11.3 Polysaccharide Degradation

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Abstract

An overview of current and potential enzymes used to degrade polysaccharides is presented. Such depolymerases are comprised of glycoside hydrolases, glycosyl transferases, phosphorylases and lyases, and their classification, active sites and action patterns are discussed. Additionally, the mechanisms that these enzymes use to cleave glycosidic linkages is reviewed as are inhibitors of depolymerase activity; reagents which react with amino acid residues, glycoside derivatives, transition state inhibitors and proteinaceous inhibitors. The characterization of various enzymes of microbial, animal or plant origin has led to their widespread use in the production of important oligosaccharides which can be incorporated into food stuffs. Sources of polysaccharides of particular interest in this chapter are those from plants and include inulin, dextran, xylan and pectin, as their hydrolysis products are purported to be functional foods in the context of gastrointestinal health. An alternative use of degraded polysaccharides is in the treatment of disease. The possibility exists to treat bacterial exopolysaccharide with lyases from bacteriophage to produce oligosaccharides exhibiting bioactive sequences. Although this area is currently in its infancy the knowledge is available to investigate further.

Keywords

Action patterns; Adhesion domains; Affinity and mechanism-based inhibitors; Catalytic domains; Classification schemes; Hydrolases; Lyases; Mechanism of action; Phosphorylases; Proteinaceous inhibitors

1 Introduction

Polysaccharides, particularly cellulose and chitin, are the most abundant carbon compounds in the biosphere. They are functionally important as structural and/or protecting materials and are the predominant storage form of carbohydrate in bacteria, protistans, algae, fungi, plants, and animals. They are also important precursors for the enzymatic manufacture of a range of bioactive oligosaccharides with application in nutrition and disease management. In this chapter enzymes involved in polysaccharide depolymerization and biosynthesis will be reviewed with special reference to their specificity, mechanism of action, and structural organization. Applications of such enzymes to the manufacture of bioactive oligosaccharides will also be discussed.

Enzymes with over one hundred different specificities have been recognized to be involved in the reactions of polysaccharide degradation and synthesis. It has not been possible to address each of them. However, particular enzymes or groups of enzymes are discussed to highlight important principles or aspects.

The first section of this chapter deals with enzymes depolymerizing polysaccharides. The reactions they catalyze are essential steps in the provision of assimilable mono- or oligosaccharides from exogenous polysaccharide substrates. The breakdown products are substrates for metabolism and energy production by bacteria, archaeans, single-celled eukaryotes, animals, and fungi. Structural polysaccharides are enzymically degraded in the course of morphogenetic changes, when cells or tissues grow and differentiate, for example, during plant cell wall modification or in the turnover of vertebrate connective tissue and arthropod exoskeletons. Polysaccharide depolymerizing enzymes produced by bacterial, fungal, insect, and nematode pathogens of plants or animals are used to gain access to host tissues. Polysaccharide hydrolases and lyases find many applications in industry, particularly the food industry. These will not all be reviewed here but the potential for manufacture of functional oligosaccharides by enzymatic degradation of polysaccharides will be discussed.

2 Polysaccharide Depolymerizing Enzymes

2.1 Introduction

Glycoside-cleaving enzymes are a diverse group, which more or less selectively, hydrolyze the wide variety of *O*-glycosidic linkages found in glycosides, oligo- and polysaccharides, and glycoconjugates. An account of one group of glycoside hydrolases; those acting on oligogalactosides, is to be found in ❯ Chap. 5.4. This section deals specifically with enzymes depolymerizing interglycosidically, *O*-linked polysaccharides, although, where relevant, information from *O*-glycoside-cleaving enzymes is discussed.

The chemistry of enzymic glycoside cleavage is a focus of attention for carbohydrate chemists and biochemists, and a considerable body of mechanistic information has accumulated, especially with regard to glycoside and polysaccharide hydrolyzing enzymes [1,2,3,4,5,6]. In parallel, there has been an explosion of DNA cloning and direct amino acid sequence determination for glycosidases [7]. In addition, the three-dimensional structures of a growing number of glycoside and polysaccharide-degrading enzymes have been determined.

2.2 Types of Glycoside Depolymerizing Enzymes

Enzyme-catalyzed glycoside cleavages (and syntheses) are considered by Hehre [6,8] to be variants of the same reaction (❯ *Eq. 1*), catalyzed by a single type of enzyme, the glycosylases, comprising glycoside hydrolases, glycosyl transferases, phosphorylases and certain lyases.

$$\text{Glycosyl} - \text{X} + \text{H} - \text{X}' = \text{Glycosyl} - \text{X}' + \text{H} - \text{X} \qquad (1)$$

This description reduces all carbohydrase reactions to an interchange between a glycosyl group and a proton (hydrogen atom) about sites X and X′ in the various potential substrates.

Thus, X may be a saccharide, a 1,2-glycal, a phosphate residue, a nucleoside diphosphate, or a polyprenol diphosphate and X′ the OH of water, an alcohol, and often a saccharide. It is convenient, however, to consider enzymes cleaving polysaccharide chains in one of four classes: hydrolases, glycosyl transferases, lyases, and phosphorylases. These follow the general reactions discussed below.

2.2.1 Hydrolysis

$$\text{Glycosyl} - \text{OR(polysaccharide)} + \text{HOH} = \text{Glycosyl} - \text{OH} + \text{HOR} \tag{2}$$

Some polysaccharide hydrolases also catalyze glycosyl transfer.

2.2.2 Glycosyl Transfer

The glycosyl transferases catalyze redistribution of glycosidic linkages between di-, oligo-, or polysaccharide donor substrates and acceptor oligo- or polysaccharides.

$$\begin{aligned}\text{Glycosyl} - \text{OR}'\text{(di-, oligo- or polysaccharide)} + \text{HOR}''\text{(oligo- or polysaccharide)} \\ = \text{Glycosyl} - \text{OR}'' + \text{HOR}\end{aligned} \tag{3}$$

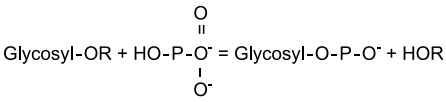
They are considered separately from the polysaccharide hydrolases, but are closely related to them, both mechanistically and structurally. In glycosyl transfer the acceptor water is replaced by a hydroxy group provided by an oligo- or polysaccharide.

2.2.3 Lyase Action (Glycal Formation)

$$\text{Glycosyl} - \text{OR} = 1,2 - \text{Glycal} + \text{HOR} \tag{4}$$

2.2.4 Phosphorolysis

Glycosidic linkages in polysaccharides may also be cleaved by phosphorolysis.
The phosphorolysis reaction is shown in ❯ *Structure 1*.



Structure 1

2.3 Criteria for Classification

2.3.1 Specificity

The Nomenclature Committee of IUB [9] classifies polysaccharide depolymerizing enzymes on the basis of the reaction catalyzed, into *O*-glycoside hydrolases [EC 3.2.1.-], glycosyl transferases [EC 2.4.1.-], lyases [EC 4.2.2.-], and the phosphorylases in [EC 2.4.1.-]. Within each

group, the individual enzymes are sub-classified according to their substrate and product specificity. The Enzyme Nomenclature list is revised continually as new enzymes are discovered and previously included enzymes are better characterized.
Each sub-category of polysaccharide depolymerizing enzymes shows specificity with respect to the configuration of the bond in the glycosidic linkage cleaved, the ring size and configuration of the glycone portion of the substrate, and most often the "aglycone" portion of the substrate. The regioselectivity for the linkage cleaved may in some cases be narrow and in others quite broad. There are also more subtle distinctions between individual enzymes of the same sub-class. Thus differences may occur in the region of the polymeric substrate attacked, i. e., the non-reducing end, the reducing end, or the interior region, the size and state of solubility of the preferred substrate [10], or the structure of the products formed. In addition, and quite fundamentally, the classification in some cases distinguishes different *O*-glycosidases on the basis of the mechanism of cleavage of the glycosidic linkage.

2.3.2 Structure

An additional classification has been introduced that disposes polysaccharide depolymerizing enzymes into glycosidase families based on their amino acid sequence relationships, made possible by direct sequencing or derived from the numerous cloned cDNA or gene sequences for the enzymes [7,11,12,13]. At present, this classification is based on about 5000 known sequences and includes 82 hydrolase, 48 glycosyl transferase families (including phosphorylases) and the ten lyase families. The determination of the three-dimensional structures, at high resolution, of a growing number of these enzymes has permitted amalgamation of some hydrolase and glycosyl transferase families into clans. Members of a clan have common structural features with respect to overall fold motifs, topology of active site residues and catalytic mechanisms, even when the enzymes are without recognized sequence identity [14].

2.4 Active Sites

The catalysis of glycoside cleavage occurs when a region of the polymeric substrate chain is bound at the active site of the enzymes to form an enzyme-substrate complex. The active sites of all types of enzymes involved in polysaccharide depolymerization are formed by catalytic amino acid residues that are directly involved in the covalent bond-breaking or -making processes and by amino acids involved in binding of substrate along an extended binding site. The former category comprises aspartic and glutamic acids in the hydrolases and glycosyl transferases [4,13] and probably arginine in at least some lyases [15]. The typical binding residues can be polar: arginine, histidine, lysine, aspartic and glutamic acids, asparagine, glutamine, serine, threonine or aromatic: tyrosine, phenylalanine, and tryptophan [4,16,17].
The substrate-binding region of the enzyme surface can be envisaged as a series of consecutively arranged binding subsites, complementary to monosaccharide residues in the substrate chain. These are often distributed asymmetrically about the site of glycosidic bond cleavage. In a widely accepted nomenclature [18] describing the monosaccharide-binding subsites in polysaccharide hydrolases, but applying equally well to glycosyl transferases, lyases, and phosphorylases, the subsites are labeled from $-n$ to $+n$ (where n is an integer) and where n

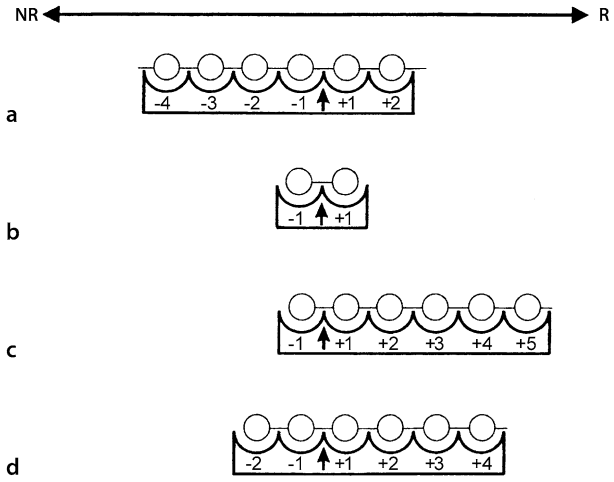


Figure 1
Schematic drawing of monosaccharide binding sites in various glycosyl hydrolases. (a) Subsites of an *endo*-hydrolase, e. g., hen egg white lysozyme, (b) subsites of a disaccharide hydrolase, e. g., ⟨α-glucosidase, (c) subsites of a monosaccharide-releasing *exo*-hydrolase, e. g., glucoamylase, (d) subsites of a disaccharide-releasing *exo*-hydrolase, e. g., β-amylase (after [18])

represents the non-reducing end and $+n$ the reducing end, with cleavage taking place between subsites -1 and $+1$ (*Fig. 1*) [18].

The physical form of the binding subsites can be seen directly from crystallographic analyses of the enzymes in complex with non-reactive substrate analogues or of inactive, mutated enzymes in complex with substrates. This allows the identification of those amino acid residues that are involved in hydrogen bond, or ionic and van der Waals interactions with the bound substrate [19]. This approach was first used for hen egg white lysozyme (reviewed in [20]) and has since been widely applied to other hydrolases, glycosyl transferases, polyuronate lyases, and phosphorylases. Information on stoichiometry and binding thermodynamics, binding kinetics, and conformational changes for substrate analogues can be obtained from solution studies using equilibrium dialysis or gel filtration, surface plasmon resonance and isothermal titration calorimetry, time-resolved fluorescence or transfer nOe NMR spectroscopy, stopped-flow spectrophotometric and temperature jump relaxation methods [21,22,23]. Models of enzyme-substrate complexes can be constructed from the three-dimensional structures using docking procedures involving conformational energy calculations and molecular dynamics. They provide useful insights into the relationship between structure and function [23,24,25,26].

Characterization of the extended substrate-binding site requires information on the number of subsites, the individual subsite binding affinities, bond cleavage frequencies and the location of the catalytic site, which can be determined by analyzing the amount and types of product formed from terminally labeled oligosaccharides [27,28]. The possibility of side reactions such as transglycosylation, and the validity of assumptions that the subsites are independent and that the intrinsic bond cleavage rate is constant, have been addressed in recent applications of the theory [29,30]. In the multiple subsite model it is envisaged that a single substrate and enzyme molecule react at one time, however, it is known that multimolecular substrate reac-

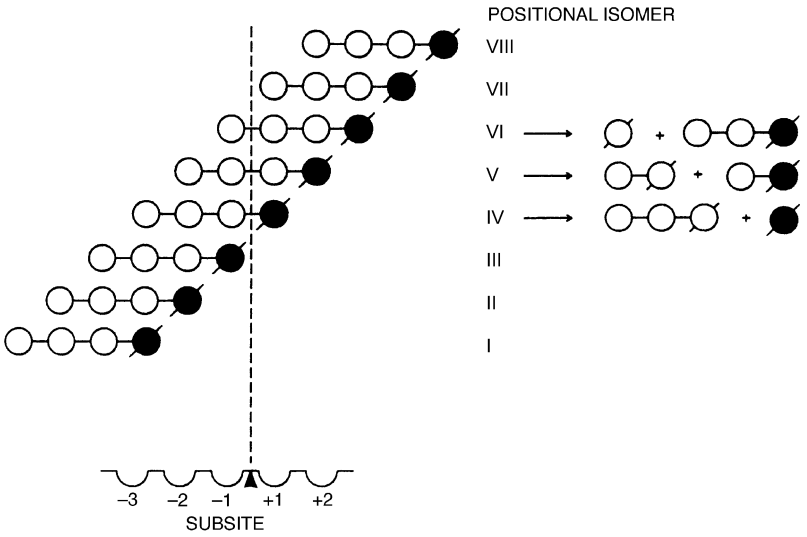


Figure 2
Positional isomers of a tetrasaccharide on a five subsite endo-hydrolase. Of the eight possible positional isomers only IV, V, and VI are productive, i. e., lie over the catalytic site. The cleavage products are shown: subsite on enzyme; position of catalytic site; monosaccharide residue; residue bearing the reducing hemiacetal in substrate; residue bearing the reducing hemiacetal in products (after [28])

tions may occur. These reactions involve, e. g., facilitated binding of one substrate molecule elicited by binding of another. Practical methods are available to assess and minimize these complications [30,31,32].

In the multiple subsite model (*Fig. 2*) [28] an oligosaccharide substrate can interact at the subsites in a number of ways. Thus, a tetrasaccharide may form eight positional isomers with a five subsite enzyme, but only the three in which substrate lies over the catalytic site can be productive. The population of the different complexes is dictated, in principle, by the energetics of interaction of the substrate monomer units with the respective subsites. Each depolymerase has its own subsite map for oligosaccharide substrates, depicting the number of subsites, the location of the catalytic site, and the binding energies contributed by the individual subsites. The latter can be calculated from the bond cleavage frequencies and Michaelis parameters determined for a range of oligosaccharide substrate lengths. A subsite map for two porcine pancreatic α-amylases deduced from kinetic analysis is shown in *Fig. 3* [33].

For this enzyme, kinetic evidence shows several weak oligosaccharide-binding sites in addition to the active site [34], in agreement with the observation that oligosaccharides in the crystal are also bound outside the active site area [35]. Thus, it is important to note that kinetically derived subsite maps may only qualitatively reflect key features of the three-dimensional structure of enzyme-substrate complexes and vice versa. However, the length of the binding site and the position of the catalytic site, as detected from crystallographic or modeled enzyme-ligand complexes generally agree with the subsite map [24,36]. Moreover, conspicuous aromatic stacking onto substrate rings, or H-bonds between charged groups of the enzyme and substrate OH groups tend to reflect high subsite affinity [19].

