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v. 43Enzymatic Hydrolysis of β -glycans

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The polymeric constituents of cell walls are polyesters, polypeptides and polysaccharides (Table 1). The esters may be polymers of a hydroxy acid, or polymers made up of alternating units of phosphate and a sugar alcohol (2). The alcohol may in turn be substituted with a sugar unit and/or alanine. (These structures resemble the nucleic acids, the latter differing in having a sugar rather than a sugar alcohol in the backbone). In general, these materials can be hydrolyzed with alkali, and the use of enzymes is restricted to removal of phosphate and of sugars from the hydrolysis products.

Polysaccharides are the most abundant and important components of the walls. They vary in complexity from those containing a single sugar and a single linkage type to those containing several sugars or modified sugars with several types of linkages (Fig. 1, Table 2). Enzymes have been used in the analysis of both the simple and the complex. Even in simple systems we are finding (as you will see) that the enzyme action is not well understood. It is wise, therefore, to be especially careful in evaluating the results of hydrolysis of the more complicated compounds.

ACIDS

Acid Hydrolysis

Before discussing enzymatic effects, I would like to say a few words about acid hydrolysis. Acids are widely used in the hydrolysis of wall materials. The action is random though

Table 1. Polymers of the cell wall

polyesters	$\left(\begin{array}{c} \text{O} \\ \parallel \\ \text{O}-\text{CH}-\text{CH}_2-\text{C} \\ \\ \text{CH}_3 \end{array} \right)_n$	poly β hydroxybutyrate
	$\left(\begin{array}{c} \text{O} \\ \parallel \\ \text{P}-\text{O}-\text{R}-\text{O} \\ \quad \\ \text{OH} \quad \text{G} \quad \text{A} \end{array} \right)_n$	teichoic acids (R=glycerol or ribitol) (and nucleic acids, R=ribose, etc.) G=sugar moiety; A=alanine)
polypeptides	$\left(\begin{array}{c} \text{NH}-\text{CH}-\text{CO}-\text{NH}-\text{CH}-\text{CO}-\text{NH}-\text{CH} \\ \quad \quad \quad \quad \quad \quad \\ \text{R} \quad \quad \quad \text{R}_1 \quad \quad \quad \text{R}_2 \end{array} \right)_n$	
polysaccharides		chitin, glucans, mannans

Table 2. Factors affecting complexity of polysaccharides

Sugar	Modifications	Linkages	Branching
glucose	oxidation: uronic acids aldonic acids	β 1,4 α 1,3	O-X branches
galactose	reduction: deoxy sugars	1,6	
mannose	terminal sugar	1,2	
fucose	alcohol		
xylose	substitution: NH_2 ; $\text{NH}-\text{C}(=\text{O})-\text{CH}_3$		
arabinose	esters: <i>o</i> -acetyl <i>o</i> -malonyl		
rhamnose	ethers: carboxyethyl (muramic)		
	ring size and shape: pyranose, furanose		
	linked to protein: lipid		

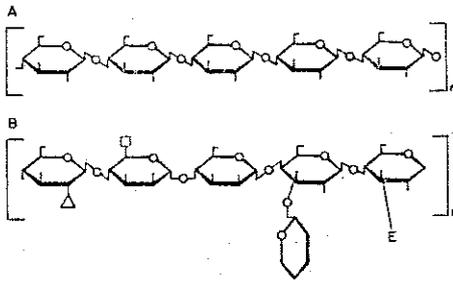


Fig. 1. A. Simple polysaccharide having one sugar type and one linkage type; B. Complex polysaccharide with substitution and branching, and with various types of linkage.

terminal linkages possibly are more resistant to hydrolysis than internal bonds. For the production of oligosaccharide products, concentration of acid and temperature are important. Reproducible results can be achieved by hydrolyzing to a pre-determined reducing value.

All types of linkages are not equally susceptible to acid hydrolysis. For the glucose disaccharides, the order from susceptible to resistant is: (α 1,2; α 1,4; α 1,3) > (β 1,2; β 1,3) > (β 1,6; β 1,4) > α 1,6. (Wolfson, 40). Whether the same sequence

occurs for the hydrolysis of these linkages in polysaccharides is not certain. It has been observed that the α 1,2 and α 1,3 links of yeast mannan are more readily split than the α 1,6 linkages (Peat, 24), acid hydrolysis yielding only the α 1,6 oligomannans.

In spite of the disadvantages of acid hydrolysis, e. g., lack of specificity and possibility of reversion, the method is useful in linkage analysis. The polymer is hydrolyzed to yield a series of oligosaccharides which are then separated on a charcoal column, and subjected to conventional methods of analysis. More widely used, however, is methylation analysis in which all free OH groups of the polymer are methylated prior to hydrolysis. From an analysis of the methylated sugars, the nature and frequency of linkages can be determined. Recently trimethylsilyl derivatives have been found useful (Sweeley, 34) for identification of the simple sugars and the dimeric and trimeric products of hydrolysis. These derivatives form quickly and are readily separated and identified by gas-liquid chromatography.

