Microbiological Process Discussion

Use of Enzymes in Isolation and Analysis of Polysaccharides[1]

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It is the purpose of this paper to explore the possibility of applying the enzyme technique to the analysis of structural polysaccharides (table 1), and to evaluate the use of enzymes in isolation procedures. Although the main structural features of a polysaccharide can usually be determined by chemical methods (Manners, 1955), these procedures are not simple and they do offer certain limitations. The successful application of enzymes to starch and protein analysis suggests that they may prove equally helpful in the analysis of other natural polymers. While we are promoting the use of enzymes, it is well to remember that they cannot do everything that an acid can do. For instance, nature has not endowed fungi with the ability to split off substituents which man has added to the cellulose molecule. Indeed, these substituents hinder the action of the enzyme cellulase on the glucosidic linkage.

Acids hydrolyze all polysaccharides without any marked preference. Enzymes are highly specific: cellulase hydrolyzes only cellulose, xylanase only xylan, and so forth. Therefore, it is theoretically possible to specifically remove any component from a mixture by means of the appropriate enzyme. This would be very difficult by chemical procedures. To be successfully done enzymatically the substrate must be accessible to the enzyme, and the enzyme preparations must have the required properties.

(Note: Much of the data presented here is previously unpublished. The procedures employed are described in earlier works of the authors.)

Major Difficulties in the Use of Enzymes

I. Accessibility of Substrate

Enzymes are large protein molecules and as such do not diffuse readily into most solids. In fresh, succulent tissues, enzymes do diffuse readily into the free spaces between cells where they digest the pectic materials binding the cells together. Such maceration has been successfully used for: (a) isolation of cuticle from leaves of plants, (b) breakdown of tobacco leaf fiber (milling increased breakdown), (c) isolation of components of vascular tissue (Wood *et al.*, 1952). However, there are no reports of successful maceration of *woody* materials. A similar action occurs on animal tissues where separation of cells and bacterial penetration have been facilitated by a "spreading agent" (hyaluronidase) which hydrolyzes intercellular materials (Pigman, 1957).

Unfortunately, most experimental materials are more woody and less porous than the above. Enzymes cannot penetrate into these, and action is limited to the surface. Complete enzyme hydrolysis can be achieved only by obtaining a maximum surface area. Some idea of the importance of grinding can be seen in the extent of the action of cellulase on cotton fiber (approximate values):

Whole fiber, 0 to 3 per cent hydrolysis
20 to 40 mesh cotton, 20 per cent hydrolysis
Ball-milled cotton, 100 per cent hydrolysis

Although ball-milling is necessary for complete hydrolysis, such an action may degrade the components which one wishes to isolate. The seriousness of this limitation will vary with the isolation problem under investigation.

A combination of chemical and enzymatic procedures may occasionally permit complete hydrolysis without the necessity of ball-milling. Partial grinding (20 to 40 mesh) followed by chemical extraction of readily soluble components may open up pores to an extent permitting enzyme diffusion. Such a procedure would minimize the depolymerization effects obtained by ball-milling.

II. Purity of Enzyme

"Pure" enzymes are hard to find, or to obtain. The enzyme systems in which we are interested are usually contaminated with unwanted, or interfering, enzymes (table 2). Even saliva, generally considered an excellent source of α-amylase, contains lipase, phosphatase, hyaluronidase, lysozyme, and probably other unsuspected enzymes.

But are pure enzymes required? In most instances, we believe not. It is only necessary that the enzyme system be selected for each specific problem, and that one recognize the limitations of the system. If the problem is to digest pure starch, it makes little differ-

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ence whether cellulase, lipase, and proteinase are present in the amylase solution. Their presence does not interfere with the hydrolysis. If, however, the problem is to remove starch from a mixture containing cellulose, fat, and protein, then, the presence of the other enzymes is not permissible. The success of commercial enzymes, most of which are crude mixtures, depends upon their application to a particular situation. Desizing of cotton cloth requires the presence of amylase and the absence of cellulase, that is, the removal of starch without destruction of the cloth. Whether other enzymes are found in the mixture is of little consequence.

