetate.
5.3.4.2 Glycosidase-Catalyzed Synthesis

Glycosidases are enzymes that are typically thought to hydrolyze glycosides, but this reaction only represents a specialized case of the transfer of a glycosyl moiety from a substrate to a water molecule acting as an acceptor. This transfer could as easily take place to a sugar acceptor (Figure 10). A wide variety of glycosidases (both endolytic and exolytic enzymes) are commercially available. These enzymes are named for the glycosyl portion of the donor substrate (Figure 10) and are usually quite specific for this sugar residue and its anomeric configuration, but are at the same time quite tolerant of a wide variety of acceptor molecules. This can lead to problems in the regioselectivity of glycosidase-catalyzed trans-glycosylation reactions resulting in mixtures of (1 → 2)-, (1 → 3)-, (1 → 4)-, and (1 → 6)-linked products.\textsuperscript{66,77}

![Figure 10](image-url) Glycosidase-catalyzed synthesis using a glucoside as the example. Transfer of a glycosyl moiety from donor to acceptor is shown. In the glucoside donor R typically represents a good leaving group such as p-nitrophenol. When the acceptor is water, glucose is the product. When a sugar or a simple alcohol (R'OH) is the acceptor, a new glucoside is formed.
As with protease-catalyzed synthesis of peptides, glycosidases can be used under either kinetic or thermodynamic control. Synthesis under equilibrium control uses high monosaccharide substrate concentrations or molecular traps to drive the reaction to glycoside formation (Figure 11). Kinetically controlled formation of glycosides using glycosidases first requires the synthesis of glycoside substrate. This substrate effectively glycosylates the enzymes which then serve as the glycosyl donors (Figure 7). This uses considerably less enzyme than does thermodynamically controlled synthesis, and it does not require the use of high substrate concentrations or the use of molecular traps. The glycoside substrate is consumed in the reaction, and its hydrolysis often represents a competing side reaction. Generally, aromatic aglycones are used in such synthesis, but inexpensive disaccharides can also be used as donors.

Compared to protease-catalyzed peptide synthesis, glycosidase-catalyzed glycoside synthesis has several serious drawbacks. First, hydroxyl groups are less nucleophilic than amino groups, leading to equilibria in glycosidase-catalyzed reactions, lying further toward hydrolysis than formation. Second, the presence of multiple nucleophiles on the acceptor molecule often results in multiple products (1 → 2, 1 → 3, 1 → 4, 1 → 6)-linked glycosides) with 1 → 6 formation being favored because the primary hydroxyl group at the 6-position is usually the most nucleophilic. This regioselective preference for the 1 → 6 linkage can be countered by using specific glycosidases that favor other linkages or by using aglycones that have demonstrated the ability to alter the regioselectivity of the transfer reaction. Third, organic solvents, which favor protease-catalyzed peptide formation, result primarily in decreased glycosidase activity and thus decreased oligosaccharide formation. This may be due to an increase in the binding affinity of polar sugar substrate (or product) to the enzymes active site, resulting in substrate (or product) inhibition of the enzyme.

Both glycosyl transferases and glycosidases can be used in concert to synthesize complex oligosaccharides. By sequentially using β-galactosidase and β-D-galactoside-3-α-alloytransferase, α-D-NeupSAc-(2 → 3)-β-D-Galp-(1 → 3)-α-D-GalpNAc-OR could be synthesized.

![Figure 11](image.png)

Figure 11. Thermodynamically controlled glycosidase-catalyzed synthesis of disaccharides and oligosaccharides. Synthesis is driven by using high concentrations of monosaccharide or by using molecular traps such as activated carbon.
Future prospects might include combined chemical and enzymatic (glycosidase and glycosyltransferase) synthesis. Genetic engineering might be an appealing approach to preparing such complex molecules as oligosaccharides and polysaccharides. Polysaccharide biosynthesis, however, is highly compartmentalized and requires many enzymes making the near term success of such an approach highly improbable. Some recent success in this area—the preparation of cyclodextrin from genetically engineered *Bacillus subtilis*—gives hope that efforts in this direction will bear fruit.