Enzymes

A. Isolation and purification of wall components.

A desired component of the cell wall can be obtained by enzymatically hydrolyzing all other materials present. This usually involves treatment with polysaccharases but the action of proteases or of lipases is often beneficial. The enzyme system is selected for the job to be accomplished. It must contain the enzymes required but must not destroy the desired product.

For successful selective degradation, the nature of the substrate is as important as the nature of the enzyme system. Since enzymes are large protein molecules, they do not readily diffuse into most solids. While they can macerate succulent leaf tissue as a result of their diffusion between cells, it is difficult for them to penetrate most woody materials. Cell walls vary in their susceptibility to enzyme attack. Those that are resistant can sometimes be made more susceptible by mild chemical action, by the extraction of fatty materials, or by mechanical disintegration.

The extraction of α -mannan from yeast is a good example of the enzymatic approach. The major component to be removed is a β -1 \rightarrow 3 glucan (Table 3).

Table 3. Enzymes for the isolation of components of cell walls

Desired Product	Wall Material of	Substance to be removed	Enzyme Source	Ref.
chitin	mushroom, after extractions with water, 2% KOH, and alcohol (=25% of dry weight)	glucan	<i>Trichoderma viride</i> QM 6a	(R)
α -mannan	yeast cells	β -glucan	β -1,3 glucanase of <i>Sporotrichum pruinosum</i>	(R)
α -mannan + β -glucan	<i>Candida</i> , <i>Trichophyton</i> , <i>Microsporium</i>	protein	Trypsin	(4)
carbohydrate + mucopeptide	<i>Streptococcus</i>	protein	Trypsin; or Chymotrypsin	(15)
carbohydrate	<i>Streptococcus</i>	mucopeptide	Lysozyme; or <i>Streptomyces</i> enzyme	(15)

Enzymes capable of this hydrolysis are widespread and readily available. At the same time, α -mannanases are currently unknown (and we have looked for them) so there is little danger of simultaneously hydrolyzing the desired product. Both the enzymatic and the alkali extraction methods give the same amount of mannan but the former contains appreciably more nitrogen (1.27 vs. 0.25%). In general, it may be expected that the milder, enzymatic methods will give less pure products, i. e., the very specificity of the reaction favors the retention of bonding. This may or may not be desirable.

B. The "pre-hydrolytic" or "activating" enzyme (C_1).

The initial disruption of wall material may be the result of a rather limited action. A number of years ago we suggested that the variable action of cellulases of different origin on cotton might be due to the presence or absence of a pre-hydrolysis factor which, because it had to act first, we called " C_1 ". There is a parallel situation in the digestion of wool by clothes moth larvae (16) where it has been demonstrated that proteases of the intestine of the larvae are unable to act until after the disulfide bridges of the wool are

reduced. The reduction permits swelling and subsequent enzyme action. We believe that C_1 action leads to a similar result but we have no idea how it works. It is detectable by submitting cotton fiber to its action, and then subsequently adding various cellulases (C_x). Without the C_1 , the cellulases are relatively inactive on cotton, although they may be highly active on soluble cellulose derivatives, or on degraded celluloses. With C_1 , the cotton is degraded and soluble sugars produced. C_1 alone produces an effect which is undetectable, except by subsequent exposure to the hydrolytic enzymes. Selby (32) has found a similar system, e. g., a factor C_A responsible for tensile strength loss, that differs from the C_x complex. The identity of C_A and C_1 has not yet been demonstrated. Both have been separated physically from the cellulolytic enzymes (C_x).

The occurrence of factors of the C_1 type may be more widespread than heretofore realized. In complex polymers, the site at which hydrolysis normally takes place may be buried within the complex, or protected by an adjacent fragment. For the enzyme to act, some conformational change must be effected. In proteins, it may be denaturation, splitting of disulfide linkages, or removal of a peptide chain as in the conversion of zymogens to enzymes. In complex structures made up of several components, it may be the removal of an encrusting substance, or the hydrolysis of a bond between diverse components.

Being unable to determine the nature of the change brought about by the C_1 component acting on cotton, we are always looking for other examples of a similar type of action. Lysis of microbial cell walls (35) may be such an example. Lysis is the conversion of an insoluble material to soluble products. The products may be polymeric, and as such the reaction limited to hydrolysis of a *very few* linkages. Indeed, I believe that most lysis is of this nature. A specific example is the hydrolysis of *Aspergillus oryzae* walls by an enzyme from *Bacillus circulans* to form a soluble polymer of glucose and galactose (12). If this explanation of lysis is correct, it must follow that it will be difficult to determine the nature of the linkage, and also of the enzyme, involved. One must be extremely cautious in attributing lysis to a particular enzyme, based on the nature of the ultimate hydrolysis products. The enzyme preparations may well contain a second component (e. g., glucanases, chitinases, etc.) capable of rapidly hydrolyzing the soluble polymer to simple sugars. This latter step must not be confused with lysis.