TABLE 1

Structure and distribution of β-glycans (after Stone, 1958)

Sugar Unit	Linkages		Name	Source
	Major	Minor		
Glucose	β-1,4		β-1,4-Glucan = cellulose	Walls of plant cells
Glucose	β-1,4	β-1,3	Lichenan	Lichens
Glucose	β-1,3		β-1,3-Glucan = pachyman	Fungus sclerotium
Glucose	β-1,3	β-1,2	Yeast glucan	Yeast cell wall
Glucose	β-1,3	β-1,6	Laminarin	Marine algae
Glucose	β-1,6		β-1,6-Glucan = pustulan	Lichen
Glucose	β-1,2		Crowngall polysaccharide	Bacteria
Mannose	β-1,4		Vegetable ivory	Palm seed
Galactose	β-1,4		Galactan	Pectins
Xylose	β-1,4		Xylan	Walls of plant cells
Fructose	β-2,1		Inulin	Plants, *Aspergillus sydowi*
Fructose	β-2,6		Levan	Bacteria
N-Acetylglucosamine	β-1,4		Chitin	Crustaceans, fungi

Preparation of Enzyme Systems

I. Production of Polysaccharases

Although plants and animals produce polysaccharases, the most important sources of these enzymes are fungi and bacteria. Our own preference is for the fungi, primarily because of ease of handling. The fungus is grown on a polysaccharide in shake cultures for several days. The digestive enzymes are secreted into the medium, and obtained free from the organism and substrate by simple filtration. Generally, a mixture of enzymes is produced (table 2), but by various means it is possible to minimize or eliminate unwanted components.

A. Selection of organism. Species of fungi differ greatly in their production of enzymes. No two are alike. Fortunately, most polysaccharases in fungi are adaptive (Mandels and Reese, 1957). The fungus produces the particular enzyme only when grown on the substrate of that enzyme (table 3). Amylase and β-1,3-glucanase are exceptions. Their appearance does not depend upon the nature of the food on which the organism grows. As a result, they are nearly always present and it is difficult to completely eliminate them from solutions of other polysaccharases.

The adaptive enzymes can usually be obtained free of each other by growing the fungus on the inducing polysaccharide. Cellulase free of chitinase is obtained by growth on cellulose; chitinase free of cellulase by growth on chitin and so forth. There is an exception in xylanase. Most fungi produce this enzyme when grown on xylan, but not when grown on glucose, starch, or glycerol (that is, it is adaptive). Yet xylanase is nearly

TABLE 2

Hydrolytic enzymes of fungi

Source		Units of Enzyme	Glucanase				Other		Glucosidases	
Fungus	QM Strain No.		β-1,4-	β-1,3-	α-1,4-	α-1,6-	Xylanase	PG*	β-	α-
Aspergillus luchuensis	873	μ/mg†	0.2	2.0	20.0	0.04	0.06	0.6	2.1	8.0
Aspergillus phoenicis	1005	μ/mg	0.0	7.5	0.1	4.0	0.12	0.4	7.0	2.5
Basidiomycete	806	μ/mg	13.0	3.4	0.1	0.0	0.4	0.3	0.1	0.0
Penicillium funiculosum	474	μ/mg	0.0	14.0	1.0	780.0	0.0	0.0	0.8	0.5
Penicillium pusillum	137g	μ/ml	150.0	32.0	0.5	0.04	0.5	1.0	0.3	0.2
Myrothecium verrucaria	460	μ/mg	0.8	0.2	0.0	0.0	0.0	0.0	0.03	0.0
Pestalotiopsis westerdijkii	381	μ/ml	20.0	9.0	0.5	0.0	0.4	0.4	6.0	0.0
Rhizopus arrhizus	1032	μ/mg	0.1	6.4	0.5	0.2	0.0	0.4	0.0	0.1
Sporotrichum pruinosum	826	μ/mg	9.0	31.0	0.4	0.0	0.5	0.3	0.1	0.1
Trichoderma viride	6a	μ/mg	9.0	0.7	0.5	0.0	0.6	0.06	0.2	0.1

* Polygalacturonase.

† Figures represent units of enzyme per ml of culture filtrate, or units per mg of dry enzyme preparation.

always found (with cellulase) when the organism is grown on cellulose, and this does not seem to be due to the presence of xylan as an impurity in the cellulose. The enzymes are different. When grown on xylan, *Trichoderma viride* produces xylanase in the absence of cellulase.

The selection of a polysaccharase system depends upon the problem to be resolved. From an analysis of the enzyme components of many fungi (table 2) it is possible to select that one which meets the requirements most closely.

