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REVIEW

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Polymer hydrolysis in a cold climate

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Abstract In this review we discuss the activity of an ecologically significant group of psychrophilic bacteria, which are involved in the hydrolysis of plant cell wall polymers. Until now these organisms have been largely overlooked, despite the key role they play in releasing organic carbon fixed by primary producers in permanently cold environments such as Antarctica. This review details a specific group of plant cell wall polymer-degrading enzymes known as β-glycanases. Studies on "cold" enzymes in general are in their infancy, but it has been shown that many exhibit structural and functional modifications that enable them to function at low temperature. β-Glycanases in particular are intriguing because their substrates (cellulose and xylan) are very refractile, which may indicate that their "cold" modifications are pronounced. In addition, mesophilic β-glycanases have been extensively studied and the current state of our knowledge is reviewed. This body of information can be exploited to enable meaningful comparative studies between mesophilic and psychrophilic β-glycanases. The aim of such investigations is to obtain a deeper insight into those structural and functional modifications that enable these enzymes to function at low temperature and to examine the evolutionary relationship between mesophilic and psychrophilic β-glycanases.

Key words Psychrophilic bacteria · Enzymes · Plant cell wall hydrolysis · β-Glycanases

Introduction

Temperature is among the most crucial of factors that determine the diversity of life in a particular environment. There are extremes of temperature, both high and low, that determine the range of biological activity. These limits are yet to be clearly defined; however, most organisms grow between 0° and 100°C, with the greatest diversity being observed between 15° and 45°C. Organisms for which optimal temperatures lie outside this narrow mesophilic range are identified as extremophiles, and the majority of studies conducted so far have focused on the organisms that can thrive in high temperatures. This work has yielded a plethora of information on how such organisms adapt to their hot habitats and has provided products and processes of extreme biotechnological significance. Despite their intrinsic interest, thermophilic environments are not abundant in the biosphere. In contrast, permanently cold habitats, such as in Antarctica where the temperature never exceeds 5°C, are widespread; indeed, because 70% of the world's surface is covered by deep oceans they are the most common conditions in the biosphere. Therefore, the ecological significance of cold-adapted organisms (psychrophiles) are clear, and those characteristics that enable them to grow at low temperature deserve further study.

Bacterial adaptation to low temperature

A wide variety of organisms are present in cold environments, displaying a diverse range of adaptations; however, for those which are unable to maintain their temperature significantly above that of the ambient environment there is a requirement for biochemical modifications to cell function. Prokaryotes certainly fall into this category; exposing bacteria to low temperatures has been shown to initiate a number of physiological modifications, which have been termed the cold shock response. After a significant decrease in ambient temperature a number of specific genes are expressed or upregulated (Jones and Inouye 1994). The resulting cold shock proteins have been demonstrated to have a variety of functions, ranging from supercoiling the DNA to initiation of translation. In addition, changes in the fatty acid composition of the cell membrane are also observed,

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which are aimed at maintaining the fluidity of the membrane (Russell and Hamamoto 1998).

Another key consideration for organisms that operate at low temperatures is the maintenance of enzyme functions that are essential for cell viability. A critical consideration is the maintenance of sufficient metabolic activity to generate energy for growth. Such activity is driven by biochemical reactions, which require enzymatic catalysis. At low temperatures, thermodynamic considerations will inevitable reduce the rate of these reactions; therefore, the enzymes employed by cold-dwelling organisms must display adaptive features to ensure sufficient metabolic activity is maintained. The functional modifications that are observed in psychrophilic (cold) enzymes have been extensively reviewed in recent times (Feller et al. 1996, 1997; Feller and Gerday 1997), and it is our intention only to outline the major structural and thermodynamic problems and relate these to the activity of plant cell wall polymer hydrolysis at low temperature.