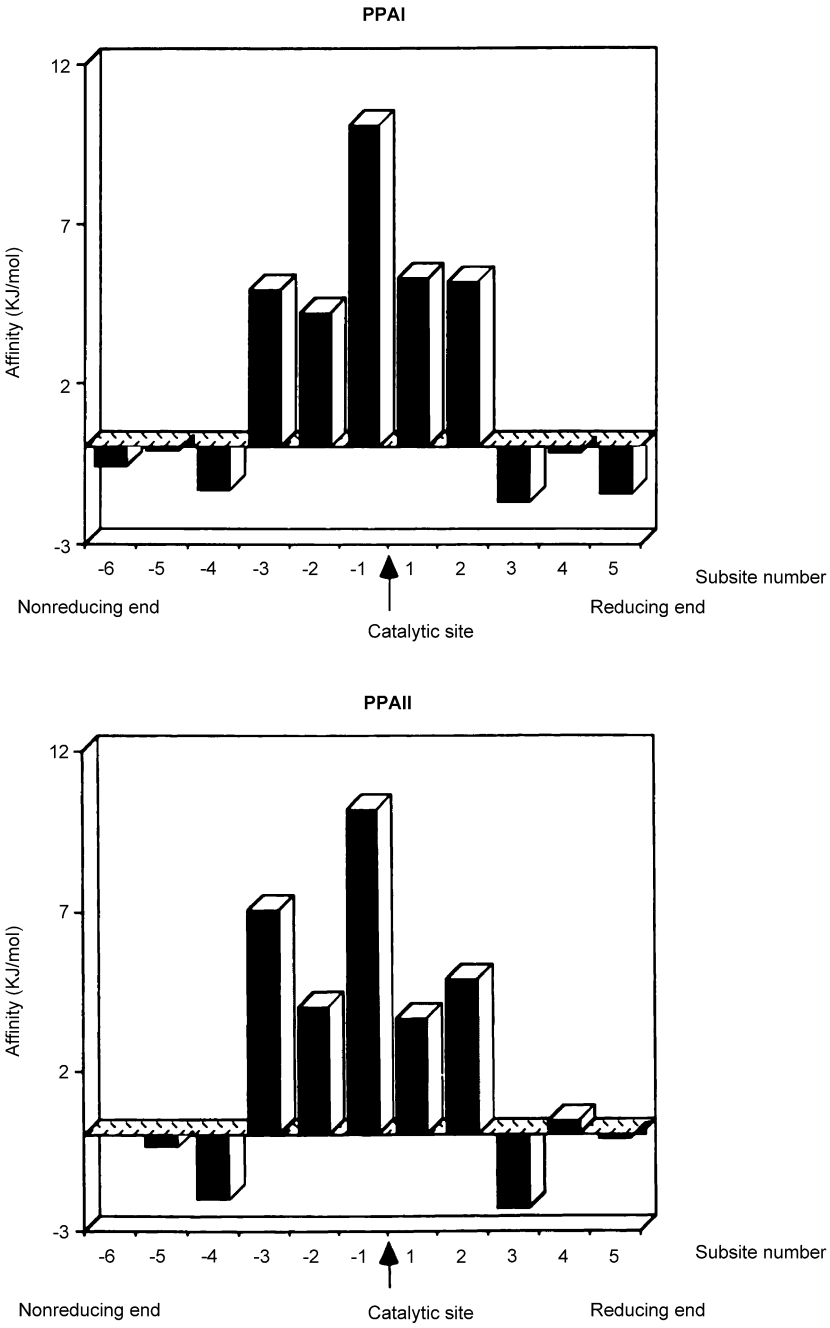


Figure 3
Porcine pancreatic α-amylase I and II energetic profiles. *Arrow* indicates the catalytic site (from [33])

2.5 Action Patterns

2.5.1 *Endo*-Action

In *endo*-cleavage the enzyme first attacks at an interior glycosidic linkage in the substrate. The *endo*-hydrolases are widely represented among the polysaccharide hydrolases and lyases. The cleavage may represent a single productive encounter with the substrate, so that both polymer fragments are released from the enzyme. This action pattern has been described as *multi-chain* attack (◉ *Fig. 4*) [37].

In other *endo*-cleavages several catalytic events may occur after the first encounter in which one polymer fragment is retained and translocated along the substrate binding site to allow

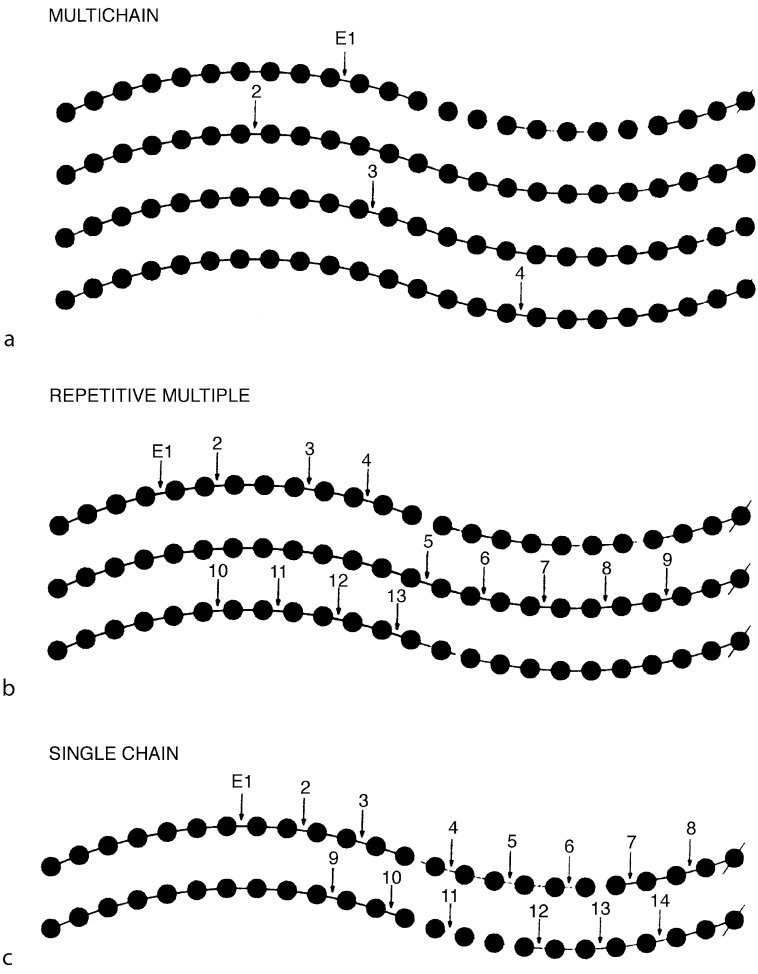


Figure 4
Types of attack patterns for polysaccharide *endo*-hydrolases (after [37])

further cleavages at the catalytic site. This mode of attack is known as *repetitive* or *multiple* attack and the action involves substrate *processivity*. Multiple attack has also been described as *endo-* and *exo*-action (see below). It was first demonstrated for α-amylases [37] but has also been demonstrated for several cellulolytic enzymes [38,39]. In the extreme case, if the enzyme-substrate complex does not dissociate, the bound polysaccharide substrate would degrade to oligosaccharides in a *single chain* attack. However, examples of this mode of attack are not known for polysaccharide degrading enzymes. The degree of multiple attack (DMA) is described by the number of cleavages following the first cleavage of the polymeric substrate. A quantitative analysis of DMA has been developed for α-amylases [37], based on the ratio between the number of oligomeric and polymeric molecules produced from amylose, a linear (1→4)-α-glucan substrate. Average values of DMA vary from 1 through 6 for Taka-amylase A, barley α-amylase, and porcine pancreatic α-amylase [37,40]. The method of Robyt and French [37] provides no information on the chain polarity of the multiple attack, i. e., which cleavage product is retained in the active site. This may be deduced either from the knowledge of the enzyme mechanism or from experiments with end-labeled oligosaccharide substrates. For *endo*-acting enzymes with a retaining mechanism one may assume that, after

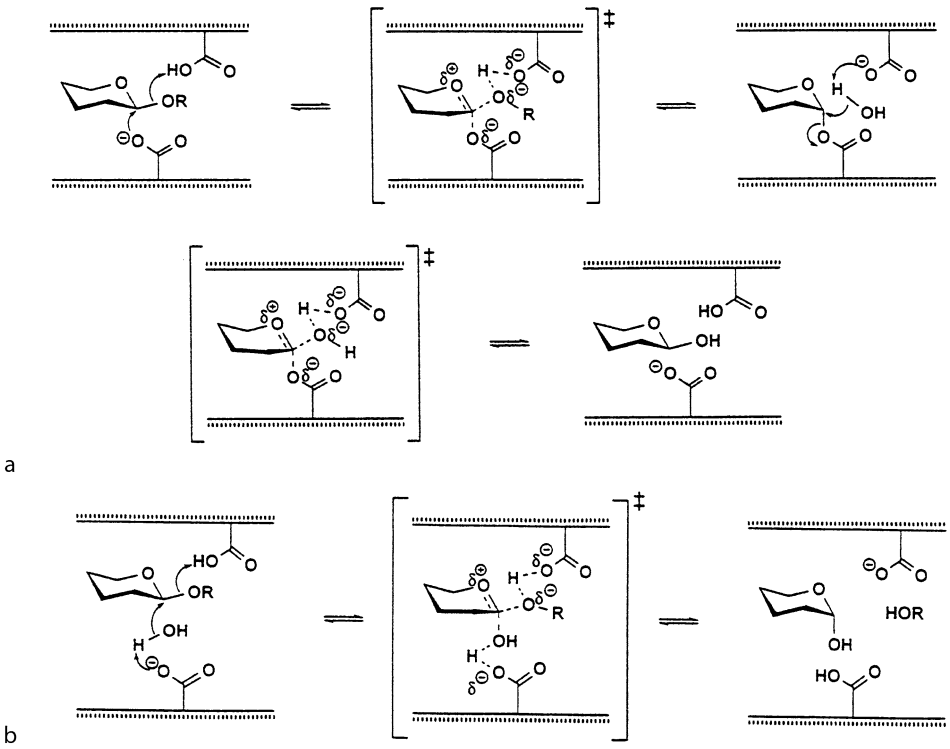


Figure 5
(a) Proposed catalytic mechanism for a retaining β-glycosidase, (b) proposed catalytic mechanism for an inverting β-glycosidase

release of the leaving product, the covalently bound glycosyl intermediate released by attack of water (Fig. 5a) is subsequently translocated in the active site for the next cleavage in the multiple attack reaction. It has been noted that the type of attack may change as the length of the substrate chains decreases during the course of the depolymerization or be dependent on reaction conditions, e. g., pH [41].

2.5.2 *Exo*-Action

Exo-action is well-recognized among the (1→4)-α-, (1→6)-α-, (1→3)-β-, and (1→4)-β-glucan hydrolases, amylolyases, and glycogen and starch phosphorylases. *Exo*-attack generally occurs from the non-reducing end of the substrate chain and is blocked by modification of the terminal residue, e. g., by periodate oxidation in the case of a (1→3)-β-glucan substrate [42,43,44]. *Exo*-cleaving enzymes, depending on the source, may release mono-, di-, or longer oligosaccharides, up to octasaccharides, from the polysaccharide substrate [45,46,47,48,49,50,51,52,53]. The active sites of *exo*-enzymes can also be conveniently described using the now generally accepted nomenclature (see Fig. 1). Thus, the active site of an *exo*-hydrolase, releasing disaccharides, is described as −2, −1, +1, +2, +3, indicating that two glycosyl binding subsites, −2 and −1, accommodate the future product, and that three binding subsites, +1, +2, and +3, interact with glycosyl residues toward the reducing end of the substrate.

Exo-attack may follow the same action patterns as described for *endo*-attack. Thus, β-amylases catalyze the liberation of maltose from the non-reducing end of starch polymer chains in a multiple attack mechanism [54]. Modeling of substrate complexes, for this enzyme, based on its three-dimensional structure, supports the view that the new non-reducing end of the (longer) chain remaining after removal of maltose is translocated to subsite −2 for subsequent attack [55]. The DMA value for the (1→3)-β-exoglucanase from *Sporotrichum pruinosum* QM 806 is 4 [42]. In rare cases, e. g., some cellulases, the *exo*-attack has been described as occurring from the reducing chain end. Thus, the cellulase from *Thermomonospora fusca* E4 [56], which corresponds to the cellobiohydrolase I from *Trichoderma reesei*, displays a preferred release of ^{18}O-labeled cellobiose from the reducing end of ^{18}O-labeled cellopentaose. In contrast, for cellobiohydrolase II the preferred attack between subsites −1 and +1 is at the non-reducing end of cellopentaose [57]. In this connection it should be noted that cellobiohydrolase II (Cel6A) from *Trichoderma reesei*, earlier believed to be an *exo*-enzyme, has been shown to exhibit *endo*-attack through its ability to cleave a bifunctionalized, fluorogenic tetrasaccharide [58]. Thus, it will be important to know whether the *T. fusca* enzyme is a true *exo*-enzyme. It may be envisaged that the attack described as *exo* from the reducing end of the substrate chain may very well be the result of an initial *endo*-action by a retaining enzyme followed by multiple *exo*-attack [38].

2.6 Mechanism of Glycosidic Linkage Cleavage

2.6.1 Hydrolases

The mechanism of enzyme-catalyzed hydrolysis of glycosidic linkages is analogous to acid-catalyzed hydrolysis. Cleavage is between the glycosyl anomeric carbon (C1) and the gly-

cosidic oxygen [59]. The general reaction pathways were first recognized by Koshland [60] who categorized glycoside hydrolases mechanistically on the basis of whether the hydrolysis occurs with retention or inversion of configuration at the newly exposed anomeric center (❯ *Fig. 5*). A distinction can also be made for hydrolases and glycosyl transferases with pyranosyl substrates depending on whether the enzymes cleave equatorial or axial glycosides [2]. Thus, four stereochemical types of glycosidase action, $e \rightarrow e, e \rightarrow a, a \rightarrow a$, and $a \rightarrow e$, can be recognized.

The most probable route for the retaining glycoside hydrolase reaction is through a general acid/base catalysis that involves two crucial carboxylic acid residues at the enzyme's catalytic site. In retaining enzymes the reaction path shown in ❯ *Fig. 5a* involves a double-displacement in which the glycosidic oxygen is protonated by one of the carboxylic acids. The other carboxylic acid acts as a nucleophile, i. e., forms a glycosyl-enzyme and the aglycone departs. A water molecule is then deprotonated and makes a second nucleophilic attack at the anomeric carbon generating a product with the same stereochemistry as the substrate. The dual role of the general acid (acid/base) catalyst in the double displacement reaction involving a covalent glycosyl-enzyme intermediate (❯ *Fig. 5a*) was demonstrated in the *Bacillus circulans* xylanase by the oscillation of its pK value with the individual steps in the reaction pathway [61]. The distance between the two catalytic carboxyls in retaining enzymes is 4.5–5.5 Å (❯ *Fig. 5a*) [62] allowing a close approach of the nucleophile to the anomeric carbon. In the action of retaining enzymes it is proposed that either an oxacarbenium ion-like intermediate or a covalent glycosyl intermediate is involved (❯ *Fig. 5a*). Both states may participate. The covalent intermediate is a carboxylic ester of the catalytic nucleophile in which the glycosyl residue is in an anomeric configuration opposite to that of the substrate. Stable covalent glycosyl intermediates have been isolated by designing substrates with good leaving groups. In inverting enzymes the reaction pathway (❯ *Fig. 5b*) involves a single direct displacement in which the glycosidic oxygen is protonated by one of the pair of carboxylic acids and the aglycone departs concomitantly with the attack by a water molecule activated by the second carboxylic acid residue. In these enzymes the two catalytic acids are separated by 9–9.5 Å (❯ *Fig. 5b*) [62] allowing the water and substrate to bind simultaneously.

There is now substantial direct evidence from crystallographic and mutagenesis studies, and from the use of mechanism-based inhibitors that the two postulated catalytic groups do indeed exist for glycoside hydrolases and glycosyl transferases. The proton donor and catalytic nucleophile are invariably carboxyl groups of aspartyl or glutamyl residues. Further evidence for the proposed reaction schemes has been adduced from kinetic isotope effects [63,64]. The bell-shaped activity-pH profiles for glycoside hydrolases (and glycosyl transferases), with optima generally in the range 4.5–8.0, are consistent with the presence of ionizing groups with pK values below and above the optimum pH. The value of the optimum is determined largely by the nature and environment of the catalytic nucleophile and acid-base catalytic carboxylic acids in the active site [65], but cases with pH optima above and below the normal range have been recorded. Using site-directed mutagenesis, the pK_a values of catalytic residues have been changed with consequential alteration of the pH-activity profile [66,67,68]. However, no correlations were found between altered pH-activity dependence, and the calculated electrostatic fields in *Bacillus licheniformis* α-amylase [67]. Although examples exist of mutated enzymes with altered pH-activity dependence, rational engineering of desired shifts in the pH-activity profiles is considered to have only a limited possibility of being successful.