B. Modification of the growth conditions. In addition to the selection of an organism and of a particular substrate, there are other techniques which can be applied to the production of the desired enzyme system. The aims are high yields of "wanted," low yields of "unwanted" components. All the variables customarily used in growth experiments can be applied. Of these, the nature and amount of the carbon and nitrogen sources, the pH, and the length of the incubation period are of primary importance. But the conditions for production and liberation of a particular enzyme are not the same as those for maximal growth.

There have been many investigations of the optimal conditions for production of a single enzyme. Much less effort has been directed toward stimulating the production of one while inhibiting, or suppressing, the development of a second. Length of the incubation period is often important. Some enzymes (the adaptive ones) appear earlier in the culture filtrate than others (the

TABLE 3

Production of polysaccharases by fungi

Constitutive
α-1,4-Glucanase (amylase)
β-1,3-Glucanase (laminarinase)
Adaptive: Produced Only when Fungus is Grown on the Proper Substrate
β-1,4-Glucanase (cellulase)
α-1,6-Glucanase (dextranase)
β-1,4-Mannase (mannanase)
β-2,1-Fructanase
β-2,6-Fructanase
Pentosanase
β-1,4-Xylanase
β-1,4-Chitinase
α-1,4-Polygalacturonase (pectic enzyme)
α-1,4-Polymannuronase

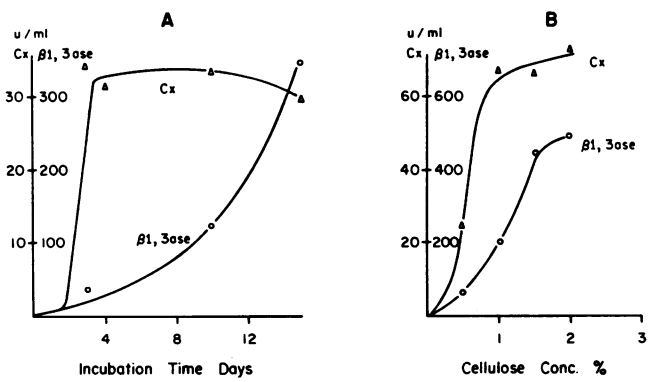

Figure 1. Production of enzymes of *Sporotrichum pruinosum* strain QM 826. *A*, Effect of incubation time; *B*, Effect of cellulose concentration. Cx = β-1,4-glucanase; β-1,3ase = β-1,3-glucanase.

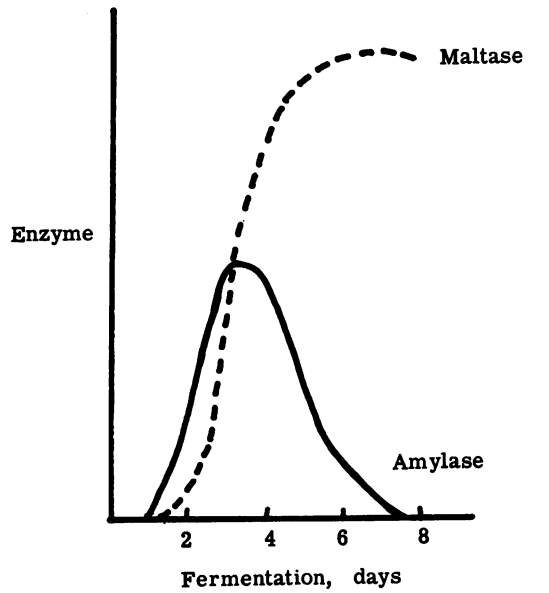

Figure 2. Production of maltase free of amylase by *Aspergillus niger* (Shu and Blackwood, 1951).

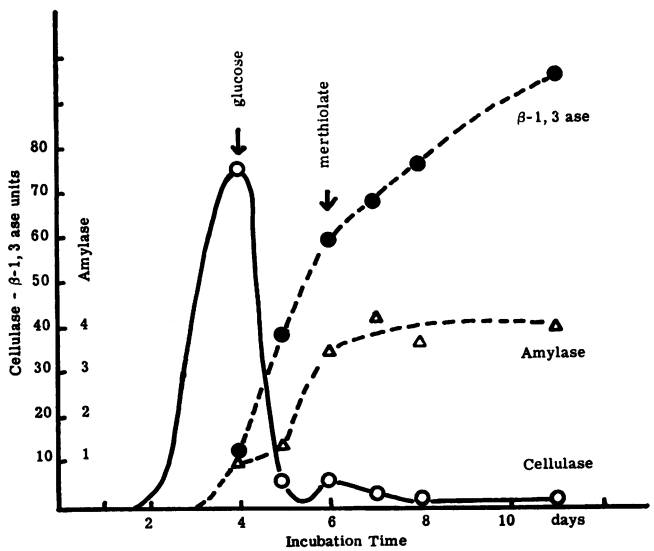

Figure 3. Control of polysaccharase appearance in cultures of basidiomycete, QM 806. Initial substrate = cellulose (1 per cent). Additions: glucose (0.5 per cent) on 4th day; merthiolate (0.02 per cent) on 6th day.