Characteristics of "cold" enzymes

There will always be a compromise between the stability and the function of an enzyme, particularly if it is adapted to function at an extreme of temperature. Amino acid residues that optimize the stability of a protein structures often reduce the catalytic or ligand-binding properties of the enzyme (Shoichet et al. 1995). Indeed, it is possible to replace catalytic residues and inhibit activity while increasing the stability of the enzyme. It has been demonstrated that cold enzymes generally sacrifice structure-stabilizing features such as the content of arginine and proline residues hydrogen bonding, and aromatic interactions (Feller et al. 1997); for example, the α-amylase of the psychrophile *Alteromonas haloplanctis* has fewer salt bridges than that of its porcine homolog (Feller et al. 1994). The result of the absence of such structural features is the rapidity with which enzymes from psychrophilic bacteria can be deactivated. For instance, the α-amylase of *Alteromonas haloplanctis* has a low thermal stability (Feller et al. 1992); similarly, three proteases derived from *Pseudomonas fluorescens* were shown to be quickly denatured at 45°C, despite the observation that two of these enzymes also demonstrated their highest relative activity at this temperature (Margesin and Schinner 1992). It is worth noting that thermal instability is not a ubiquitous feature for all cold enzymes; an aspartate transcarbamylase from a psychrophilic bacteria TAD1 was shown to be stable at 60°C (Sun et al. 1998). This probably reflects the fact that any compromise between the structural stability and activity needs to accommodate organisms which may at times be subjected to wide variation in the external temperature.

Comparative studies on homologous enzymes from mesophilic and psychrophilic organisms have also identified those functional features that are considered to be diagnostic of cold enzymes. The low levels of available free energy in cold environments necessitate that there is a reduction in the activation energy. To achieve this, the enzyme must exhibit a high degree of conformational complementarity with its substrate, which in turn would account for the high specific activity observed in cold enzymes. High conformational complementarity could be facilitated by an increase in the relative flexibility of the polypeptide chains, and given the thermolability of cold enzymes such a mechanism would seem likely; however, this flexibility has not been conclusively demonstrated and remains one of the key questions that needs to be addressed (Feller et al. 1997).

The ecological role of "cold" polymer hydrolases

In permanently cold environments such as the littoral and nearshore ecosystems of Antarctica, cold-adapted organisms must fill all the ecological niches available. This particular environment is characterized by a low temperature and a pronounced seasonal variation in primary productivity (Clarke 1988). Around the coastal regions, very large blooms of phytoplankton occur that are subsequently deposited as sediment on the seabed rather than being grazed (Clarke and Leakey 1996). The carbon fixed by primary producers is converted to structural polymers such as cellulose, xylan, and pectin by a variety of microbial photosynthetic organisms and macroalgae, up to 70% of the total dry weight of which are cell wall polymers (Kaehler and Kennish 1996). These structural polymers are presumably degraded by a process involving ingestion by marine invertebrates (Clarke et al. 1994) and direct microbial degradation, given that there are no reports of large volumes of detrital material persisting for extended periods. There has been very little work done on the hydrolysis of cell wall polymers in this environment, although clearly they are an important source of carbon. It is unlikely that the invertebrate community utilizes this material directly; rather, one assumes that they either rely on microbial processes to liberate the carbon, or simply exploit the soluble carbon fraction of the epiflora.

The evidence for microbially mediated plant cell wall (PCW) polymer degradation in Antarctic marine and terrestrial environments, although circumstantial, is convincing and would equate with many other environments where such a process is ecologically significant. To our knowledge there are no published reports of β-glycanase activity at low temperature; we therefore sought to isolate organisms capable of cellulose and xylan hydrolysis at 3°C from the marine sediment collected from the shallow waters around Adelaide Island, British Antarctica. Enrichment cultures yielded xylanase activity, but as yet cellulose degradation has still to be convincingly demonstrated. A gram-negative bacterium has been purified that has abundant xylanolytic activity in the culture supernatant. Crude enzyme activity was measured in the supernatant at temperatures ranging from −5° to 50°C. The bacterium was psychrotolerant, giving the fastest growth rate at 15°C; however, optimum enzyme activity was obtained at 37°C. The xylanase activity

showed low thermostability at elevated temperatures, which is typical of cold enzymes. Further studies on purifying the enzyme(s) and biochemically characterizing the activity are under way.