Another possible example of the C_1 - C_x type recently reported (Merrick, 18), deals with the digestion of poly- β -hydroxybutyrate (PHB) granules. "Depolymerization by purified extracts depends on the successive action of two components: a heat stable 'activator' and a heat labile 'depolymerase'. Under certain conditions, trypsin can replace the 'activator' ... No change in morphology was detected with 'activator' treated granules." (18). Whereas "native" granules are susceptible to digestion, solvent treated or heat treated granules are resistant. This is similar to the resistance developed by cotton fibers as they dry after the boll opens. The explanation of the first step in digestion of both PBH and cotton may be more a problem for physical chemists than for enzymologists.

C. The random-splitting or endo-polysaccharases.

(a) the hydrolysis products.

Table 4. Breakdown products of polysaccharides by endo-polysaccharases

Polymer	Enzyme	Products	Ref.
<u>single linkage: single sugar</u>			
cellulose (β 1,4 glucan)	cellulase of <i>Streptomyces</i>	$[G]_3: [G]_2 = 1:3$	(30)
pustulan (β 1,6 glucan)	β -1,6 glucanase of <i>Penicillium</i> *	$[G]_3: [G]_2: [G]_1 = 1:1.5:0.35$ (also $[G]_4$)	(29)
mannan, ivory nut (β 1,4 mannan)	mannanase, <i>Penicillium</i> *	$[M]_3: [M]_2: [M]_1 = 1:3.6:0.7$	(R)
laminarin (β 1,3 glucan)	β -1,3 glucanase of <i>Rhizopus</i>	$[G]_5, [G]_4, [G]_3, [G]_2$	(R)
amylose (α 1,4 glucan)	amylase of <i>Bacillus</i>	cyclic $[G]_6 - [G]_3$	
amylose (α 1,4 glucan)	salivary α -amylase	$[G]_3: [G]_2 = 1:1.6$	(38)
pectin (α 1,4 methylgalacturonan)	"eliminase"	$\Delta^{4,5}$ unsat. uronic 1 α 4 uronic acid	(1)
<u>two linkages: single sugar</u>			
levan (β 2,6 fructan)	levan polyase, <i>Azotobacter</i>	$[Fr]_{11} \pm$	(11)
lichenin (β 1,3, 1,4 glucan)	"Bacterial Amylase", Novo Terapeutisk	$G1 \rightarrow 4G1 \rightarrow 3G^*$	(28)
mycodextran (α 1,3, α 1,4 glucan)	mycodextranase of <i>Penicillium</i>	$[G]_4 = G1 \rightarrow 3G1 \rightarrow 4G1 \rightarrow 3G^*$ + $[G]_2 = G1 \rightarrow 3G^*$	(27)
glycogen; amylopectin (α 1,4, α 1,6 glucan)	salivary α -amylase	α -1,4 dextrans containing 1 or 2 α 1 \rightarrow 6 linkages; maltose; maltotriose	(38)
dextran (α 1,6, α 1,3 glucan)	dextranase of <i>Penicillium</i>	$G1-6G1-6G1-6G^*$; $G-6G-6G-6G$ $\begin{array}{c} 3 \qquad \qquad 3 \qquad \qquad 6-G^* \\ \qquad \qquad \\ G \qquad \qquad G \end{array}$ and isomaltose	(13)

* Possibly containing in addition a low level of glycosidase

The endo-polysaccharases hydrolyze their polymeric substrates in a random manner to produce smaller fragments. Hydrolysis may not be purely random, for end-linkages are often found to be resistant (38, 39). When the substrate is soluble, the rate of hydrolysis does not seem to be appreciably affected by chain length (above DP 6). When the substrate is insoluble (cellulose, etc.), the initial rate is low compared to that of the solubilized fragments.

Endo-polysaccharases differ in the nature of the end products which result from hydrolysis of the polymeric substrate (Table 4). Or it may be said that these enzymes vary in their ability to hydrolyze the shorter oligosaccharides; the lower the DP, the more resistant the fragments to hydrolysis. Thus, the relative rates of hydrolysis by *Myrothecium verrucaria* cellulase (Whitaker, 39) are

$$[G]_5 \ 500 > [G]_4 \ 83 > [G]_3 \ 16 > [G]_2 \ 1.2$$

In line with the resistance of terminal linkages, the tetramer, which is the shortest unit having a non-terminal linkage, is often the shortest unit susceptible to hydrolysis. Occasionally trimers are slowly hydrolyzed by some endo-polysaccharases but the dimers are invariably resistant. [Even the exo-enzymes, such as the glucamylase of *Rhizopus delemar* (20), may show this preference for longer chains, the dimer (maltose) being hydrolyzed at about