constitutive, figure 1*A*). Some are more rapidly inactivated after reaching a level of maximum potency. Thus, maltase can be obtained free of amylase by waiting until the latter has been destroyed (figure 2).

Recently we have found another useful procedure (figure 3). When certain metabolites are added to a culture which has grown on cellulose and produced cellulase, there is a rapid inactivation of the cellulase. When incubation is further prolonged, the cellulase reappears in an amount approaching that previously reached (not shown). The action may be a specific masking and unmasking of the cellulase. When, however, Merthiolate is added (while inactivation is complete), the cellulase activity does not recover, but the β-1,3-glucanase and amylase activities remain at a high level. By such manipulations of the cultural conditions, it is possible to enrich the filtrate in one enzyme, or to eliminate an enzyme from the mixture. These examples indicate the possibilities inherent in this approach.

II. Purification of Polysaccharases

Having produced the best possible enzyme system by selection of an organism and by control of the growth conditions, we may still be faced with the problem of removing traces of an "unwanted" enzyme component.

Successful enrichment of a desired enzyme by most enzyme-separation techniques is largely a matter of trial and error. Heat, and irradiation, are destructive, yet it is possible to completely inactivate one enzyme while leaving much of the second intact. Electrophoresis, especially block-electrophoresis, has been successful, but the samples are usually small. Adsorption and elution from columns appears to have most promise for large scale separations.

TABLE 4

Separation of glycosidases on specific adsorbents†*

Enzyme	Adsorbent	
	Enzyme adsorbed on:	
	[*p*-OH-Styrene-β-D-glucoside]$_n$	[*p*-OH-Styrene-α-D-galactoside]$_m$
	%	%
β-D-Glucosidase	45	23
α-D-Galactosidase	9	41
β-D-Galactosidase	0	0

* Almond emulsin.

† Helferich and Jung (1958).

TABLE 5

Selective inactivation and adsorption of enzymes

Enzyme	Source	Inhibition by Methocel at		Adsorption on Cellulose
		0.1%	0.02%	
		%	%	%
Cellulase	*Trichoderma viride*	46	43	89
Xylanase	*T. viride*	0	0	20
Xylanase	*Aspergillus fumigatus*	3	0	88

The use of specific adsorbents appears to be an ideal method of separation. By this technique, a substrate or modification thereof is used to adsorb only the enzyme having an affinity for that substrate, for example, amylase on starch. Helferich and Jung (1958) recently formed the β-glucoside and the α-galactoside of polyhydroxystyrene. The former preferentially adsorbed β-glucosidase, the latter α-galactosidase (table 4) from a naturally occurring enzyme mixture (almond emulsin). In a similar manner, we have selectively adsorbed cellulase on cellulose (table 5), leaving behind most of the xylanase; French and Knapp (1950) have completely adsorbed amylase on starch, leaving much of the maltase in solution; and Jeuniaux (1957) has purified chitinase by adsorption on chitin. Straub (1957) reported that the conditions for adsorption are important. These include temperature, pH, amount of adsorbent per unit of enzyme, and the nature of the impurities in the system. For amylase, Straub found maximum adsorption on starch at 13 C. The results encourage one to pursue this method as perhaps the most logical approach to enzyme purification. At the moment, however, the results are no better than those obtained by conventional methods, and the source of enzyme has proved to be a very important factor. Helferich's procedure failed entirely when the enzymes came from alfalfa, and we have found vast differences between fungi in the adsorbability of their cellulases on cellulose. Yet, we are confident that the use of adsorbents specific for each enzyme will prove to be the most successful of separation techniques. It can be used for isolation of a single enzyme, or for removal of an unwanted enzyme from a mixture. We anticipate major developments along this line.

III. Inhibition and Inactivation of Unwanted Enzymes

Inhibition and inactivation are not purification procedures. They do not remove components from the system. More often something is added. Yet they are highly useful techniques. Many problems require only that enzyme A be present in the absence of enzyme B. Where both are found, B may be removed, but it is often more simple to inactivate it. The mixture of A and inactivated B usually serves the same purpose as A free of B.