Our study has shown that the enzymes responsible for "cold" PCW polymer hydrolysis can be demonstrated in the environment, allowing a comparative study with their mesophilic counterparts. The thermodynamic limitations placed on their activity by low temperature, and the refractile nature of their substrates, even in warm environments, suggests that such enzymes, which we shall refer to as β-glycanases, possess novel characteristics that may help elucidate the mechanisms by which PCW hydrolysis is achieved. "Cold" β-glycanases also have biotechnological significance. Potentially, they could be exploited in a wide range of applications, ranging from detergents to food processing and paper production. Microorganisms capable of hydrolyzing plant cell wall polymers may also be present in less remote locations than Antarctica; for example, the spoilage of refrigerated fresh fruit and vegetables is a common problem. Increasingly there is a demand by consumers for minimally processed vegetables and fruits that are only washed, cut, and packaged. This produce is stored at refrigeration temperatures but has a short shelf life because of bacterial spoilage (Nguyen-the and Carlin 1994). The role of psychrophilic β-glycanases in this process is unknown, but may be significant and contribute to the release of nutrients, which may allow pathogenic organisms to proliferate on the produce.

The structure and function of β-glycanases

The complexity of the cell wall structures of many plants and algae is enormous, and consequently the enzymes responsible for their degradation are numerous and diverse. The major structural polymers found in plant cell walls (PCW) are cellulose, a variety of hemicelluloses, the most abundant of which is xylan, and lignin. The structure of these polymers confer the rigidity and strength to the cell wall, and these qualities exert an influence on the activity of those enzymes that utilize them as substrates. Cellulose is a linear polymer comprising of β-1,4-linked glucose subunits that form long chains bound together by numerous hydrogen bonds. This interaction between many cellulose molecules leads to the formation of insoluble crystalline microfibrils, which are characteristic of cellulose and give it its tensile strength. Microfibrils are found in cell walls organized into parallel sheets providing rigidity and strength. Often they are embedded in a hemicellulose and lignin matrix (Bégiun and Aubert 1994). Xylans are the commonest of the hemicelluloses, which constitute around a third of plant biomass. The basic chain consists of β-linked xylopyranose residues, which in certain marine algae is a mixture of β-1,3 and β-1,4 linkages (Barry and Dillon 1940), but is typically only β-1,4 linked in higher plants. Unlike cellulose, the xylan backbone carries side chains such as arabinofuranoside (Coughlan and Hazelwood 1993) which ensure that xylans are amorphous and lack the highly crystalline structure observed in cellulose.

The hydrolysis of the β-1,4 glycosidic bond found in the structural polymers of plant and many macroalgal cells is catalyzed by *O*-glycosyl hydrolases (E.C. 3.2.1), which for the purposes of this discussion are termed β-glycanases. These enzymes are widely distributed among saprophytic bacteria and fungi and have been the subject of extensive research in recent years into their mechanisms of activity, molecular biology, and structure. Typically, complete hydrolysis of plant structural polymers requires the cooperative activity of a consortium of microbial enzymes (Coughlan and Hazelwood 1993). To achieve hydrolysis, the majority of organisms involved in hydrolysis synthesize a variety of cellulase and xylanase enzymes (Fig. 1).