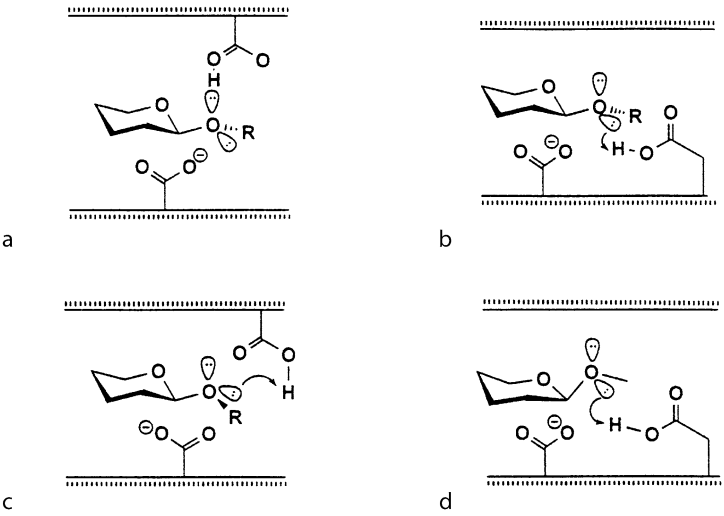


Figure 6

Protonation trajectories. (a) Protonation perpendicular to the plane of the ring, (b) *anti*-protonation, (c) *syn*-protonation, (d) lateral protonation moves the fissile C–O bond into a pseudoaxial position (after [69])

The direction of approach of the proton to the glycosidic oxygen shown in the mechanistic scheme in *Fig. 6a* [69] suggests that its trajectory is from above or below, and perpendicular to the plane of the glycone. It is now clear, however, from studies with inhibitors, analysis of the structures of enzymes in complex with ligands and by modeling, that protonation in many, if not all, glycosidases, does not follow such trajectories [69,70]. In contrast, the direction of protonation is lateral, or side-on, and in the plane of the glycosyl ring (at subsite −1) (*Fig. 6b, c*). Thus, the lateral trajectory of the proton may follow one of two directions with respect to the plane defined by O1, C1, and H1, i. e., it may be *anti* or *syn* to the pyranoside endocyclic O5–C1 bond as shown in *Fig. 6b, c*. The lateral protonation moves the fissile C–O bond into a pseudoaxial position (*Fig. 6d*). Heightman and Vasella [69] state that "It makes sense that the catalytic carboxyl group is located close to the main plane of the pyranoside ring and not above it since the departure of the aglycone in pseudo-axial direction would be restricted if the carboxyl group was located above the plane of the ring" (*Fig. 6a*). Stereoelectronic considerations require that protonation of the glycosidic oxygen atom be accompanied by a lengthening of the fissile C–O bond in a pseudo-axial direction as the incipient oxacarbenium ion develops. This is supported by two high-resolution crystal structures of the cellulases from *Fusarium oxysporum* [71] (Family 7, *syn*-protonation) and *Bacillus agaradherans* (Family 5, *anti*-protonation) [64]. They show that the pyranose ring is distorted to a skew conformation which places the glycosidic bond in a pseudo-axial orientation. A similar observation has been reported for two chitin-degrading enzymes of families 18 and 20 [72]. Similarly, the water molecule which subsequently attacks the glycosyl ester intermediate must have access from the pseudo-axial direction. The recognition of the *anti* or *syn* trajectories of approach of the proton allows families of glycosidases to be further sub-divided [69], emphasizing the relatedness of the members of the sub-divisions in respect to active site structures and mechanisms.

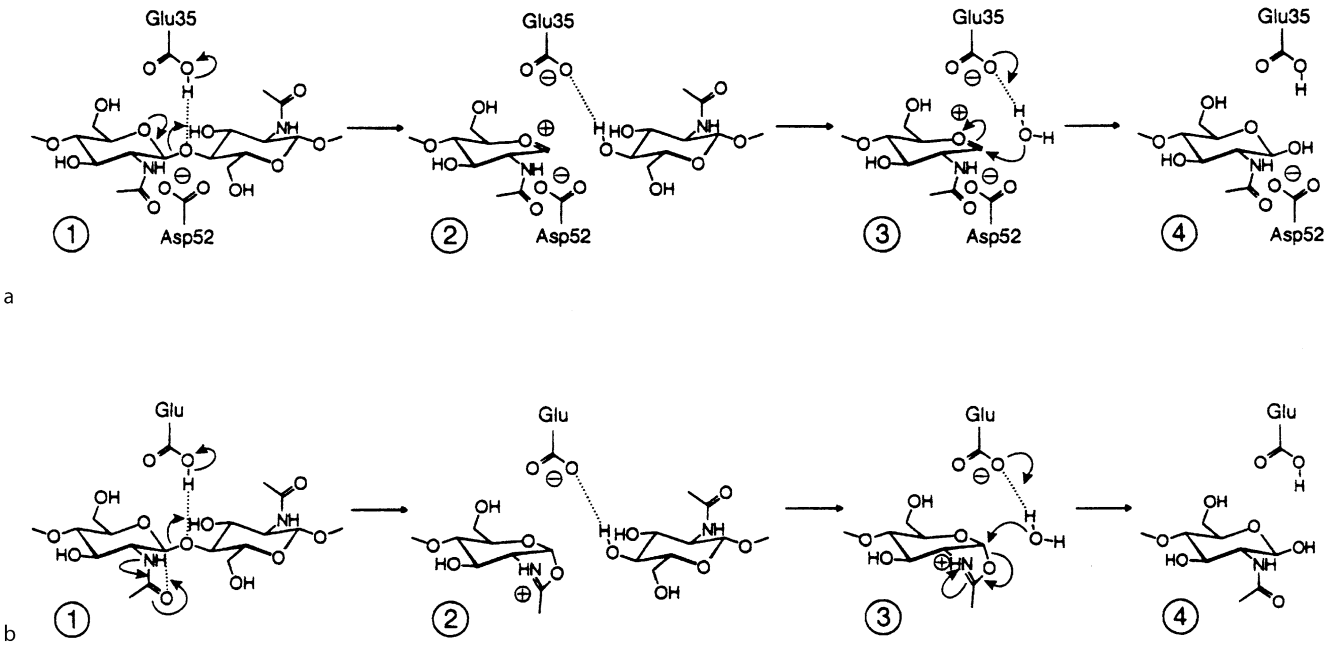


Figure 7

(a) Glycosyl hydrolysis by hen egg white lysozyme following general acid-base catalysis. Glu35 is the acid protonating the glycosidic oxygen and Asp52 is the nucleophile proposed to stabilize the reaction intermediate electrostatically. The two enzyme carboxylates have respective pKa values of 6 and 4. (b) Scheme for retaining chitinases where stabilization is proposed to occur via a covalent oxazolinium ion intermediate. The nucleophile is the *N*-acetyl group of the substrate itself. For stereochemical reasons the scheme is limited to retaining enzymes (from [72])

For a number of retaining polysaccharide hydrolases structural studies have revealed, contrary to expectation, a single obvious candidate for the catalytic acid but no candidate for the catalytic nucleophile near the anomeric carbon. These enzymes include family 18 and 20 chitinases [73], muramidase/transglycosylase [74,75,76], and some lysozymes [77,78]. To explain this it was proposed that in chitinase action the acetamido group of the *N*-acetyl-D-glucosamine monomer in subsite −1 plays the role of the catalytic nucleophile in the attack on the glycosyl intermediate (● *Fig. 7*) [72]. This was supported by X-ray crystallography of the enzyme in complex with allosamidin (see ● *Fig. 17k*), a pseudotetrasaccharide derivative substituted at its reducing end with an oxazoline-like structure.

2.6.2 Lyases

Lyases Acting on Uronic Acid-Containing Polysaccharides These enzymes, classified in [EC 4.2.2.-], are produced by bacteria, bacteriophages, marine gastropods, and a few fungi. Their substrates are all uronic acid-containing polymers with α- or β-(1→4) linkages, such as pectin and pectate (polygalacturonan), alginate, chondroitin, heparin, heparin sulfate, dermatan sulfate, hyaluronan, gellan, the side chain of xanthan and the Vi antigen of enteric bacteria [79]. The lyases so far have been classified into 10 families based on sequence homologies and folding patterns. The bacterial pectate lyases have a pH optimum around 9.5 and require Ca^{2+} for activity. The Ca^{2+} is bound in the active site [80]. The *Erwinia chrysanthemi* pectate lyase C (PelC) has been studied in great detail and generates a trigalacturonide product with a ^{4,5}-unsaturated bond in the galacturonosyl residue at its non-reducing end [81]. The β-elimination reaction (● *Fig. 8*, [82]) in pectolytic cleavage can formally be envisaged as involving three processes: neutralization of the carboxyl group adjacent to the glycosidic linkage cleaved, abstraction of the C5 proton, and transfer of the proton to the glycosidic oxygen. An arginine and an aspartic acid are presumed to play a crucial role in catalysis [15]. The complex of PelC crystallized with a Gal*p*A pentasaccharide shows the substrate fragment bound in a cleft and interacting primarily with positively charged groups: either lysine or

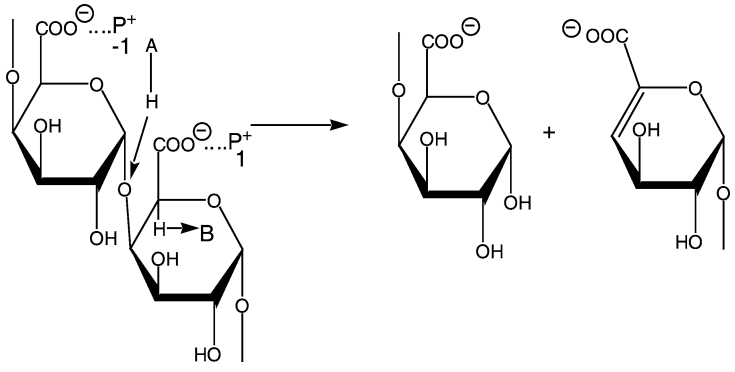


Figure 8
Proposed β-elimination reaction for cleavage of an oligogalacturonide in a pectate lyase (after [82])

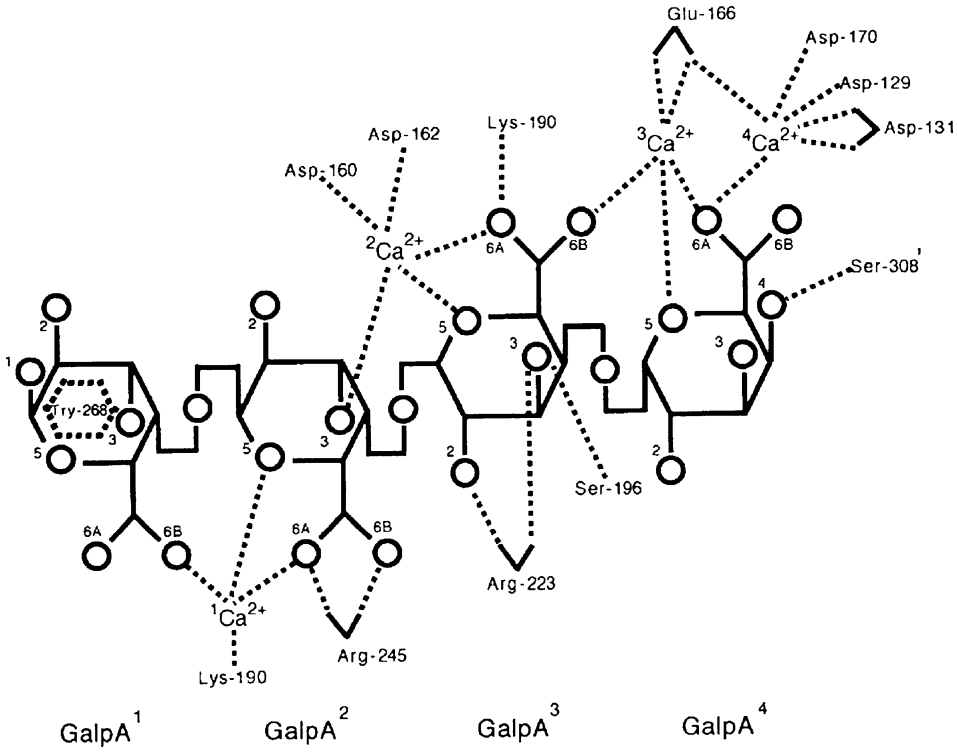


Figure 9

Schematic representation of the Ca^{2+} ion interaction with a tetraGal*p*A in the active site of the mutated (R218K, not shown) pectate lyase (PelC) showing the interactions primarily with positively charged groups: either lysine or arginine residues and four Ca^{2+} ions. GalpA^1 is the reducing saccharide, and GalpA^4 is the non-reducing terminus. The bond cleaved is most likely between Gal*p*A^3 and Gal*p*A^4. Gal*p*A^3 has the most interactions with the enzyme surface. The interactions are shown with *dotted lines*. Oxygen atoms are represented by *circles*, with the corresponding number, and the carbon atoms are assumed at the intersection of the bonds shown as *boldface lines*. Water molecules interacting with tetraGal are not shown (from [83])

arginine on the enzyme and the four Ca^{2+} ions found in the complex. The interaction of PelC with tetraGal*p*A as deduced from the crystal structure is shown in ❯ *Fig. 9* [83].

The protein-oligosaccharide interactions shown in ❯ *Fig. 9* provide a functional explanation for the occurrence of many of the invariant or conserved amino acids in this family of proteins [84]. An arginine residue (Arg218) is proposed to act as a general base in the catalysis. This is suggested by the proximity to the C5 of a Gal*p*A residue, its lower than normal pK_a, 9.5, compared with the calculated pK_a of 12.0–12.5 for all other arginines, and the impairment of activity when the residue is mutated to a lysine [15]. Members of the polysaccharide lyase superfamily (clan) are suggested to have the same reaction mechanism.

Lyases Acting on Neutral Polysaccharides Only one group of enzymes of this type is known, these are the amylolyases or (1→4)-α-glucan (starch) lyases [EC 4.2.2.13] that specifically

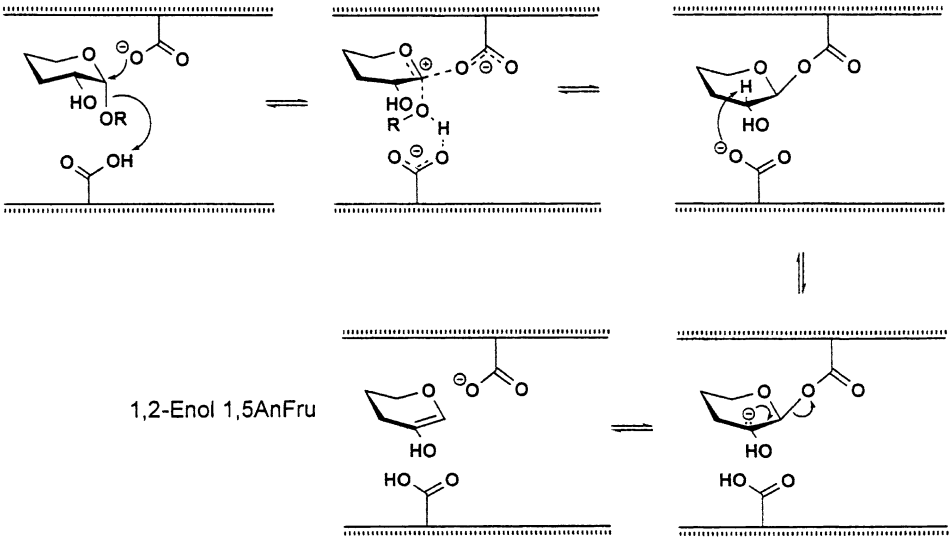


Figure 10
Proposed reaction mechanism of a (1→4)-⟨α-glucan lyase (amylolyase). The non-reducing glucosyl residue of the (1→4)-⟨α-glucan substrate chain is shown, the remainder of the chain is represented by R (after [51]). It has been suggested, however, that the catalytic nucleophile shifts position and becomes protonated by the H-C(2) in an E_{21} or E_{24} elimination (A. Vasella, personal communication)

cleave (1→4)-α-glucosidic linkages in starch or glycogen in an *exo*-action, converting the glucose residues at the non-reducing ends of the substrate to 1,5-AnFru (anhydrofructose), and ultimately liberating the reducing end residue of the substrate as free glucose [51] (● *Fig. 10*). The amylolyases are formally related to the glycosidases that catalyze the formation of 1,2-glycals from 2-deoxy-hexopyranosides detected by the reverse reaction in which a 1,2-glycal is hydrated (termed 'glycal hydrolysis') [85,86]. Amylolyases have been found in the cytosol of some fungi and in the stroma of chloroplasts of red algae, where they may be involved in the depolymerization of algal (Floridean) starch. Sequence alignment of the amylolyases has revealed that they are related to glycoside hydrolase Family 31 that includes α-glucosidases and sucrase-isomaltases. Seven well-conserved segments comprise functional residues including the catalytic groups identified in members of Family 31 [51]. This is the first case of a lyase and a hydrolase belonging to the same family and as such it may illuminate facets of the catalytic reaction mechanisms of both. Apart from sharing substrates with the amylases, the transition state analogue inhibitors, such as, 1-deoxynojirimycin (see ● *Fig. 17b*), and acarbose (see ● *Fig. 17g*), are effective with both types of enzyme. However, other common glycoside hydrolase inhibitors like glucono-1,5-lactone and the cyclodextrins, and the affinity labeling reagents, conduritol-B epoxide (● *Fig. 11a*) and 2′,3′-epoxypropyl-α-D-glucoside, do not inhibit the amylolyases. The mechanism of action [51,87] is likely to resemble that of α-glucosidases to a certain extent (● *Fig. 10*).