Enzyme inhibitors (like adsorbents) may be either general or specific. The general inhibitors affect many different enzymes although not always to the same degree. For instance, the amount of disodium ethylenebis(dithiocarbamate) required to give 50 per cent inhibition of enzymes of *T. viride* is only 0.0026 per cent for cellulase, but it requires 100 times as much to in-

activate β-glucosidase (Reese and Mandels, 1957). Although it is thus possible to find, among the general inhibitors, compounds that may be used selectively, success is largely a matter of chance and continued effort.

A. Specific inhibitors. Specific inhibitors of enzymes are usually compounds resembling the substrate of the enzyme. The best specific inhibitor is one which combines irreversibly with the desired enzyme. Usually the specific inhibitor is competing with the substrate for the enzyme. Methocel (methyl cellulose) is a case in point (table 5). It resembles cellulose and competes with it for cellulase. It is sufficiently different from xylan so that it does not compete with xylan for xylanase. Although this is an excellent inhibitor based on data for *T. viride*, it does have its limitations. Even in this organism, only part of the cellulase is susceptible to the methocel effect, that is, the specificity is too great. In other fungi, even less of the cellulase is so affected.

The lactones of sugar acids have been shown to be specific inhibitors of glycosidases (Conchie and Levvy, 1957). Thus, gluconolactone inhibits glucosidases, galactonolactone inhibits galactosidases, and mannonolactone inhibits mannosidases.

B. Naturally occurring inhibitors. These inhibitors have been found for a number of polysaccharases (table 6). They seem to have been accidentally discovered. Until the present, the number of natural inhibitors has been few, and their chemical nature unknown. It is not clear from the data how specific each may be.

C. Antisera. The introduction of enzymes into the blood of an animal leads to the production of antibodies. These antibodies are highly specific in inhibiting the enzyme used as antigen. Complete inhibition of the following polysaccharases by their respective anti-enzyme sera has been reported (Cinader, 1957): amylase (barley), hyaluronidase, carbohydrase (pneumococcus polysaccharide), lysozyme. Since the antibodies are proteins, their mode of inhibition cannot be related to their resemblance to the substrates (which are polysaccharides). The enzyme-antibody reaction is often hindered by the presence of the substrate of the enzyme (Nomura and Wada, 1958), the antibody and substrate apparently competing for the enzyme. As with the

TABLE 6

Specific inhibition of polysaccharases

Enzyme		Inhibitor		Reference
Name	Source	Source	Nature	
Cellulase	Some fungi		Methocel	Reese *et al.*, 1952
Cellulase	Some fungi	Eucalyptus		Jermyn, 1957 (personal communication)
Cellulase	Some fungi	Grape leaves		Etchells *et al.*, 1958
Polygalacturonase	Fungi	Grape leaves		Etchells *et al.*, 1958
Pectinase		Broad bean leaves		Brown, 1915
Pectinase		Pears		Weurman, 1953
Protopectinase	Fungi	Oxidized apple; oxidized potato juice	Oxidized leucoanthocyanine	Cole, 1958
Amylase	Animal; bacterial	Cereals	Protein	Kneen and Sandstedt, 1946
Amylase	All	Sorghum	High mol wt organic acid	Kneen and Sandstedt, 1946

TABLE 7

Selective inactivation of enzymes by heat

Condition				Enzyme Destroyed	Enzyme Remaining	Enzyme Source	Reference
Temp	Time	pH	Other				
C	*min*						
60	10			Cellobiase	Cellulase, 50%	Fungus	Aitkin *et al.*, 1956
100	3	6.0		Cellobiase	Cellulase, 20%	Fungus	Kooiman *et al.*, 1953
60	15			Endo-β-1,3-glucanase	Cellulase, "partly"; β-glucosidase "partly"	Alga	Duncan *et al.*, 1956
70	10	6.0	Ca^{++}	β-Amylase	α-Amylase, 100%	Malt	Schwimmer, 1947; also Kneen *et al.*, 1943
30	120	3.0		α-Amylase	β-Amylase 90% (+)	Malt (NaCl extract)	Kneen *et al.*, 1943
10	12,960	2.4		α-Amylase	Glucamylase, 80%	Fungus	Phillips and Caldwell, 1951
60	10	4.5	Ca^{++}	Glucamylase	α-Amylase	Fungus	Phillips and Caldwell, 1951

natural inhibitors, work on these agents is at an early stage.