5.3.5. Enzyme-Assisted Modification or Degradation

Enzymatic hydrolysis of oligosaccharides and polysaccharides is an important tool in structure determination, sequencing and characterization of carbohydrate composition. Precursors used in organic synthesis, representing chiral synthons, are often obtained from degradation of polysaccharides. These range from simple disaccharides such as Gal(α-1→4)-Gal(β)-OMe, obtained from the glycosidase-catalyzed hydrolysis of polygalacturonic acid, to structurally complex oligosaccharides obtained from glycoproteins.

5.3.5.1. Neutral Polysaccharides

Glycoproteins represent the final product of many recently developed biotechnological processes such as recombinant genetic engineering, monoclonal technology, and cell-tissue culturing. Although these glycoproteins have many applications, the most important so far has been as pharmaceuticals. Recent animal and human testing of these glycoprotein-based drugs has demonstrated that their glycosylation plays an important role in their biological activities. The term “carbohydrate remodeling” has recently been coined when talking about chemical and enzymatic approaches aimed at modifying the carbohydrate portion of glycoproteins. First the carbohydrate composition, the oligosaccharide sequence, and the site(s) of glycosylation in glycoprotein obtained from cell culture or recombinant organisms are determined. Then the glycosylation requirements for appropriate activity, specificity, and pharmacodynamics are established and a structure-activity relationship (SAR) is developed to understand the glycosylation requirements for ideal pharmacological activity. Once a target has been selected, the glycoprotein is remodeled, primarily by using specific glycosidases. These enzymes are tools that can be used with precision to cut and trim the oligosaccharide groups of a glycoprotein to a form which results in an ideal pharmacological agent.

These enzymes are also used for determination of oligosaccharide content and sequence. Selective hydrolysis using an exolytic glycosidase
removes one sugar residue at a time from the nonreducing end of an oligosaccharide chain. Alternatively, hydrolysis or partial hydrolysis using a specific endolytic glycosidase results in smaller oligosaccharides that can be used in structural determination. Enzymatic processes are also important in the food industry for the modification, mostly through partial hydrolysis, of a variety of polysaccharides, important as food additives and in baking and brewing. The hydrolysis of starch using amylase is an example.

5.3.5.2. Acidic Polysaccharides

Depolymerization of acidic polysaccharides using lyases catalyzes the eliminative cleavage (Figure 12) of a glycosidic linkage (distinctly different from the hydrolysis mechanism associated with glycosidases) and results in one product chain containing an unsaturated sugar residue at its non-reducing end. These enzymes can cleave acidic polysaccharides with either an endolytic or exolytic action pattern resulting in either small or large oligosaccharide products. Lyases which act on plant polysaccharides, such as pectin/pectate and alginates are used by the food industry to reduce their molecular weight or to remove these polysaccharides from complex mixtures. Complex acidic polysaccharides derived from animal tissue, including proteoglycans and glycosaminoglycans, have important biological functions. Heparin, for example, has been intensively studied for over

![Diagram](image)

**Figure 12.** Eliminative cleavage of an acidic polysaccharide by a polysaccharide lyase. X is SO₂ or H, and Y is SO₃ or COCH₃.
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fifty years by many research groups including ours, in an effort to improve its properties as an anticoagulant drug.²⁻⁴ Heparin lyases⁵⁰⁻⁹¹ have been used in the controlled depolymerization of the drug heparin to prepare low-molecular-weight heparins with improved anticoagulant properties.⁸ Heparin oligosaccharides, prepared enzymatically from heparin, exhibit other important activities including anti-atherosclerotic activity, the ability to inhibit complement activation, and also anti-angiogenic, antitumor, and antiviral activities.⁶ The chemical structures of many of these oligosaccharides have been characterized using spectroscopic methods, and this has led to a better understanding of the precise structure of the complex, microheterogeneous, polydisperse polysaccharide heparin.⁹²⁻⁹³ One of these oligosaccharides which has been produced by chemical and enzymatic controlled depolymerization of heparin⁹⁴⁻⁹⁵ as well as by de novo multistep chemical synthesis⁹⁶ is heparin's antithrombin III binding site. This unusual pentasaccharide is believed to be primarily responsible for heparin's anticoagulant activity.⁸ Although controlled enzymatic depolymerization of heparin can result in low recoveries if intact binding site,⁹⁴ exhaustive treatment with heparin lyases destroys this binding site.¹⁻⁹⁴ Work is currently underway in our laboratory to control this enzymatic reaction in an effort to prepare a structurally defined oligosaccharide having an intact antithrombin III binding site in high yield in a single enzymatic step in an effort to rival the complicated and time-consuming chemical synthesis currently used.⁹⁶ There is no reported reverse enzymic catalysis involving polysaccharide lyases. Such an approach might be extremely useful in reassembling oligosaccharides into acidic polysaccharides of defined structure and with desirable biological properties.