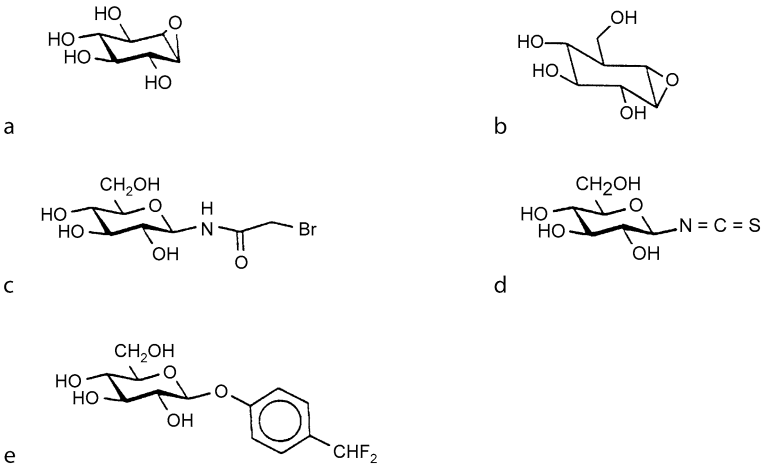


Figure 11
Structures of affinity- and mechanism-based inhibitors of glycoside hydrolases. (a) Conduritol B epoxide, (b) cyclophellitol, (c) *N*-bromoacetyl-glucosylamine, (d) glucosyl isothiocyanate, (e) *p*-difluoromethylaryl β-glucoside (mechanism-based)

2.6.3 Phosphorolases

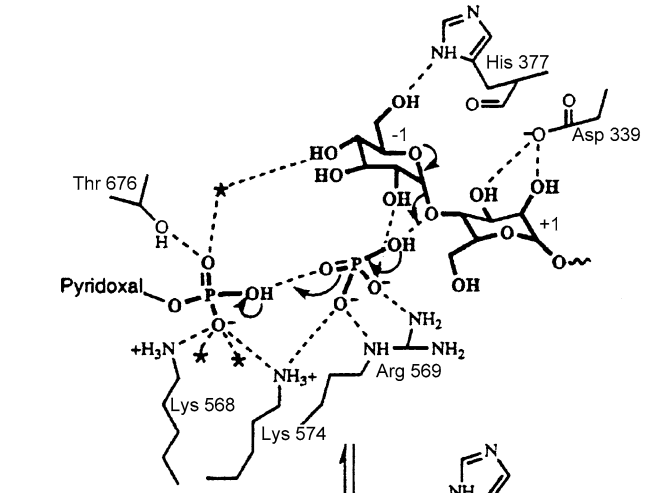
Phosphorolysis of glycosidic linkages in α- and β-glucans (*Structure 1*) is an alternative to depolymerization by hydrolysis and is used in mobilizing cellular storage polysaccharides. The glucose-1-phosphate product is a direct intermediate in hexose metabolism. Thus, the phosphorylases are found intracellularly together with their substrates: glycogen in vertebrates, invertebrates, and bacteria; starch polymers in higher plants; and (1→3)-β-glucans (paramylon) in euglenoid protozoa. Bacteria that are able to transport maltodextrins across their cell membranes depolymerize them with a maltodextrin phosphorylase and comparable eukaryotic, bacterial, and archaean phosphorylases for laminaribiose [88], cellobiose, and cellodextrins [89] have been described. The (1→4)-α-glucan phosphorylases from mammalian, plant, and bacterial sources are *exo*-enzymes catalyzing the reversible phosphorolysis of glucosidic linkages at the non-reducing ends of glucosyl chains to produce αGlc-1-phosphate, i. e., with retention of configuration. In the reverse direction, the (1→4)-α-oligoglucoside or glucan acceptor is lengthened by the transfer of the glucosyl moiety from α-glucose 1-phosphate, e. g., to its non-reducing end with the concomitant production of inorganic phosphate. The equilibrium constant for the reaction expressed as the ratio of [Pi]/[Glc-1-P] is 3.6 at pH 6.8. All phosphorylases are dependent for activity on the presence of metal ions, typically Mg^{2+} or Mn^{2+}, and have an absolute requirement for pyridoxal 5′ phosphate as a co-factor [90]. The (1→4)-α-glucan phosphorylases are classified in glycosyl transferase Family 36 and probably have similar kinetic mechanisms.

Phosphorylases from animal, bacterial, and plant sources can be distinguished by their substrate preferences. Animal and bacterial phosphorylases prefer the short outer branches of highly branched α-glucans, such as glycogen. The phosphorylase from *Corynebacterium calluna*, which accumulates a starch-like polysaccharide, is exceptional among bacterial enzymes

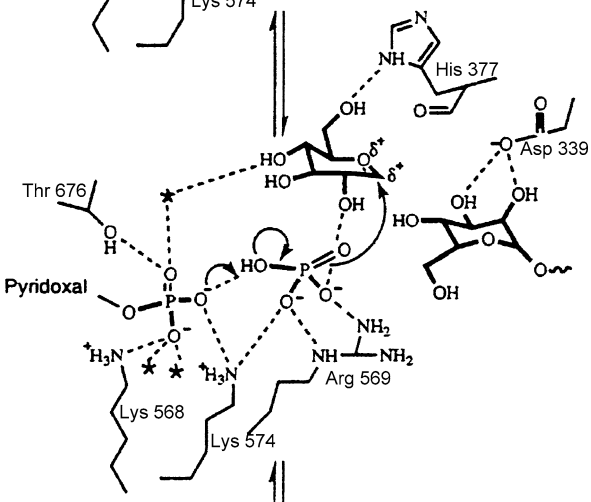
in its preference for long chain substrates [91]. *E. coli* possesses, in addition to a genuine glycogen phosphorylase, a maltodextrin phosphorylase [92] which has a high affinity for linear oligosaccharides, and less than 1% activity against glycogen. There are two isoforms of the plant phosphorylase; Pho1, from the cytosol, with a high affinity for amylopectin and glycogen, and Pho2, from plastids, that prefers amylose and maltodextrins. The three-dimensional structures of the mammalian [93,94] and yeast [95,96,97] enzymes reveals the active site in a tunnel with the Glc-1-P situated in the −1 subsite, at its end, far removed from the bulk solvent and adjacent to the essential pyridoxal cofactor [98,99]. The mammalian phosphorylases have not been crystallized in complex with their oligosaccharide substrates. However, the *E. coli* maltodextrin phosphorylase has 98% sequence identity with the mammalian enzyme in the region of the catalytic site and its structure has been determined in complex with bound maltopentaose [100] and the pseudo-oligosaccharide, acarbose (see ❯ *Fig. 17g*) [53,92]. The catalytic site is situated in the center of the large subunit and is accessible through a 20-Å-long channel that forms the oligosaccharide binding site. The non-reducing terminal maltosyl portion of the bound maltopentaose and the acarviosine portion of acarbose occupy subsites +1 and +2 and the reducing terminal maltosyl of acarbose, subsites +3 and +4.

In the *E. coli* maltodextrin phosphorylase, structural analysis of the binary complex with maltopentaose, 4-*S*-α-D-glucopyranosyl-4-maltotetraose (G*S*G4) and the ternary complex with GSG4 and phosphate show the pentasaccharide binding along the catalytic site and occupying subsites −1 to +4 with the phosphate group poised to attack the glycosidic bond and promote phosphorolysis [53]. In all three complexes, the pentasaccharide exhibits an altered conformation across subsites −1 and +1 (❯ *Fig. 12*), from the preferred conformation of (1→4)-α-linked glucosyl polymers. The oligosaccharides can find their way into the catalytic site tunnel only by alteration of the glycosidic torsion angles. Similar observations have been made with porcine pancreatic and barley α-amylase [36,101] and soybean β-amylase [55]. In starch phosphorylase [Pho2(L)] a 78 residue insertion is located beside the mouth of the active-site cleft and presumably sterically hinders the approach of the large highly branched glucan to the active site [102,103].

Muscle glycogen phosphorylase is composed of two identical ∼90 kDa sub-units. The catalytic capability of the enzyme is regulated by phosphorylation/dephosphorylation of a specific serine residue by a glycogen phosphorylase kinase/phosphatase and, non-covalently, by allosteric effectors such as AMP. The enzyme can exist in two states, an unphosphorylated, inactive form and a phosphorylated, active form. The covalent attachment of a phosphate group at serine 14 elicits conformational changes at remote sites mediated through subunit-subunit interactions. These changes lead to opening of a gate, created by a loop that closes the catalytic site in the inactive form, and by rearrangement of certain amino acids, creates a high affinity substrate-binding site. The unphosphorylated form can be activated by binding AMP at a site close to serine 14 which causes conformational changes leading to correct orientation of amino acids involved in subunit-subunit interactions [104,105,106]. By contrast, in the *E. coli* maltodextrin phosphorylase, the relative orientation of the two subunits is quite different and there are significant changes in subunit-subunit interactions. Sequence changes result in loss of the allosteric control sites resulting in the loop, which acts as a gate to control access to the catalytic site in the muscle phosphorylase, being held in the open conformation. In addition to binding (1→4)-α-glucan substrates in the active site, muscle phosphorylase has a non-catalytic, (1→4)-α-glucan-binding site, or 'glycogen storage site,' through which the enzyme may


His 377
Asp 339
-1
+1
Thr 676
Pyridoxal
Arg 569
Lys 568
Lys 574

a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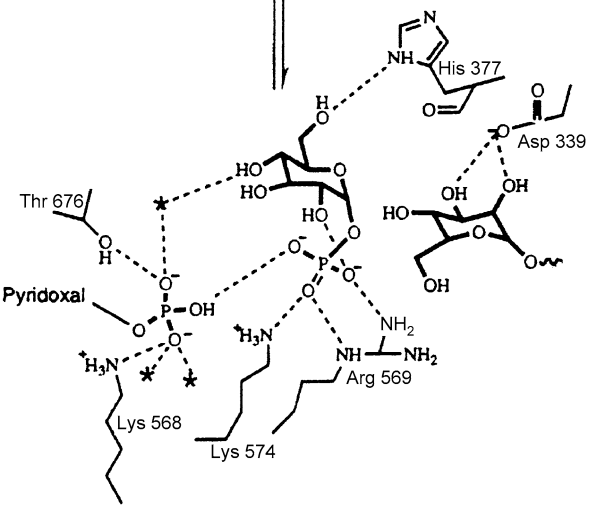

His 377
Asp 339
Thr 676
Pyridoxal
Arg 569
Lys 568
Lys 574

c

Figure 12
Proposed catalytic mechanism for a phosphorylase based on the maltose phosphorylase GSG4-P ternary enzyme-substrate complex. (a) The oligosaccharide substrate is bound across the catalytic site in subsites −1 and +1. The phosphate substrate is within hydrogen-bonding distance of the glycosidic oxygen and is stabilized in this position by contacts to Arg569 and Lys574. The phosphate substrate is within hydrogen-bonding distance of the pyridoxal phosphate 5′-phosphate group whose position is stabilized by contacts to water (shown as *asterisks*) and polar amino acid residues. The co-factor 5′-phosphate acts as a general acid to promote attack of inorganic phosphate on the glycosidic oxygen. The oxygen is protonated leading to the cleavage of the C1–O bond and formation of the oxonium-carbonium ion intermediate. (b) The carbonium ion is promoted and stabilized by the inorganic phosphate dianion, the carbonyl oxygen of His377 and the lone pair of electrons on the 3′-OH of the saccharide in subsite +1. The 2′-OH group of the saccharide at subsite −1 is polarized by contact with the inorganic phosphate. (c) The cleaved oligosaccharide is free to diffuse away. Nucleophilic attack by the inorganic phosphate at the C1 position of the carbonium ion leads to the formation of ⟨-glucose 1-phosphate (from [52])

be attached to glycogen particles in vivo [107]. The site is on the enzyme surface some 30 Å from the catalytic site. The affinity for oligosaccharides at this site is higher than for the active site itself [108]. Such a 'storage' site is also present on plant phosphorylases but is lacking in the bacterial enzymes.

Mechanistic studies on phosphorylases suggest a double displacement mechanism is operating. The reaction proceeds through a rapid equilibrium bi-bi kinetic mechanism. There is random addition of the α-glucan and inorganic phosphate substrates, but the binding of one substrate increases the enzyme's affinity for the other substrate. Phosphorylases are able to catalyze the arsenolysis of Glc-1-P and the exchange between Glc-1-P and Pi, but only in the presence of an acceptor [109,110]. The rate-limiting step in the reaction is the interconversion of the ternary phosphorylase-phosphate-glycosyl complex to the ternary phosphorylase-Glc-1-P-glycosyl-complex [111]. By analogy with other retaining enzymes it may be suggested that a glycosyl-enzyme intermediate is involved, but such an intermediate has not been isolated. Positional isotope exchange results with the potato enzyme are consistent with such an intermediate. At present the identity of the presumptive nucleophile is unknown as there is some uncertainty due to the conformational flexibility at the catalytic site, and other mechanisms have been suggested [90]. The role of the essential pyridoxal phosphate bound at Lys 680 by a Schiff base close to the substrate phosphate in glycogen phosphorylase has not been resolved [4,99,112,113,114]. However, studies with the closely related *E. coli* maltodextrin phosphorylase [53] (*Fig. 12*), show that no acidic group participates directly in catalysis, but the 5′-phosphate of the pyridoxal cofactor promotes general acid attack by the inorganic phosphate, resulting in protonation of the glycosidic oxygen of the bond to be cleaved.

Substrate analogue inhibitors of phosphorylase include the ground state analogues, glucose; glucose-1-methylenephosphate and glucose-1,2-cyclic phosphate [107,114,115,116,117]; the dead-end product, heptulose 2-phosphate [99,118] and glucose derivatives with an sp^2 hybridized anomeric center; heptenitol [118,119]; D-gluconohydroximo-1,5-lactone *N*-phenylurethane [120,121]; acarbose [122]; and isofagomine [123,124,125]; nojirimycin tetrazole [113]; glucose spirohydantoins [126,127,128]; alkyl-dihydropyridine-dicarboxylic acid [129] and *N*-acetyl-β-D glucopyranosylamine [130]. 4-Deoxymaltohexaose/pentaose are good competitive inhibitors of phosphorylase with K_m values some 40-fold and 10-fold lower, respectively, than the underivatized oligosaccharides [52]. The increased affinity may relate to a stabilizing effect of the hydroxyl group on the ground state.

2.7 Hydrolase Inhibitors

2.7.1 Reagents Reacting with Amino Acid Residues Required for Activity or Binding

Inhibition or abolition of enzyme activity by group specific reagents that modify amino acid residues with a function in catalysis, or in binding the substrate can provide a preliminary indication of the identity of these important residues. Thus, various carbodiimides and Woodward's reagent K inhibit by reacting with carboxyl groups, other examples include *N*-bromosuccinimide reacting with tryptophans, tetranitromethane reacting with tyrosines, diethyl pyrocarbonate reacting with histidines, a variety of acylating reagents that modify lysines, alkylating reagents that modify thiol groups, and phenylglyoxal that modifies arginines [25,131]. The interpretation of the results needs to be made cautiously since many of the reagents are not absolutely specific and without corroborating evidence it is not possible to assign a catalytic or binding role to the amino acids. Differential labeling with unlabeled reagent in the presence of a substrate or substrate analogue, dissociation of the ligand and reaction with labeled reagent can provide better evidence for the role of specific amino acids in catalytic activity. Thus, acarbose (see *Fig. 17g*), a pseudo-tetrasaccharide substrate analogue and inhibitor of glucoamylase, protected the essential Trp120 from reaction with *N*-bromosuccinimide [132]. Although such reagents can be useful in exploring structure/function relationships, particularly when coupled with sequencing and differential labeling, most often site-directed mutagenesis is preferred in structure/function investigations [16,17]. Formation of hybrid or chimeric enzymes is another powerful tool in structure/function and structure/stability investigations and for protein engineering [133,134,135].

2.7.2 Irreversible Active-Site-Directed Inhibitors

Affinity Labeling These reagents [1,136,137] combine a glycosyl substrate moiety that targets the compound to the catalytic site and a functional group capable of reacting to form a stable, covalent linkage with a group in the active site, sometimes a catalytic amino acid, leading to inactivation of the enzyme. One of the first of this class of inhibitors was the 2,3-epoxypropyl-β-chitobiose used to inactivate lysozyme [138,139,140,141]. These substrate analogues require an acidic group for the protonation of the oxirane ring and a nucleophile for the formation of the covalent bond. The reaction is shown in *Fig. 13*.