D. Heat inactivation. Heat has been used in instances where the unwanted enzyme is present only in minute quantities. To be sure, the desired enzyme also suffers during the treatment but often excellent results have been obtained (table 7). The temperature effect is dependent upon other conditions, conditions which have not always been reported. Enzymes are usually much less stable below pH 4.0 and above pH 8.0. α-Amylase of *Aspergillus niger* can be inactivated at pH 4.2 even at low temperatures, with little or no inactivation of maltase (Tsuchiya *et al.*, 1950). The presence of impurities in the enzyme solution affects heat inactivation. This is particularly true of proteins and of the enzyme substrates. The former have a general protective action, the latter tend to protect only the enzymes for which they are substrates. It is possible to some degree to direct the inactivation by protecting one enzyme. This can be done by addition of substrate, coenzyme or activator (that is, Ca salts for α-amylase). In this manner, general inhibitors may become specific inhibitors.

Applications

I. Isolation of One Substance from a Mixture

A desired component of a mixture 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 sometimes 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 (table 8). These systems result from the practices previously described.

A. Xylan purification. The commercial xylan intended for use as a substrate for xylanase was found to have 23 per cent glucan (table 9). Without knowing the nature of the glucan, we were able to find an enzyme system (*Rhizopus arrhizus* strain QM 1032) which

TABLE 8

Production of one enzyme in the absence of another

Enzyme		Source		Activity		Ratio, A:B
Desired (A)	Free of (B)	Organism	QM Strain No.	μA	μB	
β-1,4-Glucanase	β-1,4-Xylanase	*Penicillium pusillum*	137g	150.0	0.5	300:1
β-1,4-Xylanase	β-1,4-Glucanase	*Trichoderma viride*	6a	1.5	0.0	∝
β-1,4-Glucanase	β-1,3-Glucanase	*T. viride*	6a	9.0	0.7	13:1
β-1,3-Glucanase	β-1,4-Glucanase	Basidiomycete	806	60.0	0.0	∝
β-1,4-Glucanase	α-1,4-Glucanase	*P. pusillum*	137g	150.0	0.5	300:1
α-1,4-Glucanase	β-1,4-Glucanase	*Aspergillus luchuensis*	873	20.0	0.0	∝

TABLE 9

Purification and isolation of one compound from a mixture

Desired Product	Starting Material	Substance to be Removed			Enzyme Source
		Nature	Before enzyme	After enzyme	
			%	%	
Xylan	Xylan, commercial	Glucan	23	8	*Rhizopus arrhizus* strain QM 1032
Chitin	Mushroom, after extractions with water, 2% KOH, and alcohol (= 25% of dry wt)	Glucan	63	25	*Trichoderma viride* strain QM 6a
Lignin	Spruce wood, ball-milled (Pew, 1957)	Cellulose and hemicelluloses	70	12	Enzyme no. 19*
Lignin	Spruce wood, blocks (Pew, 1957)	Cellulose and hemicelluloses	70	6–14	Growing brown-rot fungi
Pentosan	"Squeegee" starch (Simpson, 1954)	Protein	8	1	Proteinase
		Starch	50	0	Amylase
Starch	Wheat flour (Simpson, 1955)	Pentosan			Pentosanase
Olive oil	Olives (Pineda, 1954)	Pectins			Pectinase

* Rohm and Haas Co., Philadelphia, Pennsylvania.

would hydrolyze most of the impurity without affecting the xylan. The action was not complete. The residual xylan (77 per cent recovery) still yielded glucose (8 per cent) on acid hydrolysis. This glucose probably arises from a glucan different from the one hydrolyzed, or perhaps from a mixed polysaccharide.

B. Isolation of chitin from mushroom (Agaricus campestris). Repeated extractions with hot water, with hot KOH (2 per cent), and with ethyl alcohol removed 75.2 per cent of the mushroom solids (table 9). Enzymes of *T. viride* strain QM 6a (and of *R. arrhizus* strain QM 1032) further hydrolyzed 49 per cent of the residue. The per cent glucan was reduced from 63 to 25 per cent. The product (12.5 per cent of initial dry weight) contained all of the chitin.