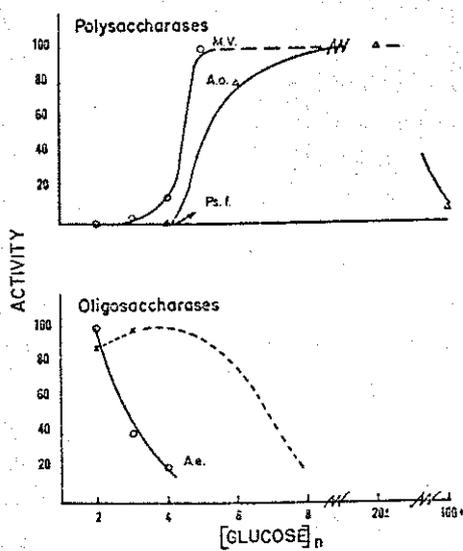


Fig. 2. Effect of chain length (DP) on enzyme action.

Enzymes: M. v.=cellulase of *Myrothecium verrucaria* (Whitaker)

A.o.=cellulase of *Aspergillus oryzae* (Grassman)

Ps.f.=cellulase of *Pseudomonas fluorescens* var. *cellulosa* (19)

A.e.= β -glucosidase of almond emulsin

x-x="cellobiases" of *Aspergillus phoenicis*, and of *Pestalotiopsis westerdijkii* untested except as shown (Reese unpublished).

minant. Tetramers and pentamers, etc., are often observed. In an unusual instance, the products are cyclic oligosaccharides (Schardinger dextrans).

Increasing the complexity of the polysaccharide leads to hydrolysis products which differ from those of the parent polysaccharide (Table 4). Very long fragments (DP 11) result from the action of levan polyase on a levan (2,6) containing occasional 2,1 links. Branches remain in the products of hydrolysis of branched polysaccharides. Dominant trimers or tetramers result from the action of specific enzymes on polymers of mixed linkage. Such products, it can be seen, are characteristic of the initial material and useful for its identification. Their production is the result of the action of specific enzymes (often of somewhat rate occurrence).

One occasionally needs to be reminded that hydrolysis is not the only enzymatic means of depolymerization and that the products of such reactions are quite different from those of hydrolysis. Phosphorylation is well-recognized in the α -1,4-glucan system, and its role in the breakdown of cellobiose has recently been demonstrated. More unusual is the action of the recently discovered "eliminases" (1) where chain scission results from the removal of a

1/7th the rate of the pentamer]. Glycosidases and other oligosaccharases, tend to show the opposite relationship, i.e., a decreasing rate as the chains increase from 2 to 3 to 4 units in length though there are a number of instances in which the rate remains nearly constant over this range. In these, the rate may begin to decrease with the pentamer or hexamer (Fig. 2).

The products which accumulate during hydrolysis are those most resistant to the action of the enzyme. In a cellulose digestion by a *Streptomyces* cellulase, the only detectable products are cellotriose and cellobiose in ratio (weights) of about 1 to 3 (Table 4). In an amylose digestion by salivary α -amylase, the products are maltotriose and maltose in a weight ratio of 1:1.6 (Whelan, 38). Here there is a deviation from purely random hydrolysis due to the resistance of both terminal linkages to the action of the enzyme.

Whereas dimers and trimers and the most common end products of endopolysaccharase action (Table 4), the reaction can be stopped at a stage in which larger products predominate.

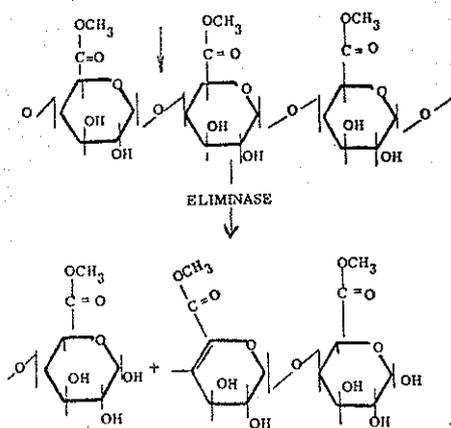


Fig. 3. Pectin "transeliminase".

having reducing properties and lowered viscosity. As a result—unless the products are further characterized—one is not likely to suspect that the action is anything but hydrolytic. There is, however, an easy way to detect the "eliminase". This is the appearance of a strong absorption of the unsaturated products at 232 $m\mu$. Another difference from the hydrolases is in the position of cleavage. In the eliminases the split it between C_4 and the bridge oxygen; in the hydrolytic enzymes, between the C_1 and the oxygen. Recently, it has been reported (17) that at least one of the eliminases acts from the *reducing* end of the polymer. If verified, this will be contrary to the action of most *exo*-poly-saccharases (which act from the *non-reducing* end).

(b) Effect of substitution on enzymatic hydrolysis (31).