Reduction of the glycosyl ester so formed converts the carboxylic acid-bearing residue to the corresponding alcohol and can be used to identify the catalytic amino acid at the site of reaction [139,140]. A panel of these reagents [142] has been used to probe the active sites of a series of β-glucan hydrolases [143] and to 'specifically' label and identify their catalytic nucleophiles [144,145,146,147,148,149,150]. The 2,3-epoxypropyl-α-glucoside reacts with a carboxylic acid in β-amylase [151]. The length of the epoxyalkyl chain influences the effectiveness of the inhibition, which depends on whether the oxirane ring is within reach of the catalytic group [145]. Because the alkyl chains show flexibility, active-site carboxyl groups in the vicinity of the catalytic nucleophile may be labeled, and it is necessary to verify the identity of the labeled group by independent methods such as X-ray crystallography of the enzyme complexed with the inhibitor. This has been achieved for hen egg white lysozyme [152] and

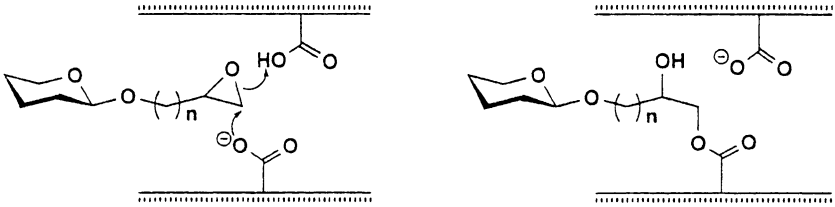


Figure 13
Reaction pathway for epoxyalkyl β-glycoside substrate analogue with a β-glucan hydrolase involving protonation of the oxirane ring by an acid group and the formation of a covalent bond with a nucleophile at the active site

the hybrid *B. macerans/B. amyloliquefaciens* (1→3; 1→4)-*endo*-β-glucanase [150] or with a bona fide mechanism-based inhibitor.

Significant differences in the stereospecificity of the interaction of the epoxyalkyl group between the (1→3; 1→4)-β-glucan hydrolases from *Hordeum vulgare* (Family 17) and *Bacillus subtilis* (Family 6), with indistinguishable substrate specificities, were shown by comparing the series of (*RS*)-epoxalkyl-β-cellobiosides with the pure (3*R*)- and (3*S*)-diastereoisomers [153]. The 3*S*-isomer was most effective for the barley enzyme whereas the 3*R*-isomer was almost without effect. The opposite was true of the *B. subtilis* enzyme. The structure of the *Fusarium oxysporium* (1→4)-β-glucan endohydrolase [154] affinity-labeled with a mixture of *R*- and *S*-forms of epoxybutyl-β-cellobioside shows the butyl moiety lying over the −1 subsite. Both isomers appear to be present, but modeling studies suggest that the electron density is much stronger for the *R*-isomer. Examples of other reagents with functional groups that have been used in affinity labeling of glycoside hydrolases are the oxiranes, conduritol B epoxide (*Fig. 11a*), and the naturally occurring cyclophellitol (*Fig. 11b*) [155], and various glycosyl epimines (aziridines) [156,157], the *N*-bromoacetyl-glycosylamines (*Fig. 11c*) [158], the glucosyl isothiocyanates (*Fig. 11d*) [159], and diazoketones [160]. As is the case with the epoxyalkyl affinity reagents the labeled amino acid residue may not necessarily be the catalytic nucleophile.

Bromoketone *C*-cellobioside and *N*-bromoacetyl-β-D-cellobiosylamine [161] differentially inactivated *Cellulomonas fimi* β-glucanases. The bromoketone derivatives inactivated the (1→4)-β-glucan endohydrolases, CenD and CenA, with stoichiometric labeling of the enzymes, but not the (1→4)-β-glucan exohydrolase Cex, whereas the *N*-bromoacetyl derivatives inactivated CenA and Cex, but not CenD. This is attributed to the different modes of hydrogen bonding of the amide moiety. The amino acid residues labeled were not identified. However, stoichiometric labeling of the putative acid/base, Glu276, in *Saccharomyces cerevisiae* α-glucosidase was obtained using the 1′-bromo-3′-(α-D-mannopyranosyl)-2′-propanone [162]. This Glu is completely conserved in other Family 13 α-glucosidases and α-amylases whose three-dimensional structures have implicated this residue as the acid/base catalyst, i. e., the proton donor. Thus, the bromoketone derivatives are good candidate reagents for labeling the general acid/base catalyst.

Mechanism-Based Inhibitors Another class of active-site-directed inhibitors are glycoside derivatives that require mechanism-based activation in order to react covalently with

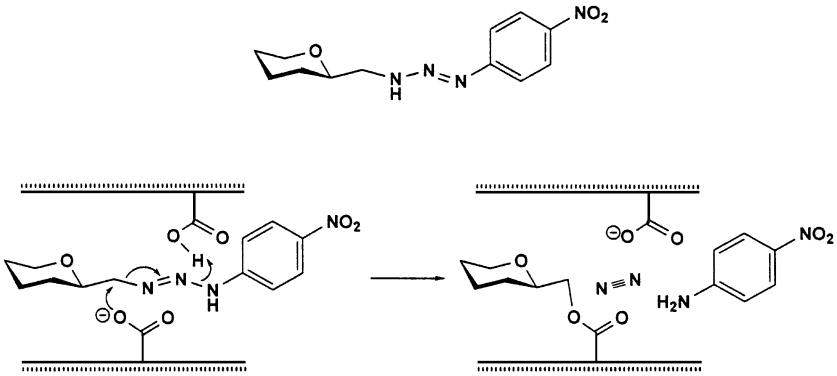


Figure 14
Reaction pathway in glycosyl methyltriazene activation [163]

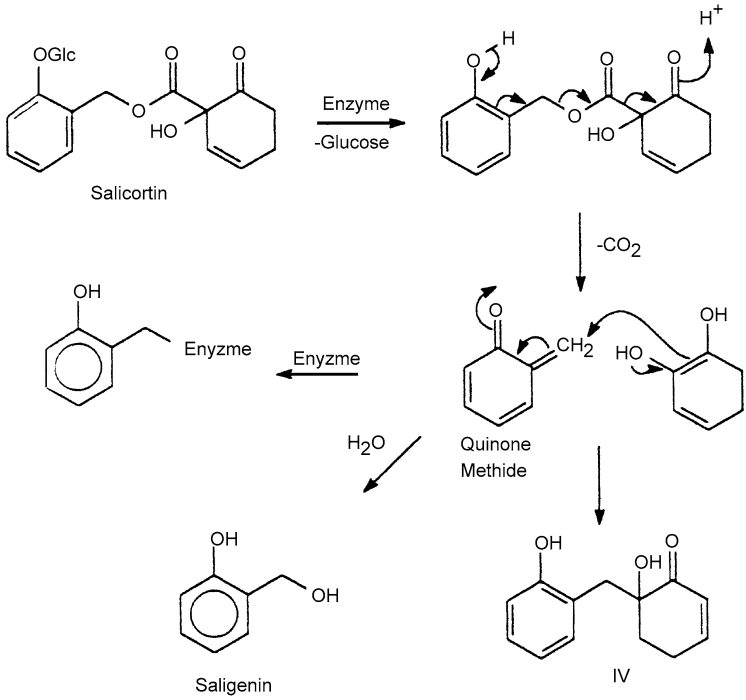


Figure 15
Reaction pathway for salicortin inhibition (after [165])

the enzyme. One class of such nucleophile-specific reagents are the glycosylmethyl-triazenes [163] that require protonation after binding, and decompose to yield a very reactive glycosylmethyl-carbenium ion (*Fig. 14*).

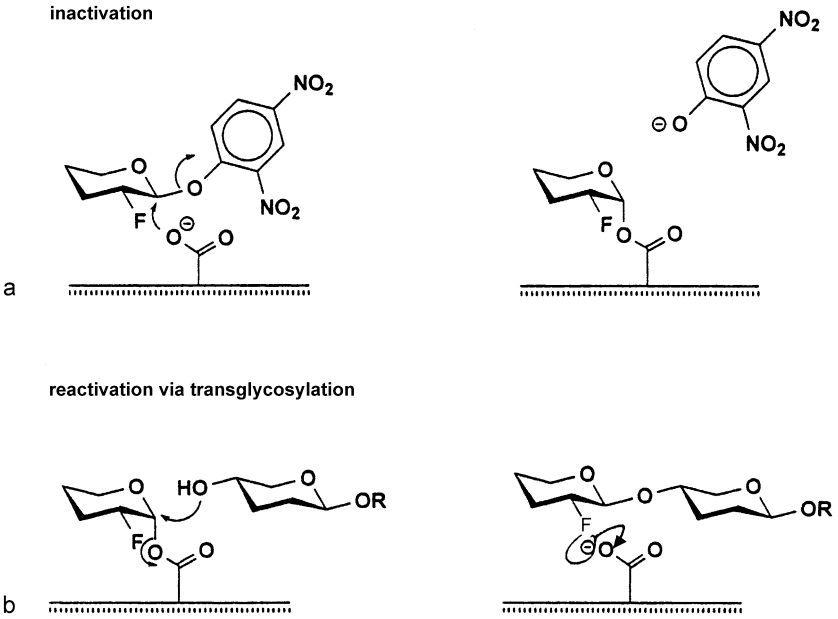


Figure 16
Reaction pathway and mode of activation of a β-glucosidase by 2,4-dinitrophenol 2 deoxy-2-fluoro-D-glycoside (after [137,167])

A second class of mechanism-based inhibitors are those that in the course of their cleavage by the enzyme release an aglycone that rearranges to give a reactive species. Two examples may be cited. Salicortin [164], a natural product, is hydrolyzed by β-glucosidases to produce, by rearrangement, a reactive quinone methide as shown in ❯ *Fig. 15* [165].
The *o*- and *p*-difluoromethylaryl β-glucosides (❯ *Fig. 11e*) [166] are further examples of this class of mechanism-based inhibitors. They are cleaved by β-glucosidases to yield a fluorohydrin that decomposes to hydrogen fluoride and an acyl fluoride that reacts at the active site. A third class of mechanism-based inhibitors are the 2-deoxy-2-fluoro-D-glucosides with good leaving groups, e. g., 2,4-dinitrophenol [167] and the 5-fluoro-D-glucosyl fluoride [168,169]. These reagents form reactive species only after cleavage of the glucosyl moiety and hence are highly specific inhibitors of β-glucosidases and have been used successfully to label a number of $e \rightarrow e$ enzymes. In the reaction (❯ *Fig. 16*) "the covalent intermediate formed is stabilized against hydrolysis, on the one hand, by the inductive destabilization of the positively charged oxacarbenium ion-like transition states by the electronegative fluorine and, on the other, by loss of crucial transition state hydrogen-bonding interactions" [4]. When a good leaving group is incorporated, the first step in the reaction proceeds at a reasonable rate. In some $e \rightarrow e$ enzymes the turnover rate of the intermediate is measurable, but the intermediate is stable enough to allow identification of the nucleophile. The identity of the amino acid residue labeled by affinity- or mechanism-based inhibitors has been determined using sensitive, mass spectrometric methods without the need for radiolabeled reagents [137,170,171].

Many of the affinity- and mechanism-based inhibitors were designed for use with glycoside hydrolases, however, they have potential applications with glycan hydrolases. Their effectiveness may be improved by extending the glycosyl portion to provide tighter binding in the active site. This was demonstrated to be the case in a family of epoxyalkyl β-glucosides and oligoglucosides of various chain lengths [170]. Those with longer oligosaccharide chains were more reactive and the inhibition more specific (see [1,5,137] for a discussion of the operation of these mechanism-based inhibitors).

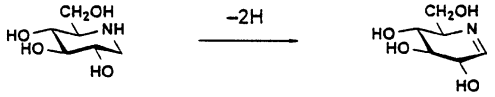
2.7.3 Transition State Inhibitors

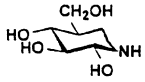
Transition-state analogues represent a class of inhibitors distinct from the active site-directed inhibitors. They are reversibly bound in the active site, and compete with the substrate by mimicking the shape and/or the charge of the transition state. A large number of transition-state inhibitors is available for glycosidases, some of which were developed for therapeutic or other applications, some are effective with polysaccharide depolymerizing enzymes. Transition-state inhibitors are useful tools for elucidating important residues in the enzyme-substrate interaction by protein crystallography. For glycoside hydrolases, an important transition state is believed to be a planar glycosyl-oxacarbenium ion [4,69]. Examples of transition state-analogue inhibitors are aldonolactones such as D-glucono-1,5-lactone (*Fig. 17a*) [1], basic sugar analogues such as the glycosylamines [1], glycosylimidazoles [1], cyclic aminoalditols, such as 1-deoxynojirimycin, (*Fig. 17b*) and isofagomine (*Fig. 17c*) [123,124]; glycotetrazoles, e. g., glucotetrazole (*Fig. 17d*) [69,172,173], pseudosubstrates such as glycals, e. g., D-glucal (*Fig. 17e*) [1], and the aminocyclitols, e. g., valienamine [1], carbacyclic pseudosaccharides incorporating valienamine or an analogue into an oligosaccharide chain, e. g., methylacarviosin (*Fig. 17f*), acarbose (*Fig. 17g*) [35,36,45,101,174,175,176], adiposin (D-*gluco*-dihydroacarbose) (*Fig. 17h*), methyl β-acarviosin (*Fig. 17i*), and methyl β-acarboside (*Fig. 17j*) [177,178,179] and the oxazoline-like pseudotrisaccharide, allosamidine (*Fig. 17k*) [72,74].

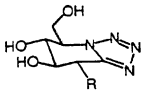
From a structural point of view some of these compounds are not perfect transition state analogues, but have closely related structures to, and behave kinetically as, transition state inhibitors [180]. One way to test the validity of a compound as a transition state analogue is by determining the relationship between K_i and k_{cat}/K_m for a series of mutated enzymes such as, e. g., for the acarbose-glucoamylase system [181] where these parameters gave a linear relationship in a double logarithmic plot. The pre-steady state kinetic analysis [177] indicated, however, that the interaction with acarbose elicited a unique conformational change in the enzyme as the final step in its binding mechanism [21]. Thus, thorough analyses of specific inhibitor-enzyme pairs may reveal aspects that are not evident from conventional kinetics. For Family 18 chitinases that follow a substrate-assisted catalytic mechanism involving a neighboring substrate C2′ acetamido group stabilization, molecular mechanical calculations have shown that oxazolines are potential inhibitors [73]. This is supported by the structure of the complex with the natural oxazoline, allosamidin (*Fig. 17k*) [74].

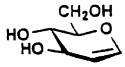
Figure 17
Structures of some transition-state inhibitors of glycosyl and glycan hydrolases

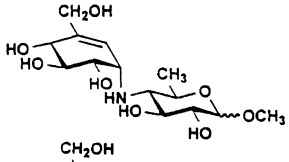
a 

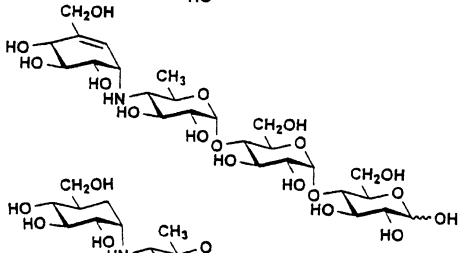
b 

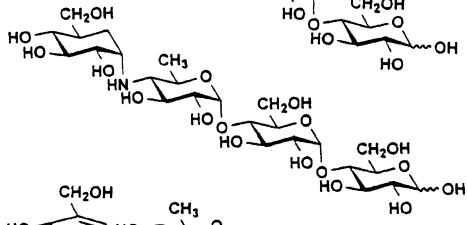
c 

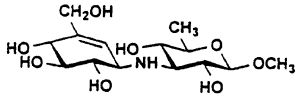
d 

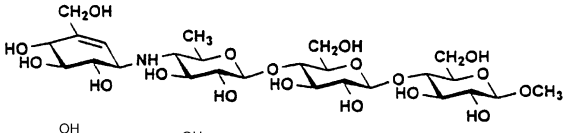
e 

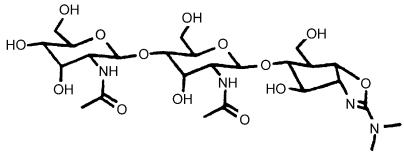
f 

g 

h 

i 

j 

k 

Recently, a series of tetrazoles, including *gluco*tetrazole (◗ *Fig. 17d*), was designed as neutral, stable lactone analogues and correlations were found between k_{cat}/K_m of various glycosidase-substrate pairs [172]. The tetrazole transition-state inhibitors have been used to classify hydrolases according to the *syn* or *anti* in-plane-protonation of the oxygen of the glycosidic bond cleaved [69,173].