C. Isolation of lignin. Enzymes can effectively remove the carbohydrate complex from wood, provided that the wood is finely ground (Pew, 1957; figure 4*A*). About 70 per cent of the spruce wood was digested. The action is not complete. The lignin residue contains polysaccharide to the extent of 12 per cent. This last trace could not be removed by a variety of enzyme systems tested (unpublished data). The reducing sugars produced during the early stages of hydrolysis contained little glucose (figure 4*C*). Chromatograms (not shown) revealed a predominance of xylose and xylo-oligosaccharides. Xylan thus appears to be more rapidly hydrolyzed than cellulose by the enzyme systems used.

The limitation to this procedure is obviously the necessity for fine grinding (figure 4). Such action tends to degrade lignin to some extent. An alternate procedure employs an actively growing fungus to remove the polysaccharides, a method which does not necessitate grinding. The objection to the latter is contamination of the lignin by mycelial residue.

II. Preparation of Hydrolysis Products

A. Preparation of products of polysaccharide hydrolysis. A variety of enzymes is involved in polysaccharide hydrolysis. Those that involved phosphate are found inside of the fungus, and not in the extracellular solutions. Those that hydrolyze the oligosaccharides are found both within and without, whereas those that hydrolyze the polysaccharides are predominantly outside of the organism.

Enzymes can be used to produce the dimer, the disaccharide component of a polysaccharide. In digestion by most polysaccharase systems of fungi, the simple sugar appears as the dominant product at all phases of the incubation period. In these it is an easy matter to produce monomers (simple sugars) from polysaccharides. In other polysaccharase systems, the dimer is the usual hydrolysis product (isomaltose from dextran by dextranase; maltose from starch by β-amylase). The reaction stops here when enzymes capable of acting on the dimer are lacking. For the hydrolysis of most polysaccharides, it is possible to find an organism having an enzyme system such that the reaction can be halted at the disaccharide stage (table 10). Most of these hydrolyzates also contain trimer, and perhaps longer fragments (particularly so in xylan-, and in fructan-hydrolyzates), separation of which can be accomplished on carbon columns. In our own studies, we have prepared several of the disaccharides, one (cellobiose) in rather appreciable amount (15 g) and others (including β-2,6-difructose) in quantities sufficient for use as reference materials in paper chromatography. (Commercially, some corn syrups are the result of enzyme hydrolysis of starch.)

These principles, that is, the use of selected enzyme systems (table 10) and of carbon column separations, are being widely applied for the preparation of oligosaccharides. Here we will mention only one other example, the galacturonic acid oligosaccharides prepared by hydrolyzing pectic acid with polygalacturonase (Phaff and Luh, 1952; Demain and Phaff, 1954).

B. Preparation of products of glycoside hydrolysis. There are times when one wishes to hydrolyze a glyco-

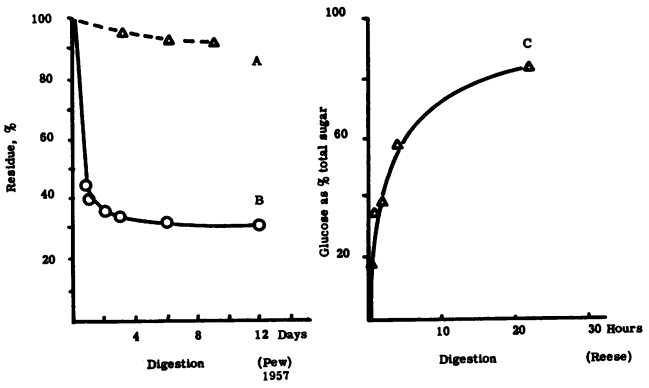

Figure 4. Removal of polysaccharides of spruce wood by enzyme action. *A*, Wood coarsely ground; enzyme no. 19 (Rohm and Haas); Pew (1957). *B*, Ball-milled wood; enzyme no. 19 (Rohm and Haas); Pew (1957). *C*, Ball-milled wood; enzyme of *Trichoderma viride* strain QM 6a.

TABLE 10

Production of dimers from polysaccharides

Polysaccharide	Enzyme Source	QM Strain No.
β-1,4-Glucan = cellulose	*Streptomyces* sp.	B814
β-1,4-Xylan = xylan	*Myrothecium verrucaria*	460
β-1,4-Chitin	*Aspergillus fumigatus*	45h
β-1,3-Glucan = laminarin, etc.	*Rhizopus arrhizus*	1032
α-1,4-Glucan = amylose	*Pestalotiopsis westerdijkii*	381
α-1,6-Glucan = dextran	*Penicillium funiculosum*	474
β-2,1-Fructan = inulin	*A. fumigatus*	45h
β-2,6-Fructan = bacterial levan	*Streptomyces* sp.	B814

side only for the purpose of obtaining the products. Here one need not worry about the presence of other enzymes, just as long as they do not affect the reaction. Recently, we had the problem of finding a fungal enzyme capable of hydrolyzing mustard oil thioglucosides in connection with a food flavor project. This we were able to do (Reese *et al.*, 1958). The fact that the preparation also contained a variety of other hydrolytic enzymes was of little consequence.