5.4. NUCLEIC ACIDS

5.4.1. Biosynthesis

Nucleic acids, deoxyribonucleic acids, and ribonucleic acids (DNA and RNA) are informational molecules that are biosynthesized by all cells. The template-directed synthesis of DNA takes place in the nucleus of eukaryotes and in the cytoplasm of prokaryotes. The DNA polymer backbone consists of furanose residues linked 3' → 5' through a phosphate diester linkage. In DNA synthesis, the diester linkage is constructed by coupling a deoxyribonucleotide triphosphate to a short oligonucleotide primer sequence bound to a DNA template with the release of inorganic pyrophosphate. Synthesis is catalyzed by a DNA-directed DNA polymerase exclusively in the 5' → 3' direction.⁹⁷ The specificity of each step
(i.e., which nucleoside is added next to the growing chain) is determined by the DNA template.\textsuperscript{98}

The biosynthesis of RNA from a DNA template is called transcription. The enzymes involved (DNA-dependent RNA polymerases) are responsible for the catalysis of the transcription reaction. RNA polymerases are also responsible for synthesizing cellular RNA. The activated intermediates are the ribonucleoside triphosphates. Polymerization starts with the formation of a phosphodiester bond between the 5'-phosphate of the incoming nucleotide triphosphate and the 3'-OH group of the growing RNA chain. The RNA polymerases, unlike DNA polymerases, do not require a primer and do not have nuclease activities. The overall reaction is irreversible due to the release and subsequent hydrolysis of the pyrophosphate group. As with DNA, the synthesis of RNA proceeds in the 5' → 3' direction. RNA is not a self-replicating molecule, and thus any mistakes in its sequence made during its synthesis do not become genetically inherited. RNA synthesis is highly regulated, promoters direct the binding of RNA polymerase and the unwinding of the DNA double strand to reveal the template, and the transcription process stops once a termination signal is reached.

5.4.2. Chemical Synthesis

In the last two decades, numerous synthetic methods have been developed and modified for the chemical synthesis of nucleic acids. The rapid advances in nucleic acid chemistry can be ascribed to the great biological potential of and the high demand for synthetic oligonucleotides.

The key step in oligo- and polynucleotide synthesis is the preparation of ultrapure reagents and the use of stable and protected nucleotides. Most of these required nucleotides and reagents are now commercially available.

The classical method for the chemical synthesis of oligonucleotides is the solution-phase phosphotriester approach which was popular prior to the introduction of solid-phase synthesis. This method was complicated, usually requiring an experienced organic chemist to perform the complex synthetic steps. A major achievement of this method was the chemical synthesis of a specific gene for somatostatin, which was expressed in \textit{E. coli} to produce protein product.\textsuperscript{99}

The introduction of solid-phase synthesis has resulted in its application throughout synthetic organic chemistry for the preparation of oligomers and polymers of known sequence through the stepwise addition of monomers.\textsuperscript{100}

The advantages of solid-phase synthesis are many; the most important is the isolation of final product in high purity by simply washing the resin and releasing the product. High reaction yields can be obtained by using excess reagents, and all of the reaction and addition steps can be carried
out in one flask, minimizing product loss. Finally, the introduction of automated synthesizers makes it possible for any scientist, even one without a background in organic synthesis, to prepare the desired products in high yield and purity.