Substituents on the sugar units of linear polysaccharides affect hydrolysis by interfering with the approach of the enzyme to the site to be hydrolyzed. The most common substituents are other sugars or chains of sugars, and the result is a branched polysaccharide. The size or nature or even the position of the substituent appears to be relatively unimportant.

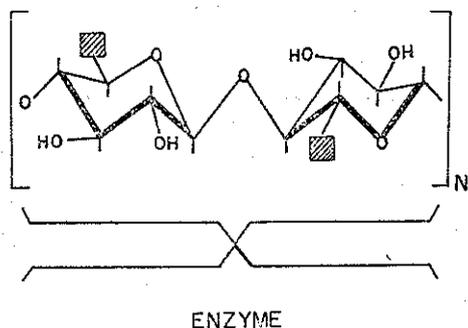


Fig. 4. Disaccharide moiety for which enzyme has an affinity:

▨ = H, xylanase; = CH_2OH , cellulase, etc.

hydrogen from the carbon α to the carbonyl and elimination of a group from the β carbon, with the production of a disaccharide having unsaturation in the non-reducing sugar moiety (Fig. 3).

At present, eliminases seem to be restricted in their action to polymers containing a uronic acid group: hyaluronic acid, pectic acid, pectin (1) and alginic acid (i. e., polymers containing glucuronic, galacturonic or mannuronic acids). The linkage may be α or β . The hydrogen on C_5 may be *cis* or *trans* to the oxygen on C_4 . These enzymes bring about depolymerization, the products

It is the presence of a substituent, or the absence of a substituent, which is significant. Xylanase acts on a chain of xylose units where the C_5 substituent is H, but does not act when the C_5 substituent is COOH , CH_2OH , etc. Cellulase acts on a similar chain when the C_5 substituent is $-\text{CH}_2\text{OH}$, but not when this group is modified either to a smaller ($-\text{H}$) or large size ($-\text{CH}_2\text{O}-\text{CH}_2-\text{CO}-\text{ONa}$). These examples, of course, merely emphasize the fact of specificity (Fig. 4).

The frequency of the substituent is of

great importance. The introduction of a group does not necessarily slow down the rate of hydrolysis. When the initial substrate is insoluble, as in cellulose, substitution may improve solubility by preventing aggregation of linear chains, and thus greatly increase the rate of enzymic hydrolysis. At the same time, these groups inhibit hydrolysis in the neighborhood of the sugar unit on which they occur and do limit the extent of hydrolysis. As the frequency of their presence (degree of substitution, DS) approaches one for every sugar unit, the extent of hydrolysis approaches zero (Fig. 5). The hydrolysis of carboxymethyl

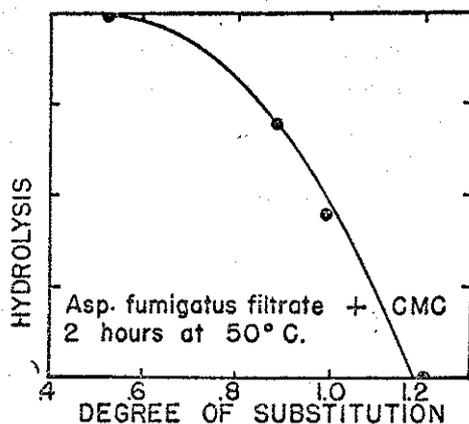


Fig. 5. Effect of degree of substitution on susceptibility of carboxymethyl cellulose to enzyme hydrolysis.

cellulose by cellulase illustrates these points. The flattening of the curve at low DS is an indication that hydrolysis is limited here by solubility. The absence of hydrolysis at high DS is due to interference by the carboxymethyl (or hydroxyethyl-, or methyl-, etc.) groups. That a DS greater than one is required for complete resistance is merely a reflection of the fact that the distribution of groups is such that at DS 1.0 a few glucose units have 2 substituents and a few have none. It is this randomness of distribution, coupled with the lack of uniformity in the substrate being treated that makes it difficult to deduce from this experiment the minimum number of adjacent unsubstituted glucoses required for enzyme hydrolysis. At least *one* unsubstituted glucose unit is required. But is one such unit enough? Dr. Perlin attacked this problem (26) using a *Streptomyces* enzyme on an arabinoxylan, by determining whether the xylose end units of the fragments resulting from hydrolysis were branched or unbranched. Both the reducing and non-reducing end units of the products were unbranched. Therefore, this enzyme has a minimal spacial requirement of *two* adjacent-xylosyl units between branches (Fig. 6).

Our recent results (unpublished) with guar support this interpretation. Guar fractions, of the configuration $-M-M-M-M-M-$, are resistant to hydrolysis by β -mannanase. There is not



enough room between branches for the enzyme to fit. These data force us to a revision of our earlier estimate that a DS of 1 is required if a substrate is to be resistant to enzyme hydrolysis (31). The behavior of guar and the data of Perlin indicate that a DS of 0.5 (i.