The structure of β-glycanases

β-Glycanases are found in both aerobic and anaerobic bacteria. Such enzymes from aerobic bacteria are somewhat different from those of anaerobes, and it is postulated that this is because of the rather different energy availability under these two conditions. Aerobic bacteria possess β-glycanases that are nonaggregated; essentially there are multiple enzymes acting in a synergistic fashion to degrade the substrate. In contrast, the anaerobic enzymes involved in

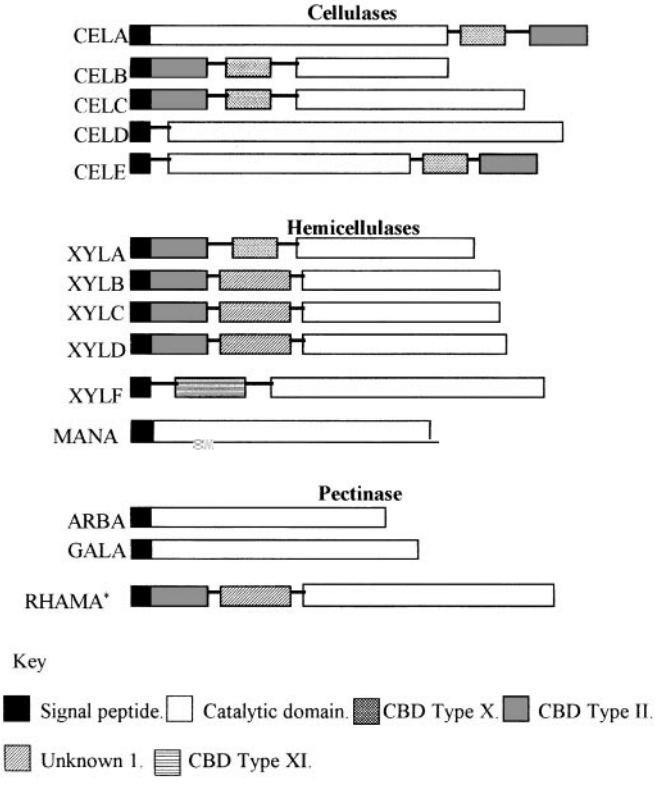

Fig. 1. The molecular architecture of *Pseudomonas fluorescens* subsp. *cellulosa* plant cell wall hydrolyzing enzymes. *, GW Black, unpublished data

plant cell wall polymer hydrolysis can be aggregated into high molecular weight multienzyme complexes. Probably among the best studied of these anaerobic systems is the cellulosome of *Clostridium thermocellum.* This structure contains several polypeptides that have distinct structural, catalytic, and substrate-binding functions which only act optimally when in a synergistic association (Garcia-Campayo and Béguin 1997). Indeed this modularity is the unifying feature of β-glycanases under both conditions as aerobic enzymes are similarly composed of several functionally discreet domains (Fig. 1). Aerobic β-glycanases are composed of three or more domains; however, only the catalytic domain and the cellulose-binding domain (CBD) are ubiquitous. Each domain can be joined by a flexible linker region, often rich in hydroxyamino acid or proline residues (Gilkes et al. 1991); the function of this region may be to confer structural flexibility on the enzyme, enabling the catalytic domain to have to have access to more glycosidic bonds (Black et al. 1996; Hazelwood and Gilbert 1998).

The enormous diversity in the structure of plant cell wall polymers is reflected by an equal diversity in the structure of the catalytic domains of β-glycanases. Therefore, to stem confusion, a nomenclature has been adopted based on amino acid sequence similarities within the catalytic domain, which has allowed division of the β-glycanases into a number of families (Henrissat 1998).

Mechanisms of activity

Despite the complexity of the substrates and the diversity of the enzymes, common themes relating to the activity of β-glycanases have emerged. Kinetic studies have demonstrated that many glycosyl hydrolases are active toward a range of soluble substrates; for instance, the xylanase A of *Pseudomonas fluorescens* showed optimal activity against oat spelt xylan, but was also capable of hydrolyzing various glycoside derivatives (Black et al. 1997). This lack of specificity, often seen among cellulases and xylanases, probably reflects the complexity of the plant cell wall, which may favor organisms that produce enzymes with activity against several related polymers.