2.7.4 Proteinaceous Inhibitors

A variety of polysaccharide hydrolases is inhibited by endogenous or exogenous proteins. These include α-amylases and limit dextrinase [182,183,182,185,186,187,188,189], fungal xylanases [190,191] and polygalacturonases [192]. Most of these inhibitors are from plants [183,185,186,187,192] but some are of microbial origin [182]. Structures of the enzyme-inhibitor complexes are available in some cases, in others only the structure of the free inhibitor is known. Five distinctly different α-amylase-inhibitor complexes have been reported including two for the pancreatic enzyme, one for the barley and two for insect α-amylases. Structural mimicry of the substrate has been observed in the bean (*Phaseolus aureus*) inhibitor-pancreatic α-amylase complex and in the complex of the small amaranth (*Amaranthus hypochondriacus*) inhibitor of the knottin family and the yellow mealworm (*Tenebrio mollitor*) α-amylase [183,187]. For the barley enzyme-inhibitor complex, contacts between the side chains and the carboxylic acid residues at the catalytic site of the enzyme involve a hydrogen bond network mediated through structural water molecules and a fully hydrated calcium ion at the protein-protein interface [186]. The role of the individual amino acid side chains in complex stabilization has been investigated by mutational analysis [135].

2.8 Structural Organization of Glycan Hydrolases

Many microbial polysaccharide hydrolases are secreted in a soluble form into the extracellular environment where their target substrates are found, whereas some bacterial cellulases are components of high molecular mass ($2.0–2.5 \times 10^6$ Da) cellulosome complexes that are anchored to the outer cell surface [193]. The α-amylases found in mammalian saliva and intestinal tract are soluble components of parotid gland and pancreatic secretions, respectively. The aleurone cells of germinating cereal seeds secrete a range of soluble glycanases concerned with mobilization of endosperm cell wall polysaccharides and starch. Some plant cellulases of unknown function are membrane-anchored [194]. In mammalian tissues polysaccharide hydrolases (e. g., lysozyme) may be compartmentalized in membrane-bound organelles (lysosomes), where they are concerned with turnover of cellular constituents and degradation of foreign material. Delivery to, and maintenance of the enzymes in, these various cell, tissue, and extracellular locations, involve specific structural features beyond those participating in substrate-binding and catalysis. Structural analysis of polysaccharide hydrolases (and glycosyl transferases) has shown that most are globular proteins carrying a catalytic domain. Additional non-catalytic domains involved, for example, in substrate binding, association with structural proteins, with membranes or with other catalytic monomers are known. In many instances the presence of a non-catalytic domain has been recognized but the function has not been identified.

2.8.1 Catalytic Domains

The various folding patterns of the polypeptide chain constituting the catalytic domains of glycoside hydrolase families have been reviewed [4]. At the time of writing three-dimensional structures are available for members of over 31 hydrolase families and one unclassified hydrolase and three families of glycosyl transferases and one unclassified (Bernard Henrissat, personal communication).

Topology of Active Sites Most often the active site is confined to one domain but there are examples where the active site is contributed by amino acid residues from two different domains [195,196]. The topology of the active sites of glycan hydrolases may be described in one of three general shapes: a pocket, a cleft or groove, or a tunnel, irrespective of whether the enzyme is inverting or retaining. Examples of the three types are shown in ❯ *Fig. 18*.
In some enzymes of the first group, the pocket may be quite shallow, but in others the pocket is quite deep [45,196,197]. The cleft or groove topology is shown by many polysaccharide hydrolases [46,101,174,176,198,199,201]. The length of the cleft will define the number of glycosyl binding sub-sites and hence the length of the polysaccharide chain that can be accommodated. In cellobiohydrolase II (Cel6A) from *Humicola insolens* [173] and cellulase Cel6A from *Trichoderma reesei* [202] the groove-type topology has been modified to form a tunnel by covering the open cleft with a polypeptide loop [203]. A tunnel-shaped active site would appear to be conducive to multiple attack. It is envisaged that the substrate is threaded through the tunnel, and after a productive binding and cleavage, one product is released and the other remains bound and processes in the active site to give a new productive association allowing repetitive (multiple) attack. How the enzyme-substrate complex is formed is not understood, but may involve opening the 'lid' over the cleft to allow binding. In *T. reesei* Cel 6A, Trp272 at the entrance of the tunnel is critical for the degradation of crystalline cellulose and has been proposed to have a role in threading the glucan chain into the tunnel [204]. In bacterial cyclodextrinase (Family 13) the shape of the active site pocket is altered by homodimer formation in which the *N*-terminal domain of one subunit covers the entrance to the catalytic site of the other, thus causing a change in substrate specificity [205,206].

Substrate Recognition at the Active Site The specificity of glycan hydrolases is determined by the ability of monosaccharide residues in the polysaccharide substrate to be recognized by, and be bound to, contact amino acid residues in the binding site. Generally, the binding of monosaccharide residues is promoted by the presence of planar hydrophobic groups on the enzyme surface, contributed by tyrosine, phenylalanine, or tryptophan side chains, which form a platform on which the hydrophobic face of the glycosyl ring sits. This appears to be a general strategy for protein-monosaccharide ring associations as was first shown for the bacterial arabinose transporting protein [19,207]. However, specificity is determined by substituents on the ring that make hydrogen bonds, either directly or through water molecules and polar interactions, with an array of specific amino acids lining the active site, as illustrated in ❯ *Fig. 19* [208].
There is absolute specificity with respect to the anomeric configuration of the monosaccharide residues in the substrate and the regioselectivity for linkage position is usually absolute for the glycone, but the 'aglycone' binding interactions may be much less stringent. Quite subtle dif-

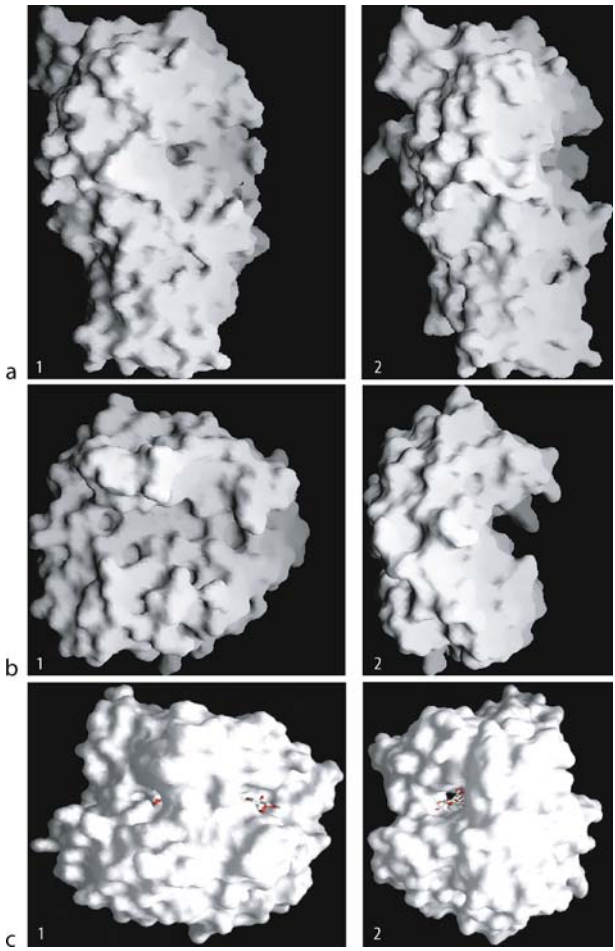


Figure 18

Three types of active sites found in glycan hydrolases. (a) *Hordeum vulgare* β-glucan exo-hydrolase [196]; 1. Viewed from above the catalytic face. 2. Rotated 90° along the long axis. The site appears as a compact hole in the middle of the molecule. (b) *Hordeum vulgare* (1→3;1→4)-β-glucan endo-hydrolase [199]; 1. Viewed from above the catalytic groove, that runs east-west. 2. Rotated 90° perpendicular to the groove axis. (c) *Trichoderma reesei* Cel6A (1→4)-β-glucan cellobiohydrolase (formerly cellobiohydrolase II) [200]; 1. Side-on view of the tunnel containing a substrate analogue. 2. End-on view of the same complex as in 1

ferences in the contact amino acids lead to changes in substrate specificity. This is illustrated by the (1→3)-β- and (1→3;1→4)-β-glucanases from barley, that both belong to Family 17, and have an almost identical folding pattern, with a binding site in the form of a deep cleft (*Fig. 18b*). Both require a laminaribiosyl residue in the −2 and −1 subsites, but the (1→3)-β-glucan hydrolase accepts only a 3-linked glucosyl in the +1 site, whereas the (1→3;1→4)-β-glucan hydrolase accepts only a 4-linked glucosyl residue in this site. Modeling studies suggest that the determinants of substrate specificity are identifiable amino acids in the active

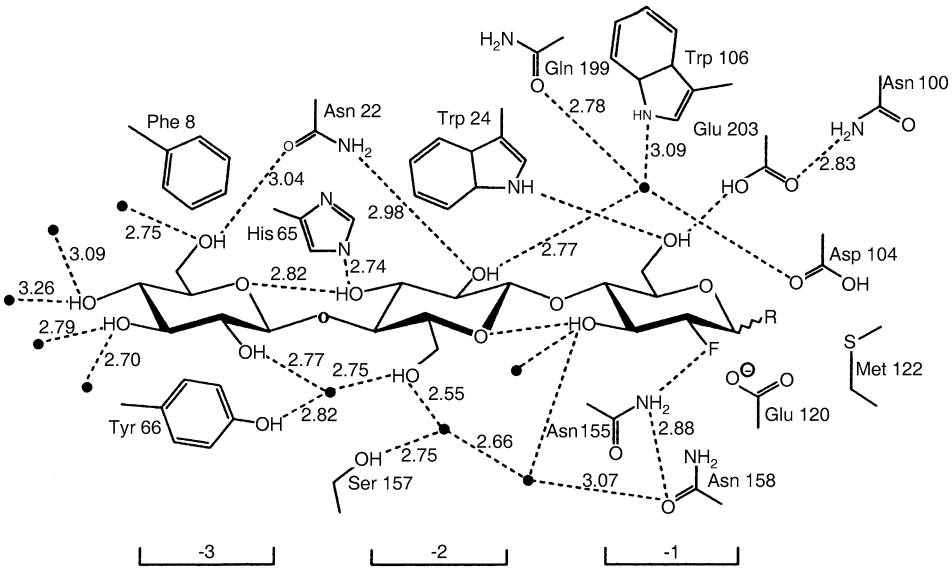


Figure 19
Active site of the *Streptomyces lividans* endoglucanase CelB2 showing the non-bonded interactions between the covalent 2-deoxy-2-fluorocellotriosyl intermediate complex with the nucleophile Glu120 and adjacent amino acids. The F-cellotriosyl portion sits in subsites −3, −2, and −1 (from [208])

site near the conserved catalytic amino acids [199]. The *Rhizopus arrhizus* β-glucan hydrolase has the same glycosyl requirement as the barley and *Bacillus* enzymes but will accept either a 3- or a 4-linked glucosyl residue in the +1 site suggesting a less stringent "aglycone"-binding interaction [209]. The homomorphous (1→4)-β-glucans and (1→4)-β-xylans are sometimes discriminated by cellulases and in other cases they are not [210]. Some chitinases that catalyze the hydrolysis of chitin, a β tGlcNAc homopolymer, also cleave the (1→4)-linkages in murein the homomorphous polysaccharide backbone of the bacterial cell wall peptidoglycan. In murein every other residue is muramic acid, the lactyl ether of GlcNAc, and this is the substrate for animal and bacteriophage lysozymes [211]. Lysozymes, e. g., from hen egg white may also hydrolyze chitin [212]. By an approach involving systematic removal of hydroxy groups [213], on disacccharide substrate it has been possible to determine which OH groups on maltose and isomaltose are needed for binding and hydrolysis by glucoamylase [214,215], but the difficulties of synthesis makes this an impractical strategy for most polysaccharide hydrolyzing enzymes. There are several examples where conformational changes of the protein are seen to accompany substrate binding but it is beyond the scope of the present chapter to include a detailed description of these events. Some well-established cases include: cyclodextrin glycanotransferase where the recognition of the substrate at the active site has been shown to be achieved by concomitant alteration of the conformation of both the substrate and the protein [216]. Structural evidence for a conformational change on substrate binding has been provided for porcine pancreatic α-amylase [36], soybean β-amylase (binding maltose) [197], and the cellobiohydrolase II, Cel6A, from *Humicola insolens* [173] and from *Trichoderma reesei* [217].

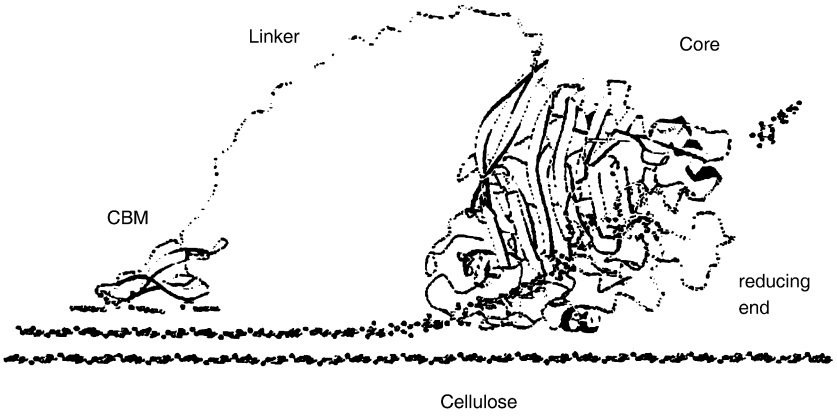


Figure 20
Model of intact cellobiohydrolase 1 (CBH1) from *Trichoderma reesei* bound to a cellulose microfibril surface. The model is based on the crystal structure of CBH1 in complex with cellohexaose, the NMR structure of the cellulose-binding module (CBM) and a modeled linker region. CBH1 is believed by some to act repetitively from the reducing end towards the non-reducing end of the cellulose chain (from [231])

Non-Catalytic, Carbohydrate-Binding Modules Hydrolases acting on large, and sometimes insoluble, substrates [10] such as starch [218,219,220,221], cellulose [222,223,224], xylans [225] and (1→3)-β-glucans [226]. The (1→3)-β-glucan binding modules are conserved in non-catalytic (1→3)-β-glucan-binding proteins from invertebrates [227]. An arabinofuranosidase has been reported with a (1→4)-xylanase-binding module [228] and a xylanase with a cellulose-binding module [229]. A xylanase from a thermophilic bacterium has a binding module for soluble xylans and (1→3;1→4)-β-glucans [230]. The binding module confers thermostability on the enzyme. In cellulases that depolymerize crystalline cellulose, the catalytic domain is connected by a linker peptide to a functionally independent, cellulose-binding module. This module associates very tightly with cellulose microfibril surfaces either in crystalline or non-crystalline areas, depending on the enzyme source, and anchors the associated catalytic domain to the solid substrate thus bringing and maintaining a high local concentration of the enzyme on the cellulose microfibril surface (*Fig. 20* [231]). Removal of the CBM from the catalytic domain results in a significant decrease in the ability to hydrolyze solid substrates.

The smallest CBM, in Family 1, consists of fewer than 40 amino acids and its binding is governed by a planar strip of three tyrosine residues which stack on every second residue of the cellulose chain [232]. The binding is quite tight and not very sensitive to changes in pH but, after replacing two of the tyrosines with histidine by site-directed mutagenesis, binding is still tight but is now pH-sensitive [233], supporting the involvement of the aromatic residues in the binding. A planar binding strip is also a feature of several other CBM families [222]. Some CBMs, in addition to binding the enzyme to the cellulosic surface, appear to disrupt non-covalent interactions between cellulose chains in the crystalline substrate and may potentiate the action of the cellulase [234]. A Family II CBM has been shown to move laterally on the cellulose surface [235] and this may aid in the translocation of the substrate

molecules in the active site in a repetitive action. The CBM of the *Thermomonospora fusca* E4–68 cellulase does not exhibit a planar binding strip topology, but binds to individual chains and is postulated [57] to act in feeding the cellulose substrate into the active site allowing the repetitive (multiple) attack (*Fig. 20*). Non-catalytic, chitin-binding-proteins that adhere to crystalline α-chitin, but not to β-chitin (chitosan), with high affinity, are produced by certain bacteria [236] and the binding involves specific Trp residues. The three-dimensional structure of an isolated starch-binding domain (SBD) from *Aspergillus niger* glucoamylase has been described [221,237,238] and a homologous SBD is seen in the three-dimensional structure of the *Bacillus circulans* CGTase [47]. The SBD is considered to disrupt polysaccharide chain interactions on the starch surface [239] since the release of glucose during solid starch degradation by a proteolyzed form of glucoamylase lacking its SBD is increased by addition of isolated SBD. A mutated SBD from *A. niger* glucoamylase was used to demonstrate that the two binding sites for β-cyclodextrin depend on aromatic residues for function and that they have slightly different binding affinities [238]. Replacement of a CGTase SBD with the one from *A. niger* glucoamylase caused a drastic decrease in activity suggesting that efficient interplay between the binding and catalytic domains is structure-dependent. CGTase has no flexible linker region, as in glucoamylase, but two globular domains connecting the binding and catalytic domains [214,215]. The crystal structure of the SBD of *Bacillus cereus* β-amylase complexed with maltose has been determined [240]. It is homologous to the *A. niger* SBD.
In many cellulose and starch hydrolases with multidomain architecture, highly *O*-glycosylated linker regions connect the catalytic domain and binding and module are considered to provide flexibility in the relative position of the domains on the substrate. The three-dimensional structures of the whole molecules are not known but dynamic light scattering studies of glucoamylase in solution using ligands having a catalytic site ligand, acarbose, connected by spacers of varying length to an SBD ligand, β-cyclodextrin, show that the two domains can come into close contact in solution [241]. Isothermal titration calorimetry indicates that strain is required to accommodate the two target moieties on the two domains in a single molecule of glucoamylase [242].