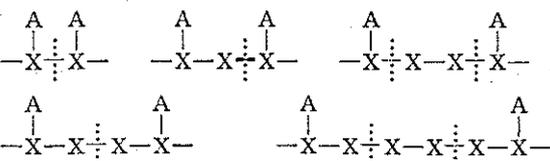


Fig. 6. Possible sites of action of xylanase on an arabinoxylan (after Perlin, 26).

e., one substituent on every other unit) is sufficient to establish resistance, provided, of course, that the substituents are uniformly distributed (See, however, a later section for an exception involving *exo*-glucanase).

The earlier results with cellulose derivatives are not contradictory to these recent findings. One should note that in all instances the requirement is "at least" so much space for the enzyme, and that even more may actually be needed.

In line with the inhibitory effect of substitution is the general observation that the more highly branched polysaccharides are more resistant to enzymatic hydrolysis than the unbranched. The more branching in dextran, for example, the more resistant it is to endo- α -1,6 glucanases (5,25). Analysis of the hydrolysis products of a branched dextran (Table 4) shows that one linkage on the non-reducing side and two linkages on the reducing side of the branch are resistant to hydrolysis (Hutson, 13). Here the spacial requirement for enzyme action may exceed two unsubstituted units between branches.

(c) Specificity of endo polysaccharases

Recent investigations of glucans (7, 21, 25, 28) having both β -1,3 and β -1,4 linkages have led to a reconsideration of the factors affecting the specificities of the glucanases. Two important facts have emerged from this work:

(1) The β -glucanases are *not* specific for the linkage which they split. Cellulases may split β -1,3 or β -1,4 bonds in lichenin. The enzyme which randomly splits the β -1,3 links of laminarin preferentially splits the β -1,4 bonds of lichenin, even though β -1,3 bonds are also present.

(2) All products of hydrolysis of a β -glucan by a particular enzyme have the same terminal linkage between the two glucose units at the reducing end of the chain. The products of hydrolysis by a cellulase and by a β -1,3 glucanase are:

	<u>Cellulase</u>		<u>β-1,3 glucanase (endo-)</u>
from cellulose	<u>G1 \rightarrow 4G</u>	from laminarin	<u>G1 \rightarrow 3G</u>
	G1 \rightarrow 4 <u>G1 \rightarrow 4G</u>		G1 \rightarrow 3 <u>G1 \rightarrow 3G</u>
			G1 \rightarrow 3 G1 \rightarrow 3 <u>G1 \rightarrow 3G</u>
from lichenin	<u>G1 \rightarrow 4G</u>	from lichenin	<u>G1 \rightarrow 3G</u>
	G1 \rightarrow 3 <u>G1 \rightarrow 4G</u>		G1 \rightarrow 4 <u>G1 \rightarrow 3G</u>
	G1 \rightarrow 4 G1 \rightarrow 3 <u>G1 \rightarrow 4G</u>		G1 \rightarrow 4 G1 \rightarrow 4 <u>G1 \rightarrow 3G</u>

Common to all of the cellulase products is a cellobiose (1 \rightarrow 4) moiety at the reducing end of the oligosaccharide; common to all β -1,3 glucanase products is a laminaribiose (1 \rightarrow 3) moiety.

"The data favor describing the action of these enzymes in terms of *the structure of the glycosyl unit that becomes the reducing end-unit of the product liberated*". (Perlin, 25). Since the substituent on the glycosyl unit is always glucose (in the work reported), we now propose extending the above concept by substituting "disaccharide" for "glycosyl". In a specific instance, cellulase has an affinity for a cellobiose fraction of the polymeric chain, hydrolysis occurring at the adjacent cellobiosyl bond. The cellobiose itself is protected from hydrolysis. We conceive of this protection as being due to a binding of dimer to the enzyme. Since the bond which is broken may be β -1,3 or β -1,4, it appears that the aglycone portion of the polymer is relatively less important. Endo- β -1,3 glucanases behave in an analogous fashion,

“grasping” a laminaribiose portion of the chain, and splitting the adjacent β -1,3 or β -1,4 bond. A similar affinity of β -amylase for a terminal maltose unit in the amylose chain has been reported by Thoma and Koshland (37), thus indicating that this mechanism may be generally applicable to glycanases forming dimeric and trimeric products, whether they be of the endo- or exo-type.

The fragment of the chain for which there is a site on the enzyme need not be limited to a dimeric unit. A specific “lichenase” (of *Bacillus subtilis*), acts on lichenin to give predominantly the trimer, 3-O- β -cellobiosyl D-glucose (28). In forming this trimer, the *B. subtilis* enzyme (A) resembles closely the β -1 \rightarrow 3 glucanases (B), but its specificity cannot be related *only* to the presence of a β -1 \rightarrow 3 dimer unit in the polymer, for it is unable to attack a long chain of such units (i. e., laminarin). The substrate in lichenin differs from that in a β -1 \rightarrow 3-glucan in having 4-substituted dimeric units rather than 3-substituted units. This difference has little apparent effect on B (which attacks both β -1 \rightarrow 3 glucan and lichenin,) but markedly affects A. The hydrolysis of lichenin by A might conceivably be viewed as a β -1 \rightarrow 4 glucanase since it splits 1 \rightarrow 4 linkages. However, that its specificity is *not* determined by a structure of this type is evident from the fact that it cannot hydrolyze a β -1 \rightarrow 4 glucan.