There are two general methods used in solid-phase synthesis; the phosphotriester method and the phosphite triester method (Figure 13). In the phosphotriester method the activated nucleotide, protected at its 5' position, reacts with the 5'-hydroxyl group of a nucleoside which is already linked to the solid support to form a new 3' → 5' phosphotriester bond. The protecting group is then removed, exposing a new reactive 5'-hydroxyl group ready for the next coupling step. Details about the coupling reaction, capping, deprotection, support type, and purification procedures can be found in many recent articles. The phosphotriester method is similar to the phosphotriester approach, except that the phosphodiester product formed in the coupling reaction is subsequently oxidized to the desired 3' → 5' phosphodiester linkage (Figure 13). Both of these synthetic methods are reliable, easy to perform, and have been automated. The major application of synthetic oligonucleotides is as primers to prepare recombinant DNA, but they are also valuable as probes for analysis and in site-directed mutagenesis.

5.4.3. Enzymatic Synthesis and Modification

Compared to other biopolymers, the use of enzymes in the synthesis and modification of nucleic acids is most well-developed. This is primarily due to three factors: (1) the importance of nucleic acids as informational molecules; (2) the uncomplicated nature (compared to the linkage chemistry of polysaccharides) of the 3' → 5' linkage; and (3) the availability of a variety of inexpensive, pure enzyme tools. This section will only examine the more important aspects of enzymatic synthesis and modification of nucleic acids.

5.4.3.1. Types of Enzymes Used on Nucleic Acids

Biologically-based synthesis of DNA and RNA using recombinant technology is beyond the scope of this review. Two types of enzymes used by molecular biologists—ligases and restriction endonucleases—play an important role in the synthesis of nucleic acids. Ligases and endonucleases that act on DNA are readily available and are widely used in genetic engineering. Restriction endonucleases recognize specific sequences and act to break phosphodiester bonds in DNA at these sites. Ligases are used to rejoin these restriction-endonuclease-modified DNA
Figure 13. Solid-state chemical synthesis of nucleic acid polymers. The phosphotriester method couples an ortho-chlorophenol-activated nucleotide phosphate protected at the 5' position (R') with a nucleotide bound to a polymeric support (P). B₁ and B₂ are two different bases.
oligomers through reformation of the phosphodiester linkage. Small quantities of DNA oligomers are prepared in vitro and are then inserted into a microbe which can be grown in a fermentor to high cell densities resulting in the in vivo production of large quantities of the desired DNA or gene product. Large-scale enzymatic synthesis of DNA oligomers using these enzymes will become practical only when suitable quantities of stable ligases and endonucleases become available.107

5.4.3.2. Enzymatic Synthesis

For biological studies, oligonucleotides longer than the ones obtained using chemical methods and having well-defined sequences, are required. This has led to the frequently used approach which combines the chemical synthesis of small oligomer sequences with recombinant technology to produce larger polymers such as genes.

DNA ligase is used to join or stitch together two synthetic oligonucleotide fragments. DNA ligase, typically produced from T4-phage-infected E. coli, catalyzes the covalent coupling of two oligonucleotide molecules. The synthetic oligonucleotides are first phosphorylated at their 5'-hydroxyl group with T4 polynucleotide kinase, and a phosphodiester bond is formed between the 5'-phosphate and 3'-hydroxyl groups in the presence of ligase catalyst and a complementary template.115 Many different oligonucleotide fragments can be linked or ligated together to produce the desired sequence having a variety of different restriction (sticky) ends. The newly formed polynucleotide gene can then be cloned by insertion into a plasmid (a small open strand of DNA having the same sticky ends, produced using the same restriction enzyme) and joined by DNA ligase. The gene contained within the plasmid (also containing a suitable antibiotic marker for selection) can be inserted into E. coli (or other suitable organism), which can be used to express the desired gene and produce its protein. Genes can also be isolated directly by digestion of native DNA with restriction enzymes. The genes corresponding to the yeast alanine tRNA,116 insulin A chain,117 human growth hormone,118 and interferon119 are among the many that have been synthesized using this recombinant technology. The use of ligases in the formation and synthesis of biologically active oligonucleotide fragments and genes has been described in detail.120