Adhesion Domains The core of the cellulosome in *Clostridium thermocellum* and *C. cellulolyticum*, is a large (210 kDa in *C. thermocellum*) multidomain, non-enzymic, cellulosome-integrating protein (Cip) (or scaffolding protein, 'scaffoldin'), that binds multiple *endo*-cellulases through high affinity ($K_D = 2.5 \times 10^{-10}$ M) interactions with multiple receptor ('cohesin') domains on the scaffold protein and 'dockerin' domains on the cellulases [243]. The Cip also has a single CBD [222,244]. There is significant synergism in the attack on crystalline cellulose so that the depolymerization of individual cellulose crystals appears to occur completely once attack has been initiated [245].

Other Non-Catalytic Domains. Many polysaccharide depolymerizing enzymes are secreted by bacteria and fungi from their intracellular site of synthesis across the periplasmic space and cell wall into the surroundings. These exported enzymes reach their destination via the general secretory pathway [246] which involves membrane protein complexes such as the bacterial ABC transporters [247] that recognize domains on the secreted proteins. There are several examples of non-catalytic domains on microbial carbohydrases that may play a role in transport from the cells. These include a repeat domain on a chitinase involved

in cell wall/membrane association [248] and cell membrane-associated pullulanase [249]. Fibronectin type III-like repeats found in several microbial carbohydrases may play a role in secretion [250].

Structure-Stability Relationships The thermostability and pH stability of polysaccharide hydrolases and glycosyl transferases vary according to the source. Generally, the enzymes originating from animal, plant, and mesophilic organisms are irreversibly inactivated above about 50 °C. Thermophilic bacteria and archaeans are a source of thermostable polysaccharide hydrolases with stabilities up to 80 °C and exceptionally up to 100 °C; and some of these enzymes are also halotolerant [251,252]. Polysaccharide hydrolases are normally stable in the pH range 4–8 but again there are acidophilic and alkalophilic organisms whose enzymes are stable at lower or higher pH values, respectively. The range of catalytic rate constants and half-lives of the extremophiles makes them candidates for application in industrial processes. Although metal ions do not participate directly in the catalysis by polysaccharide hydrolases, some e. g. a-amylases require Ca^{2+} and Na^{+} for activity [253,254]. One or more Ca^{2+} ions bind to and stabilize certain folding elements that are necessary for maintaining the active conformation of the enzyme. The requirement is usually detected by abstraction of the metal ion with a chelator such as EDTA or EGTA [255].

3 Application of Polysaccharide Depolymerases to Manufacture Bioactive Oligosaccharides

3.1 Manufacture of Nutritional Oligosaccharides

Recent years have seen an increase in interest in oligosaccharides that can play a role in optimizing health (See Chap. 5.3). These include the prebiotic oligosaccharides [256,257] and also oligosaccharides designed to inhibit adhesion of bacteria and toxins to human cells [258,259,260,261]. Whilst synthetic chemistry can manufacture almost any carbohydrate structure, large scale manufacture will probably not be based on traditional chemistry techniques. Most manufacturing technologies for bulk carbohydrates rely on enzymes, mostly polysaccharide depolymerases [262]. Polysaccharides represent a huge resource of chemical structures that can be liberated by enzymes.

A rapidly developing class of functional carbohydrates are the prebiotics (Chap. 5.3). Currently these selectively stimulate health-positive bacteria in the colon [256]. There are, however, possibilities to enhance their functionality. One such functional enhancement would be persistence of the prebiotic fermentation to the distal colon [263]. One way to approach this is to produce oligosaccharide mixtures with controlled molecular weight distributions.

3.1.1 Hydrolysis of Inulin

Inulin is a recognized prebiotic carbohydrate in its own right but is also used as a source of fructo-oligosaccharides by hydrolysis. Inulin, a fructan polymer is widely distributed in plants [264] and chicory (*Cichorium intybus*) is extensively used as a source of inulin for commercial purposes [265]. The roots of chicory contain 15 to 20% inulin and 5 to 10%

oligofructose, low amounts of glucose, fructose and sucrose have also been reported [266]. Inulin derived from chicory has a degree of polymerization of 2 to 60 with an average DP of 12, whereas the oligofructose component has a DP between 2 (F2) to 5 (GF4). A polydisperse carbohydrate, inulin exists as a linear β-(2→1) linked D-fructofuranose terminating in a glucose residue at the reducing end. The demand for fructan type compounds such as inulin and oligofructose is increasing due to the reported physiological benefits conferred on the host when these products are consumed. Such benefits include; modulation of the colonic microflora as inulin has purported prebiotic effects in vivo [256,267], enhances mineral absorption [268,269], modulates either the digestion and/or absorption or the metabolism of lipids [270] and exerts a positive influence on the body's defense mechanisms [265]. Although inulin occurs naturally in many fruits and vegetables, its quantity may be too low to beneficially influence the host, additionally cooking of these foods may influence the amount of inulin subsequently available, thus an attractive approach is the large scale manufacture of inulinoligosaccharides (IOS), a term which can be used interchangeably with fructooligosaccharides (FOS). It must be mentioned that FOS can be produced by one of two methods; (1) the hydrolysis of inulin by endoinulinases and (2) by enzymatic synthesis using sucrose as the substrate and a β-fructosidase possessing transfructosylation activity.

Using inulin as a substrate can yield two very different products depending on the enzyme used. An exo-inulinase (EC 3.2.1.26 β-D-fructofuranoside) is primarily used for the complete hydrolysis of inulin to fructose to produce an ultra high fructose syrup, whereas an endo-inulinase (EC 3.2.1.7 2→1-β-fructan-fructanohydrolase) attacks the internal linkages of inulin to produce oligosaccharides of varying DP. While extensive research is available on the production of fructose syrups there is less literature available on the manufacture of IOS, however this trend is changing due to the realization of the health benefits associated with IOS. Yazawa and Tamura [271] produced IOS through the acid treatment of inulin, although not an enzymatic process, IOS of different DP was produced. Results from a subsequent in vitro study suggested these oligomers of DP4 to 25 were selectively metabolized by *Bifidobacterium infantis*, a beneficial bacteria associated with host well being. Yeast, filamentous fungi and bacteria are all sources of inulinases their properties and applications have been reviewed elsewhere [272,273,274] and it is the action of the endo-inulinases which are of particular interest here. Such enzymes have been isolated from *Aspergillus*, *Pseudomonas* and *Xanthanomonas* sp.

Free enzyme versus immobilized enzyme can influence the yield of IOS, additionally an immobilized system would be favorable economically as the biocatalyst can be reused, enables continuous production and the end product is free of contamination. Kim et al. [275] intended to make a comparison between the reaction kinetics of free and immobilized endo-inulinases in a batch reactor however significant differences were observed in the reaction behavior and product composition due to the form of enzyme used and the initial concentration of substrate. Yun et al. [276] investigated the effect of inulin concentration on the production of IOS by free and immobilized endo-inulinase from *Pseudomonas* sp. Their findings corroborate those of Kim et al. [275] whereby different products are formed depending on the form of enzyme; a soluble enzyme yielded inulobiose and DP3 products, whereby the immobilized form predominantly produced inulobiose. As the concentration of inulin increased the yield of IOS did not increase in the soluble system and in the immobilized the yield remained the same. Although the enzyme was derived from *Pseudomonas* the immobilized form required a differ-

ent optimal pH to the soluble, pH 3.5 as opposed to pH 6, the lower pH resulted in a faster rate of hydrolysis of inulin, in addition a higher quantity of enzyme was used as the effectiveness factor is significantly decreased following immobilization. The yields of IOS produced by the free and immobilized endo-inulinase were 72 and 83%, respectively.

Form of enzyme and concentration of substrate influence the yield and type of IOS produced, however another study by Yun et al. [277] used whole cells of *Escherichia coli* HB101 whereby the gene for an endo-inulinase from *Pseudomonas* sp was expressed. In a batch reactor the production of IOS using the recombinant enzyme was compared to that of the native and there were differences in the DP distribution of the products formed although the yield of IOS was comparable. The native form produced less inulobiose and more DP4 and >DP4 products compared to the recombinant. Additionally Yun et al. [277] studied the effect of different reactor conditions; batch processing versus continuous using immobilized recombinant cells but no differences in product composition were observed.

Nakamura et al. [278] used immobilized endo-inulinase from *Aspergillus niger* in a continuous process to produce IOS. This particular enzyme was isolated from an endo-inulinase hyperproducing mutant 817 from the wild type strain *A. niger* 12 [279] which had previously been used in the production of fructose syrups by complete hydrolysis of inulin. Although the extracellular inulinase was partially purified, exo-inulinase activity remained, albeit at lower levels to the endo type. Various I/S ratios (I = endo-inulinase activity, S = exo-inulinase activity) were investigated (I/S = 4.5, I/S = 25 and I/S = 50) and the higher I/S ratio of 50 at the maximal flow rate of 6.0 ml min^{-1} produced a mixture of oligomers with a DP of 3–5.

Purified and partially purified endo-inulinases from *Aspergillus ficuum* for production of IOS were studied by Zhengyu et al. [280]. The yield of IOS was markedly different depending on the biocatalyst purity, the partially pure endo-inulinase produced an IOS yield of 50%, whereas the purified form produced an IOS yield of 70% in the same operating conditions. The product composition was also different; inulobiose – triose and tetraose where the predominant products of the partially purified form and the DP distribution for the purified enzyme ranged form DP2 to DP8 with relatively high levels of DP3 and DP4. The optimal substrate for the purified endo-inulinase was also determined. Pure inulin, Jerusalem artichoke powder and Jerusalem artichoke juice were used and the latter substrate produced an IOS yield of 80% during a 72-h reaction with a wide DP range (2–8), however no DP 6 was produced. A mixed enzyme system using endo-inulinases from *Xanthanomonos* and *Pseudomonas* sp is an interesting approach in the production of IOS [281].

3.1.2 Partial Hydrolysis of Dextran

Isomalto-oligosaccharides are commercial prebiotics in Japan [257] (Chap. 5.3). These are (1→6)-α-gluco-oligosaccharides with a degree of polymerization of two to five. The low DP means that the commercial product is partially metabolized by humans. Larger oligodextrans would be expected to have lower digestibility and increased colonic persistence. A promising manufacturing route to controlled molecular weight oligodextrans has been proposed using enzyme membrane reactors [282]. Such reactor systems use ultrafiltration membranes to retain enzymes in the system and to control the molecular weight of the product oligosaccharides (*Fig. 21*).

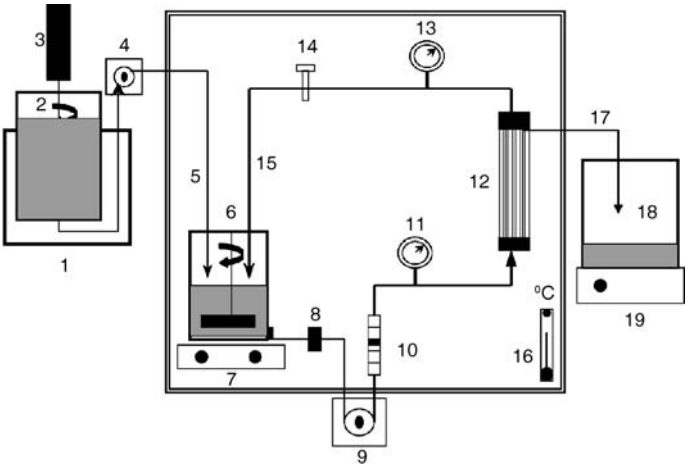


Figure 21
The continuous enzyme membrane reactor (CMR). (1): Temperature-controlled water-bath; (2): Feed tank; (3): Stirrer motor for feed tank; (4): Feed pump; (5): Feed inlet line to the reaction vessel; (6): Reaction vessel; (7): Magnetic stirring table; (8): Prefilter; (9): Recycle pump; (10): Flowmeter; (11): Membrane inlet pressure gauge; (12): Hollow fiber membrane cartridge; (13): Membrane outlet pressure gauge; (14): Pressure adjustment valve; (15): Retentate recycle line; (16): Air bath environment; (17): Permeate (product) line; (18): Permeate collection vessel; (19): Electronic balance

Table 1
Carbohydrate composition (%) of commercially available IMO product [285]

DP*	Components	IMO+
DP1	Glucose	3.8
DP2	Maltose	4.5
	Isomaltose	22.8
	Others**	13.1
DP3	Maltotriose	0.9
	Panose	11.6
	Isomaltotriose	16.7
DP4	Isomaltotetraose and others	17.7
DP5	Isomaltopentaose and others	7.2
≥DP6	Isomaltohexaose and others	1.7

+Isomalt 900® from Showa Sangyo Co. Ltd, Tokyo, Japan. *Denotes degree of polymerization. **Nigerose and kojibiose

In conventional production of IMO, starch is first hydrolyzed to maltooligosaccharides by α-amylase (EC 3.2.1.1) and pullulanase (EC 3.2.1.41). Then, α-glucosidase (EC 3.2.1.20) is added to convert the $\alpha 1 \rightarrow 4$ linked maltooligosaccharide into $\alpha 1 \rightarrow 6$ linked isomaltooligosaccharides. Finally, glucose is removed, giving a maximum IMO concentration of around 40%. About 40% glucose remains in the mixture [283,284]. ❯ *Table 1* [285] lists the composition of a commercial IMO product.

Oligodextran is a collective term used to indicate carbohydrate mixtures derived from dextran. It consists of an IMO part and a dextran part (< 70 kDa) [282], because of the resulting higher DP, oligodextrans are less digestible than IMO [286]. Commercially, they are either produced by acid hydrolysis of the native dextran from *Leuconostoc mesenteroides*, or by direct in vitro synthesis catalyzed by dextransucrase (EC 2.4.1.5) [287,288,289]. The dextran produced by *L. mesenteroides* NRRL B512F consists of 95% $\alpha 1 \rightarrow 6$ bonds, constituting the backbone of the molecule, and 5% side chains attached to the backbone through $\alpha 1 \rightarrow 3$ bonds [289]. Dextranases (1,6-α-D-glucanhydrolases EC 3.2.1.11) belong to the general class of hydrolytic enzymes and their name is derived from their catalytic function of hydrolyzing dextrans. These enzymes are used for the production of isomaltose or higher IMO's.

Dextranases from different sources exhibit diverse action patterns, but fundamentally they specifically hydrolyze the $\alpha(1 \rightarrow 6)$ glucosidic linkages of dextrans to produce primary isomlatose and some D-glucose as final products [290]. They are further divided into two classes, namely endodextranases and exodextranases. Endodextranases catalyze the rapid and random hydrolysis of the dextan $\alpha(1 \rightarrow 6)$ glucosidic linkages, irrespective of the position of these linkages in the dextran molecule (some selectivity exists), whereas endodextranases attack the $\alpha(1 \rightarrow 6)$ glucosidic bonds endwise liberating glucose [291]. The selectivity and limitation of the hydrolytic action of dextranases is attributed to their inability in hydrolyzing the branch linkages of dextrans ($\alpha(1 \rightarrow 3)$ bonds) [292]. In the case of endodextranases presence of excess branch linkages to a dextran molecule constitutes the polysaccharide highly resistant to degradation since the enzyme binding necessary for the hydrolytic action is inhibited [293]. The number of binding subsites of the endodextranases vary between 5–12 and a similar number of continuously $\alpha(1 \rightarrow 6)$ linked glucose residues, without any branch linkages in between are necessary for the attachment to take place [290]. Moreover the $\alpha(1 \rightarrow 6)$ glucosidic linkages in the vicinity of the branch linkages possess a greater degree of resistance towards hydrolysis compared to more distant ones [294]. Endodextranases have been isolated from extracellular fluids or cell extracts of fungi (e. g. *Penicillium funiculosum*, *P. lilacinum*, *Aspergillus carneous* and some of their mutants) and bacterial sources (*Lactobacillus bifidus*, *Cytophaga*, *Pseudomonas)* [292,293,295,296,297,298]. The most commonly used sources of endodextranases are from the Penicillium species in particular *P. funiculosum*, *P. lilacinum*, and *P. notatum* since they exert high enzyme yields and are easily purified [293,295,297].