We have attempted to carry over this principle to α -glucans (27). Mycodextranase is an α -glucanase which splits mycodextran (=nigeran), a fungal polysaccharide, having alternating α 1 \rightarrow 4 and 1 \rightarrow 3 linkages. The only dimer produced is nigerose (α 1 \rightarrow 3); no maltose (Fig. 7). The enzyme splits only 1 \rightarrow 4 bonds, yet amylases have no effect on mycodextran. From analogy with the β -glucan story, the accumulation of nigerose suggests that the enzyme is an α -1,3-

glucanase, with an affinity for the nigerose moiety of the polymer. Unfortunately for our proof, no α -1,3 glucan is known. Whether the theory is applicable to all polysaccharases remains to be seen. Our work on simple systems suggests the likelihood of broader application. In considering the action of enzymes on the more complicated polymers, it may be useful to realize that the moiety bound to the enzyme is of greater significance than the linkage being hydrolyzed. Release from the “bond” concept will stimulate the investigation of substrates and of enzymes that might otherwise have been overlooked. Certainly one should now be wary of describing the nature of a certain linkage simply because the enzyme used has been known to split that linkage in other polymers.

D. The end-wise or exo-polysaccharases

Removal of terminal units.

Terminal units are those found at the ends of long chains and those appearing as simple branches of the main chain. These units may be attached to the main chain through a glycosidic, ester, ether, or an amide bond. All act as foreign substituents as far as endo-

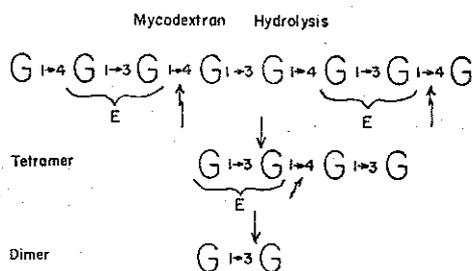


Fig. 7. Mycodextran hydrolysis by enzymes. (27).

polysaccharases are concerned, and interfere with hydrolysis of the main chain. And all may, in turn, be susceptible to removal from the chain by specific enzymes.

When the terminal group is a sugar moiety, the enzymes capable of their removal act only from the non-reducing end of the molecule. Two types are known: the exo-polysaccharases, and the glycosidases. While most polysaccharases act in a random fashion (as described above), exceptions are found among the

dextranases: *Lactobacillus bifidus* α -1,6 glucanase removing successive glucose units from oligomers of 2—9 units (Bailey, 3)

amylases: glucamylase removing successive glucose units;

β -amylase removing successive maltose units

exo- β -1,3 glucanase: fungal enzymes (14) removing successive glucose units

exo- β -1,4 glucanase: *Stachybotrys atra* "cellobiase" removes glucose units from oligosaccharides DP 2—11 (Youatt, 41) at comparable rates.

Of these, at least two act on relatively short chains and should probably be called oligosaccharases (Fig. 2), resembling the glycosidases in their selective removal of single units from the non-reducing chain end; yet differing from the glycosidases in their high activity on the DP 3—5 oligomers.

The specificity of some of these enzymes appears to be even less closely related to the linkage split than was observed above for some of the endo- β -glucanases. Glucamylase (diazyme) which cleaves successive terminal α -1,4 linkages in amylose, can also split (less rapidly) other α -linkages in short chain materials, e. g., α -1,6 linkages in panose, isomaltose, and isomaltotriose (Pazur, 22, 23) and α -1,3 links in partially degraded mycodextran (23, 27). However, its action on native dextran (all α -1,6) and mycodextran (α -1,3, α -1,4) is negligible. The exo- β -1,3 glucanases behaves differently. While normally splitting terminal β -1 \rightarrow 3 linkages, it appears to be incapable of acting on a terminal linkage which is β -1 \rightarrow 6. Instead, it reaches over this bond and hydrolyzes the penultimate β -1,3 linkage. Thus, a β -1 \rightarrow 3 glucan with β -1 \rightarrow 6 glucose side groups was hydrolyzed to glucose and gentiobiose by such an enzyme (Fig. 8).