DNA polymerase, a major enzyme in eukaryotic cells, replicates the polynucleotide template by the sequential addition of deoxyribonucleotide monophosphate residue to the 3'-hydroxyl terminus of the primer. Catalytically it interacts with deoxynucleotide triphosphate (dNTP) substrate, the primer, template and divalent cations.
Oligonucleotides of moderate size can be used as template-primers and converted enzymatically into a gene in the presence of DNA polymerase (usually prepared from *E. coli*) and four deoxynucleotide triphosphates. This DNA can then be digested with suitable restriction enzymes and ligated to a similarly restriction-enzyme-digested plasmid. This transformed plasmid containing the desired gene can be identified using complementary synthetic oligonucleotide hybridization probes. AMV reverse transcriptase has been used instead of DNA polymerase in the complete synthesis of enzymatically produced insulin A chain gene.121

Application of artificial genes to problems in biochemical and biological systems is increasing. These genes can be used to probe for a defective sequence, to regulate gene expression, to produce proteins, and to recognize the sites of interaction between proteins and nucleic acids. Many proteins are difficult and often impossible to isolate and purify from their natural biological systems. Many of these proteins can be more easily prepared in microorganisms.116,115,122 An understanding of the gene expression mechanism in *E. coli* has been due in part to the preparation and study of regulatory proteins. Various *E. coli* promoter sequences have been constructed including tyrosine suppressor tRNA promoter,123 trp promoter,124 and lambda PR promoter.125 The interaction of these promoters with DNA polymerase has also been investigated.123-125

5.4.3.3. Enzymatic Modification

Site-directed mutagenesis developed rapidly because of the ready availability of restriction nucleases and DNA ligases, which made it possible to prepare specific recombinant DNA molecules.

A wide variety of techniques using site-specific mutagenesis has been reported. The basic approach (Figure 14) uses a synthetic primer that contains a single mismatched codon in the middle of its sequence. Despite this single mismatched site, the primer will usually hybridize sufficiently to its template to permit its extension, thus providing a powerful tool for introducing any type of mutation into a specific target area.126 An entire DNA sequence can be deleted and replaced (excision and repair) through the action of endonucleases and ligases. Endonucleases have other uses; for example, DNA phosphorylated pyrimidine dimer is recognized and removed by a specific endonuclease enzyme.127 As the immune system provides protection and resistance against infection, DNA repair processes also perform a protective function by restoring damaged DNA to its original state. Carcinogens such as benzo[a]pyrene and benzo[e]pyrene, for example, are usually found linked to DNA through the N7 of guanine.128,129 Chemical modification has also been exploited resulting in
Figure 14. Site-directed mutagenesis. DNA is denatured and broken into small fragments using restriction enzymes. Among these DNA fragments the desired gene is present, and it is inserted into a nicked plasmid. A synthetic template is prepared with a complementary sequence to the desired gene but containing a single base mismatch. This is annealed to the plasmid containing the desired gene and extended to form a closed circular DNA molecule. After a series of steps that enrich for closed circular DNA, transform cells, isolate single-stranded DNA, and screen for mutations, an organism producing a gene product with a single altered amino acid residue can be found.

the development of antiviral and antibacterial drugs that produce modification of a single nucleotide or within nucleic acids.

One important application of chemical and enzymatically synthesized oligonucleotides is as hybridization probes. Nucleic acid hybridization probes offer a powerful technique and a sensitive tool for the detection of pathogens and for diagnosing genetic diseases that often cannot be detected by any other method. A nucleotide triphosphate can be labeled with a reporter group such as $^{32}$P or biotin and then incorporated into a
synthetic nucleic acid sequence through nick translation to produce the required hybridization probe. Alternatively, the intact nucleic acid sequence can also be labeled with the reporter group. The nucleic acids from the sample (target nucleic acids to be examined) are typically immobilized on a solid matrix and probed with a reporter group containing template having a complementary sequence. Histochemical and enzymatic methods are then used to detect sample containing hybridized probe.

5.5. CONCLUSION

This review has attempted to examine the important recent literature on the use of enzymes in the synthesis and modification of biopolymers. It is clear that enzymatic catalysis has a secure place among the methods used by molecular biologists and nucleic acid chemists. Whether the use of enzymes will have the same impact in protein and carbohydrate chemistry remains to be established. The need to improve the regio- and stereoselectivity in peptide and oligosaccharide chemistry represents a potent force dictating the increased use of enzymes. The two major requirements for expanded application of enzymes in these areas are increased enzyme availability and the development of new ways to apply these enzymes to current problems in protein and carbohydrate chemistry.

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