Endodextranase type enzymes due to their rapid and random hydrolysis of dextrans are of interest for oligosaccharide production. Their inability to hydrolyze the branch linkages of dextrans determines the degree of hydrolysis and consequently gives rise to a variety of products depending on the frequency and the length of these branch points [292]. Bourne et al. [295,299] reported that *P. lilacinum* and *P. funiculosum* dextranses act upon a virtually unbranched dextran and the final products are glucose isomaltose and isomaltotriose with isomaltose being the main product and with the *P. funiculosum* enzyme producing slightly more glucose. Branched dextran hydrolysis results in a greater variety of products, which in the case of *L. mesenteroides* dextran are in the range between tri- to octasaccharide, linear or containing a branch linkage [294,299]. These products of the first hydrolysis can act as substrate for further hydrolysis but as the molecular weight decreases the rate of hydrolysis decreases [294]. In more recent studies [300] it was reported (for dextranse degradation of dextran hydro-gels) that the action of the enzyme could be divided into two steps. In the first step long unsubstituted chains (18 or

more) of glucose residues are hydrolyzed and in the second the hydrolysis products become the substrate for further breakdown. In studies concerning the rate of hydrolysis it was reported that the hydrolysis increases at relatively low substrate concentrations due to decreased viscosity and reduced product inhibition. High enzyme concentrations decreased the hydrolysis efficiency due to increased transglycosylation reactions, and presence of other low molecular weight sugars in the digests did not affect the enzyme activity or the total yield of hydrolysis products [301].

Mountzouris et al. [302] investigated the types of oligodextrans that would be generated via controlled enzymatic depolymerization of dextran using an endodextranase (Dextranase 50 L from *Penicillium lilacinum*). The reaction was carried out in an ultrafiltration stirred-cell membrane reactor fitted with a 10,000 MWCO membrane. It was found that the DE (dextrose equivalent) increased with increasing enzyme concentration and decreasing substrate concentration. At high enzyme concentrations, product DE values ranged from 18–38 and the oligosaccharide content of the products ranged from 27–82% (w/w). The oligosaccharides consisted mainly of isomaltose and isomaltotriose. At medium and low enzyme concentrations, the oligosaccharide yield was lower (30–40%) and the DE values of the oligosaccharides produced were also lower (in the range of 18–22). Anaerobic batch culture fermenters showed that dextran and oligodextrans supported growth of bifidobacteria with high levels of persistence for 48 h [303].

In a later study, Mountzouris et al. [282] investigated the continuous production of oligodextrans in a continuous stirred tank (CSTR) membrane reactor system. Substrate (industrial grade dextran from *Leuconostoc mesenteroides* B 512F) and enzyme were the same as those used previously. Oligodextran yields ranged between 84.4 and 98.7%. DE values ranged from 22–41 and the oligosaccharide content ranged from 55.9 to 93.4% (w/w), which was higher than in the batch membrane reactor. Three types of oligosaccharides with respect to their polysaccharide content were produced: a. < 15%, b. 15–30% and c. > 30% and < 44 %. The oligosaccharide content was found to be affected, not only by substrate and enzyme concentrations, but also by the residence time, higher residence time resulting in products with higher oligosaccharide content. The oligosaccharide content was fairly constant at 21.7–24.8% in all products, DP 4, 7, and 8 being the predominant ones. Isomaltotriose ranged from 21.6 to 35.2% (w/w). The novel oligodextrans generated in this work are expected to have lower digestibility than the already commercially available IMO and to give different physicochemical properties. Tanriseven and Dogan [304] produced IMO by using *L. mesenteroides* B 512 FM dextransucrase immobilized in alginate fibers. The product profile was the following: fructose (20.4%), glucose (21.2%), leucrose (3.8%), DP2 (4.7%), DP3 (5.1%), DP4 (6.6%), DP5 (6.4%), DP6 (5.7%), DP7 (4.4%), DP8 (2.2%), DP9 (2.6%), DP10 and bigger (16.9%). The authors concluded that the use of alginate fibers rather than beads leads to better performance and repetitive use.

3.1.3 Hydrolysis of Xylan to Produce Xylo-Oligosaccharides

XOS are manufactured by enzymatic hydrolysis of xylan from corn cobs, oat spelt xylan or wheat arabinoxylan [257]. Xylan, a principle type of hemicellulose exists as a linear polymer of β-D-xylopyranosyl units linked by 1→4 glycosidic bonds. Depending on the origin, different substituents such as arabinfuranosyl, 4-*O*-methylglucuronosyl and acetyl groups are present. Typical raw materials for XOS production include hardwoods, corn cobs, straws,

bagasses, hulls, malt cakes and bran [305]. Three different approaches exist for production of XOS: 1. enzyme treatment of lignicellulosic material (LCM), 2. chemical fractionation of LCM, with subsequent enzymatic hydrolysis and 3. hydrolytic degradation of xylan by steam, water or dilute acid solutions [305].

In the enzymatic production of XOS, low enzymatic activities are preferable in order to avoid degradation down to monosaccharides, di-, tri- and tetrasaccharides being the required products [305]. The enzyme used for XOS production is an endo-1-4-β-xylanase (1,4-β-D-xylan

Table 2
Summary of xylanase characteristics

Enzyme source	MW	Opt. pH	Opt. T (°C)	Best yield conditions	Products	Yield XOS
P. chyrosporium, G. trabeum, H. sheweinitzii, T. viride	Nd	4.5–5.0	40	Substrate: 1 g of hylglucuronoxylan	Aldotetraouronic acid from *T. viride**	75.7–91.3%
Trichoderma longibrachiatum	37.7 kDa	5.0–6.0	45	Oat spelt	Xylobiose, xylotetraose and higher DP XOSs	Nd
Robillarda sp.	23,400 Da	4.5–5.5	55		Xylobiose	
Irpex lacteus				Xyloglycans and aryl-xylooligosaccharides	1,4-β Glycosidic linkages, or 1,3 and 1,4 β xylosidic linkages	
T. viride				23 g reduced hardwood xylan at 40 °C for 24 h	4-*O*-Methyl-α-D-glucopyranosyl-(1-2)-D-xylotriose and 4-*O*-methyl-α-D-glucopyranosyl-(1-2)-D-xylotetraose	Neutral and acidic products were 13.0 g and 2.1 g, respectively
A. oryzae	Nd	Nd	Nd	Xylan (3 g)	Xylotriose, xylobiose, and xylose	1 g/L each
T. reesei	Nd	Nd	Nd	Unbleached birch kraft pulp, with endoxylanase 40 °C for 24 h at pH 5.5, with β-xylosidase at 40 °C for 24 h at pH 5.5 and then α-glucuronidase for 48 h at 40 °C	Acidic : (4ΔUA)-β-D-xylotetraose, (4ΔUA)-β-D-xylopentaose, (4-*O*-methyl-α-D-glucurono)-β-D-xylotetraose and (4-*O*-methyl-α-D-glucurono)-β-D-xylopentaose Neutral: were D- xylose, β-1,4-D-xylobiose and β-1,4-D-xylotriose	Nd

xylanohydrolase EC 3.2.1.8). This endo- acting enzyme cleaves the glycosidic bonds in the xylan backbone subsequently reducing the degree of polymerization of the substrate. Factors influencing the extent of hydrolysis include chain length, the degree of branching and presence of substituents. Additionally, purity of the enzyme is critical to reduce or prevent complete hydrolysis to xylose by contaminating β-xylosidases. *T. reesei*, *T. harzianum*, *T. viride* and *T. koningii* are known to produce xylanases [306]. *Table 2* lists other reported sources and extensive reviews on xylanases are available [307,308]. Fungal endo xylanases have an activity between 40–80 °C and between pH4–6.5. Extracellular enzymes from *Thermobifida fusca* were successfully used to produce XOS from various lignocellulolytic agricultural waste, however physical pretreatment of the substrate was a prerequisite to expose the xylosidic linkages to enable enzymatic hydrolysis [309]. Although various treatments are used (alkaline extraction, acidic treatment and cooking), for the production of XOS the acidic approach is undesirable as high quantities of xylose may be produced. An endo xylanase from *Aspergillus versicolor* has also been reported for production of XOS using xylan as substrate, interestingly no xylobiose or xylose were detected [310]. Techniques used to characterize the XOS include capillary zone electrophoresis and MALDI-TOF-MS [311].

3.1.4 Partial Hydrolysis of Pectins

Pectins are classified as dietary fiber and are reported to exert a beneficial effect on the gastrointestinal tract of the host (Chap. 5.3), thus the oligosaccharides derived from these may have similar or additional functionalities. Also, the oligosaccharides would be of a lower molecular weight, possibly more soluble than the native pectin as well as less viscous making them more amenable to be incorporated into foodstuffs. The approach of controlled partial hydrolysis applied to dextrans above has also been used to manufacture pectic oligosaccharides [312]. Enzyme membrane reactors were established to hydrolyze high methoxy citrus pectin (60–68% methylation) and low methoxy apple pectin (8% methylation) to low molecular weight oligosaccharides. A commercial endo-polygalacturonase (EC 3.2.1.15) from *Aspergillus pulverulentus* (Pectinas DL "Amano") chosen after screening several available preparations for the absence of pectin methylesterase (EC 3.1.1.11). Conversion to product was dependent on the degree of methylation where higher product conversions were achieved when the apple pectin was used as substrate compared to the citrus pectin. Yamaguchi et al. [313] studied the partial hydrolysis of citrus pectin (75% degree of esterification) using a crude enzyme mix from *Kluyveromyces fragilis*. Although a low molecular weight pectin was formed, the extent of hydrolysis was not comparable to that achieved by Olano-Martin et al. [312] as the molecular weight was 66,000 Daltons compared to the 3500 Dalton product from the continuous hydrolysis of a 60–60% methylated citrus pectin. It has been suggested that the pectins with a high degree of esterification are less susceptible to hydrolysis by endo-polygalacturonases than the pectin esterases, however other parameters such as temperature, enzyme concentration, origin and purity of enzyme, substrate concentration, pH, time and reactor will no doubt also influence the hydrolysis. The functionalities of the products produced by Yamaguchi et al. [313] and Olano-Martin et al. [312] have been investigated in vitro and although results are promising further in vitro studies would be required to substantiate a health claim before clinical trials are performed. The product oligosaccharides were found to have prebiotic potential using in vitro model fermentation systems [314]

and were characterized for their ability to inhibit the adhesion of *E. coli* verocytotoxins to human cells [315]. Lipid accumulation by the liver of rats was repressed, however serum cholesterol was not suggesting the molecular weight of the oligosaccharides could influence this.

3.1.5 Degradation of Bacterial Exopolysaccharide (EPS)

Bacterial EPS are a diverse group of polysaccharides comprised of a wide range of monosaccharide residues such as glucose, galactose and mannose. Historically most attention has been given to the EPS of Gram negative bacteria, particularly those of the various serotypes of *Klebsiella* spp. and *E. coli*, where the EPS carries the K antigenic specificity. Many of these EPS sequences carry potentially bioactive oligosaccharide sequences cryptically encoded in their primary structures. Many recognized receptor-active sequences can be found in these EPS molecules (*Table 3*). Economic methods of hydrolysis and isolation of these sequences would facilitate large scale production of receptor active oligosaccharides for subsequent development into more potent therapeutic derivatives [259,260].

Bacterial EPS tend to be rather refractory to most polysaccharide hydrolases [79]. A promising approach to preparation of oligosaccharides, however, is the use of bacteriophage. Many bacteriophage produce capsular depolymerase enzymes to degrade the EPS capsule and gain access to the bacterial cell surface. These enzymes are capable of reducing the EPS of their host organism to the repeating oligosaccharide unit. Many of these enzymes have been characterized [79,316] and they frequently display a very high degree of specificity for one particular linkage in the EPS sequence. They can either be hydrolases or lyases [79] although deacetylases (carboxylic ester or amide hydrolases) can also be found [317]. These cleave the acetyl substituents from the EPS.

No pure depolymerase enzymes are available commercially, and they have to be isolated from the bacteriophage particles [318]. Bacteriophage may be found in the natural habitat of their host bacteria, for example bacteriophages of the *Enterobacteriaceae* can be isolated from human or animal feces or in sewage [317,319]. It is in the base plate of the bacteriophage where the depolymerase is thought to be located [317]. However, it has also been discovered that in many of the systems studied the enzyme also exists as a soluble protein, found in the cell lysates following viral maturation. Yurewicz [320] has investigated the catalytic and molecular properties of enzyme from each source, finding that both are similar.

The exopolysaccharide can be broken down rapidly or slowly by endo- or exo- acting enzymes respectively. The majority of enzymes investigated to date are endoglycanases and endo- acting polysaccharide lyases [79]. Exceptions to this are the findings by Mishra et al. [321] who identified a polysaccharide which required both endo- and exo- acting enzymes to be degraded. Although phage enzymes are highly specific, it has been observed that some enzymes can act on more than one polysaccharide. Depolymerases from *E. coli* systems have been found to be active against *Aerobacter cloacae* polysaccharide and vice versa [322].

Various parameters need to be considered before performing EPS hydrolysis, namely substrate specificity, temperature, reaction medium, duration, and pH. Carboxyl reduction of EPS [323,324] prior to hydrolysis renders the bacteriophage enzyme inactive against the EPS, even though the polymer structure remains intact. A preliminary indication of the presence of depolymerase activity can be seen by the presence of a translucent halo surrounding the phage

Table 3
List of oligosaccharide repeating units resulting from bacteriophage hydrolysis (modified from Sutherland, 1999). Highlighted linkages represent anti-adhesive sequences

Oligosaccharide	Source
Glc[1]β1→4GlcAβ1→2Rhaα1→3Rhaα1→2Rhaα1→3Gal	Klebsiella K36 EPS
Gal[1]β1→4GlcAα1→3**Manα1→2Man**α1→3Gal	Klebsiella K74 EPS
Glcβ1→2Rhaα1→4GlcAα1→3Rha 3 ↑ Rhaα1	Klebsiella K17 EPS
Glcβ1→3Man[1]β1→4GlcAα1→3Manα1→3Galα1→3Gal	Klebsiella K46 EPS
Glcα1→4GlcAβ1→3Galα1→3Manα1→3Glc ↑ ↑ 2 2 Glcβ1 Glcβ1	Klebsiella K60 EPS
Galα1→3GalA[2]α1→2Fuc	Klebsiella K63 EPS
Man[1]β1→4GlcAα1→3Fucα1→3Glc	Klebsiella K6 EPS
Man[1]α1→4GalAα1→3**Manα1→2Man**α1→3Gal	Klebsiella K3 EPS
Gal[1]β1→4Glcβ1→6Glcα1→4GlcAα1→3**Manα1→2Man**α1→3Gal	Klebsiella K26 EPS
Manβ1→4GlcAβ1→2**Manα1→2Man**α1→3Gal	Klebsiella K43 EPS
Gal[1]α1→3Rhaα1→3**Galα1→4Gal**[1]α1→3Rhaα1→3Gal	E. coli K103 EPS
Manβ1→4GlcAβ1→3**Manα1→2Man**α1→3Glc	Klebsiella K24 EPS
Glcβ1→4GlcAβ1→2Manα1→4GlcAβ1→2Manα1→3Glc	Klebsiella K39 EPS
GlcAβ1→2Rhaα1→3Rhaα1→3Glcβ1→4Glc	Klebsiella K44 EPS
Glcα1→4GlcAβ1→2Manα1→3Glc 3 ↑ Galα1	***E. coli*** K39 EPS
GlcAβ1→2Rhaα1→4GlcNAcα1→6GalNAc	***E. coli*** K44 EPS
Gal[1]β1→4GlcAα1→3Manβ1→4Glcα1→3Glc	Klebsiella K13 EPS
GlcAα1→3Manβ1→4Glcα1→3Glc	Klebsiella K2 EPS
GlcA[1]β1→4Fucα1→3Glc	Klebsiella K1 EPS
Manα1→4GalAα→2Manα1→3Gal	Klebsiella K57 EPS

[1]Pyruvylated, [2]acetylated and formylated

plaques on confluent agar cultures of the host organism. In some studies the phage induced enzymes were recovered from cell lysates [323,324] for hydrolysis of the EPS and in others, crude preparations of phage were used [325,326,327]. Different temperatures have been used for EPS hydrolysis, 35 °C [328], 37 °C [323,325,326,329], and 40 °C [318]. The duration of the reaction has also varied. Dutton et al. [326] investigates hydrolysis at 24, 67, and 72 h.
Oligosaccharides prepared using this manufacturing technology can be used in in vitro assays to determine their anti-adhesive properties and might be suitable for anti-adhesion therapy as

they are likely to be non-immunogenic, are of low molecular weight and are soluble [258]. It is hoped that these compounds would be specific for the pathogen to be eliminated, unlike antibiotics which are not as specific, however extensive trials in vitro and in vivo would be required to confirm this.

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