In many polysaccharides, the substituent side group is a simple sugar and it has been suggested that these are removed by glycosidases. Some time ago, Deuel (8) and later Courtois (6) and others indicated that an α -galactosidase removes the galactose group from the side of a β -1 \rightarrow 4 mannan chain (carob gum; guar gum). This is a system we are currently re-examining. There is no doubt that galactose is liberated by most of the complex mannanase systems. When α -galactosidase is absent, action of mannanase is limited to those

regions of the chain where there are few galactose units. The galactosidase responsible for the removal of the side groups is quite specific for the galactomannan oligosaccharides, being relatively inactive on α -methyl galactoside, melibiose, raffinose, and stachyose. (There are other α -galactosidases

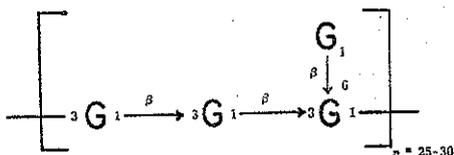


Fig. 8. β -1 \rightarrow 3 glucan with β 1,6 glucosidic side group. (14)

in which the specificity is just the opposite). After removal of the galactose units, the mannanase is again able to function, the combination of enzymes (with β -mannosidase) hydrolyzing guar completely to galactose and mannose. Unfortunately, we have not as yet been able to isolate the α -galactosidase, so that its activity on galactomannan chains of various lengths remains unresolved.

The ability of enzymes to remove *ester* groups from the side of a long polysaccharide molecule is unusual if indeed it occurs. The esterase which hydrolyzes off the acetyl groups from cellobiose octaacetate, is unable to remove the same groups from cellulose acetate (31). Similarly, sulfate is removed from chondroitin sulfate only after appropriate degradation (bacterial enzyme) of the polysaccharide chain (Dodgson, 9). On the other hand, it has been reported that a sulfatase from *Charonia lampas* does indeed remove sulfate groups from cellulose sulfate and that this enzyme is different from the one which removes the same group from glucose-6-sulfate (Fig. 9; Takahashi, 36).

Obviously, in the substituted polysaccharide there is greater likelihood that the substituents will interfere with the approach of enzyme to the site of hydrolysis, than there is in enzyme action on smaller fragments, but this type of argument can scarcely bear much weight in

the presence of the "*fait accompli*". If there exist in nature, polysaccharides having a substituent on every unit of the chain, then there must also be enzymes capable of coping with their hydrolysis.

Ether linkages are generally considered to be resistant to enzyme hydrolysis, and indeed *ether* linkages are of infrequent occurrence in nature. One example is that between lactic acid and the 3-OH in *N*-acetyl muramic acid (Fig. 10). Recently it has been claimed (Singer, 33) that this *ether* bond is hydrolyzed by a bacterial enzyme to yield a soluble non-dialyzable

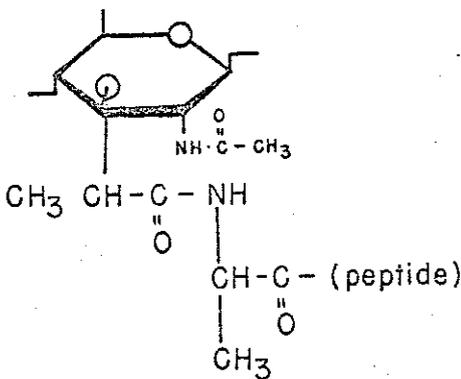


Fig. 10. Poly *N*-acetyl-muramic acid.

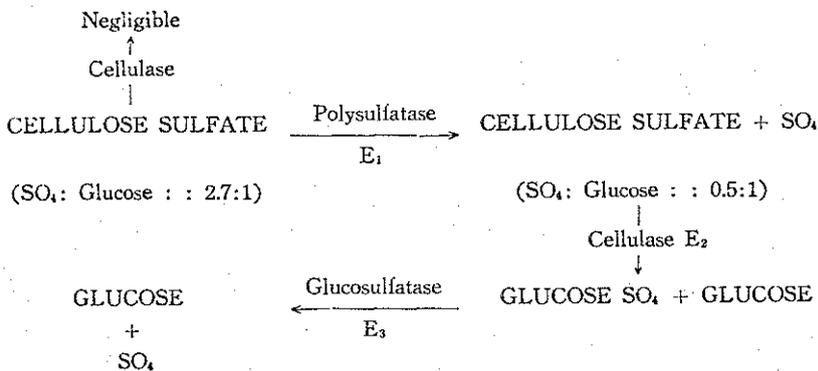


Fig. 9. Enzymatic breakdown of cellulose sulfate. (36)

polymer of *N*-acetylglucosamine, and lactate-peptides. If confirmed, this offers a choice of two explanations: (a) that *ether* α -hydrolyzing enzymes do exist or (b) that the assumed structure of the substrate is incorrect.

The hydrolysis of the *amide* linkage, connecting the peptide with the lactic acid side group (Fig. 10) has been demonstrated numerous times. It would be interesting to know whether this amidase can act on the lactate-peptide link in the product obtained by Singer and Church; and, conversely, whether the enzyme of Singer and Church can remove the lactate, after liberation of the peptide from the complex by the amidase.

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要 約

細胞壁の重合成分は polyester, polypeptide, polysaccharide で, その中 polysaccharide はあらゆる細胞壁に