The mechanisms by which hydrolysis of plant cell wall polymers are brought about are not fully resolved. Hydrolysis of cellulose has received the most attention, and forms the basis of the current paradigm. Complete degradation is achieved by three types of enzymes acting synergistically. Initially, endo-acting cellulases hydrolyze β-1,4 glycosidic bonds within the cellulose chain producing shorter oligosaccharides. The free ends of the oligomers are recognized by the active sites of exo-acting cellulases, which hydrolyze the bonds to produce disaccharide fragments. β-Glycosidases subsequently degrade these disaccharides into their component subunits (Wood and McCrae 1972). Although the simplicity of this model is attractive, the synergistic cooperation involved in polymer degradation now appears to be rather more complex. Recent studies have shown that exo-acting β-glycanases such as cellobiohydrolases (CBH) have endo-acting activity (Davies and Henrissat 1995), resulting in observations of synergistic degradation between two exo-acting CBH activities (Henrissat 1994). It has become evident that there is no kinetic requirement among cellobiohydrolases for cellulose chain ends, and thus the terminology of exo-acting and endo-acting enzymes is redundant. The activity of β-glycanases acting on polymer chains is probably more satisfyingly explained by the concept of processivity (Boisset et al. 1998). The topology of the active site of β-glycanases has one of three configurations; a pocket, found in β-glycosidases, a groove, typical of endo-acting cellulases, or a tunnel, as observed in cellobiohydrolases (Davies and Henrissat 1995).

Enzymes that have a pocket or groove with only a few sugar-binding subsites will not remain bound to the polysaccharide chains after hydrolysis has occurred. In contrast, if the enzyme has an extended groove or tunnel topology to the active site, many more sugar-binding subsites are available; therefore, after the glycosidic bond has been hydrolyzed, release of the polysaccharide chain is unlikely, promoting processive degradation of the substrate (Divine et al. 1998). Processive degradation is most commonly observed in CBH; however, endoglucanases can also have extended active sites that allow processive degradation (Reverbel-Leroy et al. 1997). The endo-activity observed in some CBHs can be explained by the polypeptide loops forming the "lid" of the active site tunnel opening, allowing access to the substrate without the requirement for chain ends (Davies and Henrissat 1995). Enzymes involved in the hydrolysis of polymers such as cellulose and xylan can, therefore, be described as preferentially exo-acting or endo-acting, which promotes the efficiency of polymer hydrolysis (Warren 1998). Hence, CBHs can create chain ends by endo-attack if none are available.

It is the widely held view that these enzymes are efficient catalysts of the hydrolysis of crystalline cellulose (Teeri et al. 1998). However, cellobiohydrolases may serve more than a simple catalytic role. There is evidence that CBHs are also involved in disrupting the structure of crystalline cellulose by utilizing the energy obtained from the hydrolysis of the glycosidic bond. CBHs display rather unusual kinetic characteristics; for instance, k_{cat} is low compared to that observed in typical "exo-"acting glycanases. They can also change their kinetic parameters profoundly as the result of a single mutagenic event, which implies these enzymes are very inefficient (Sinott 1998). This has led to the suggestion that the role of CBH is to couple the energy released by the hydrolysis of the glycosidic link to the disruption of crystalline cellulose, an activity they share with certain non-catalytic binding domains.

An understanding of the mechanism such as processivity by which β-glycanases work has been facilitated by determining the three-dimensional structure of such enzymes using X-ray crystallography. To date, 74 different *O*-glycosyl hydrolase structures (mostly cellulases and xylanases) have been solved (Henrissat 1998). Within the groove or tunnel are located the substrate-binding subsites. Each subsite is responsible for binding an individual glucose residue of the cellulose molecule. β-Glycosidic bond cleav-

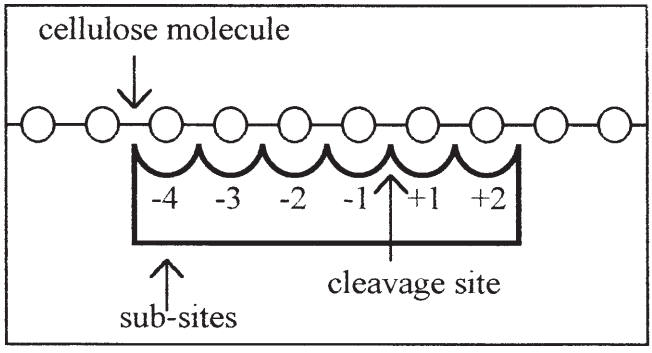

Fig. 2. Schematic representation of the binding of the active site of a β-1,4-glycanase to a cellulose molecule. The substrate is bound to the active site at a number of subsites labeled −4 to +2; the hydrolytic event occurs at subsite +1

age occurs between subsites −1 and +1 (Fig. 2) and can be affected by distant substrate-binding subsites, such as −2 and +2. Binding involves the formation of hydrogen bonds between the hydroxyl groups of the glucose residues and the polar side chains of the amino acid residues located in each subsite, or hydrophobic stacking of the glucose residues and the aromatic side chains of amino acids located in each subsite. This binding energy is used to distort the glucose residue in subsite +1 from a chair formation to a skewed-boat formation; a conformational change that is necessary to allow in-line nucleophilic attack of the anomeric carbon atom of the glucose residue in subsite +1 and subsequent cleavage of the glycosidic bond via acid–base catalysis.

Hydrolysis occurs at the reducing end of the sugar residue at the −1 site and can proceed via two mechanisms. "Inverting" enzymes employ two catalytic residues separated by 9–10 Å; one interacts with a water molecule, leading to the production of an hydroxyl ion, which launches a nucleophilic attack on the anomeric carbon of the sugar ring. The second residue acts as an acid–base catalyst and protonates the leaving group. The configuration of the two catalytic residues is therefore inverted. "Retaining" enzymes also employ the same two acid–base and nucleophilic residues; however, they are separated by 5–6 Å which enables simultaneous protonation by the acid–base residue and direct nucleophilic attack by the second residue, which forms a glycosyl-enzyme intermediate. The acid–base catalytic residue then activates an incoming water molecule to launch a nucleophilic attack on the intermediate. After the resulting hydrolysis, the catalytic sites are retained in their original configuration (Bégiun and Aubert 1994; Davies 1998).

Binding domains

Binding domains are structurally independent noncatalytic domains that appear to mediate the attachment of the enzyme complex to the polysaccharide substrate. They are found on many carbohydrate hydrolases including amylases, chitinases, and β-1,4 xylanases (Ferreira et al. 1993). The biological role of binding domains has yet to be established; however, they do enhance cellulase activity against insoluble cellulose (Hall et al. 1995). In *Pseudomonas fluorescens* the CBDs of the xylan-degrading enzymes XYLA and XYLC were deleted; subsequently, activity against xylan contained within insoluble cellulose-hemicellulose complexes was reduced compared to the wild type enzymes, whereas activity against soluble substrate was unaffected (Ferreira et al. 1990; Black et al. 1996). Family II CBDs of *Cellulomonas fimi* have been demonstrated to bind irreversibly to its substrate. Despite this, the catalytic domain has been demonstrated to diffuse across the surface of the substrate (Boraston et al. 1998); perhaps CBDs prevent the secreted enzyme from diffusing away from the bacterium, which may itself be adsorbed to the substrate. Some family II CBDs of *C. fimi* disrupt the surfaces of cotton fibers, releasing small particles, although they lack hydrolytic activity (Warren 1996). This disruption of crystalline cellulose has also been proposed as one of the functions of the catalytic domains of CBHI and CBHII of *T. reesi* (Sinott and Sweilem 1998), but whether CBDs and CBH can act cooperatively to achieve disruption of crystalline cellulose has not been explored.

Cellulose-binding domains can, like the catalytic domains, also be grouped into distinct families based on their amino acid sequence similarities; 180 putative CBDs have been identified that are currently divided into 13 families. Structural data have been obtained for representatives of 5 of these families (I–V), indicating that they are all antiparallel β-strand polypeptides. A more detailed study on *C. fimi*, which expresses a number of β-glycanases possessing CBDs from families II and IV, has indicated those features present in the CBDs of these enzymes that appear to contribute toward their activity in vivo. The surface of the CBDs from families II, which binds to crystalline cellulose, has highly conserved aromatic residues linearly arranged on a planar face. The presence of these residues presumably enables this planar surface to bind to the cellulose via hydrophobic interaction. Significantly, family IV CBDs did not possess a planar face and were shown not to bind to crystalline cellulose; instead, they adsorbed to amorphous cellulose and soluble cellulose derivatives (Boraston et al. 1998).

"Cold" enzymes as tools in elucidating β-glycanase activity

Despite the extensive research being carried out on the degradation of plant cell walls, many of the processes of polymer hydrolysis are still unresolved, and the molecular analysis of "cold" β-1,4-glycanases has an important role to play in the continuing development of our understanding of enzymatic hydrolysis of these complex macromolecules. Because of the paucity of information about those organisms which undertake the degradation of cellulose and hemicellulose at low temperature, we do not have informa-

tion on basic structural properties of the enzymes involved, such as whether cold low-energy environments have selected for either highly efficient complexes of aggregated cellulases and xylanases, resembling those found in similarly energy-limited anaerobic environments, or the nonaggregating enzyme systems, typical of energy-rich aerobic environments.

The other key question that needs to be addressed is how do cold cellulases and xylanases achieve bond cleavage? As chemical reactions are temperature dependent, at low temperatures extra energy is required that is not available from the environment. It may be that binding is more efficient with cold cellulases and xylanases such that extra binding energy is released. As has been discussed, bond cleavage requires that the glucose residue in subsite +1 has to be distorted into a skewed configuration; what is not obvious is where the energy to achieve this comes from at such low temperatures. Perhaps the enzymes are so flexible that binding generates sufficient energy to distort the glucose residue. If this is the case, then we need to identify the structural features that allow this to occur. Finally, the large body of data collected on mesophilic β-glycanases will enable meaningful comparative studies to be conducted between them and their psychrophilic counterparts. Such studies may also illustrate the evolutionary relationships between these enzymes.

Biotechnological applications of cold β-glycanases

The biotechnological applications of "cold" enzymes have been discussed in several recent reviews (Margesin and Schinner 1994; Brenchley 1996; Russell and Hamamoto 1998). The major advantages of such enzymes are that they enable efficient catalysis to occur at lower temperatures, reducing the requirement for energy. Efficient low-temperature processes would also be useful in biotransformations or synthetic processes involving volatile substrates. In addition, such enzymes can also be easily denatured to halt a process by moderate heating. Interest in β-glycanases has included their use as additives to detergents. Cellulases can improve the performance of detergents by removing fibers that cause fuzzing of frequently washed garments. Such enzymes, which operate at a low temperature, would therefore improve the quality of cold washing, which in turn has cost savings associated with reduced energy consumption.

There is also a role for β-glycanases in the paper industry. Cellulases are used in paper manufacture to increase the beatability of pulp (Viikari et al. 1998), and xylanases can aid in bleaching the paper pulp by enhancing lignin removal (Paloheimo et al. 1998) and may also find a role in the bioremediation of the wastewater produced during this process. In the food industry, pectinases capable of functioning at low temperature could have a role in the clarification of fruit juices and enhance the ripening of cheeses. In the long term, an understanding of why cold enzymes are capable of rapid catalysis at low temperatures, coupled with knowledge of how enzymes from thermophiles are stable at elevated temperatures, could make it possible to stabilize cold enzymes so that they do not inactivate at higher temperatures and therefore create highly efficient enzymes which work at elevated temperatures.

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