For hydrolysis of both α- and β-glucosides we have found preparations from the following fungi to be highly satisfactory: *Aspergillus luchuensis* strain QM 873 and *Aspergillus phoenicis* strain QM 1005.

Commercially available enzymes rich in one or the other (or both) of these glucosidases are lipase, (GBI);[2] 19AP, (Rohm and Haas);[3] Hemicellulase (NBC).[4]

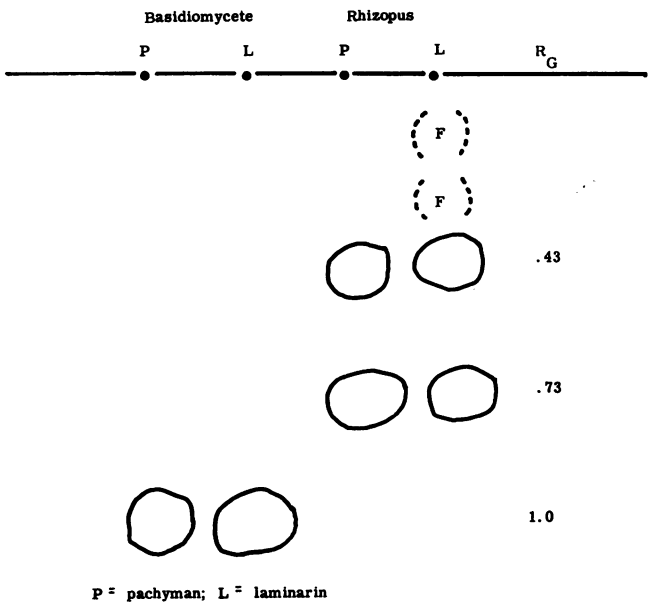

Figure 5. Enzyme hydrolysis of pachyman and of laminarin. Basidiomycete = enzyme solution basidiomycete, QM 806. Rhizopus = *Rhizopus arrhizus* strain QM 1032. Conditions: 40 min at pH 4.7; 50 C (after Reese and Mandels, 1959).

III. Identification of Polysaccharides and Oligosaccharides

A. Polysaccharides. We will cite two examples from our own work to illustrate the methods used. Pachyman may be considered as a glucan of unknown linkage. Recent chemical evidence (Warsi and Whelan, 1957) indicates it to have a β-1,3-bond. We, therefore, attempted to confirm this by hydrolysis with enzymes known to attack this linkage. At the same time, we hydrolyzed a known β-1,3-glucan, laminarin (figure 5). Fortunately, two β-1,3-glucanase systems were available (Reese and Mandels, 1959). That of basidiomycete strain QM 806 is an exoenzyme attacking chains from the end and yielding only the monomeric unit, glucose. The rhizopus β-1,3-glucanase is an endoenzyme hydrolyzing the chains at random and yielding predominantly the β-1,3-linked di- and trisaccharides. Hydrolyses of both polysaccharides gave identical monomer, dimer, and trimer. Pachyman is thus a β-1,3-glucan.

In a similar manner, we have identified the linkages in unknown fructans from fungi and bacteria (Loewenberg and Reese, 1957). The job, of course, is to find suitable enzyme systems. Once found, they are easily and quickly applicable to the problem.

Identification of a compound is simplified if it can be broken into smaller units without other modification. Here enzymes, because of their gentle action and specificity, are quite useful. Agarase acts on agar to produce characteristic disaccharides (Yaphe, 1957). Leucosin on enzyme hydrolysis yields products which characterize it as a β-1,3-glucan (Archibald and Manners, 1958).

It is obvious that chemical hydrolysis can similarly

[2] General Biochemicals, Inc., Chagrin Falls, Ohio.
[3] Rohm and Haas Company, Philadelphia, Pennsylvania.
[4] Nutritional Biochemical Corp., Cleveland, Ohio.

TABLE 11

Determination of linkage type in glucosides

Enzyme and Source	QM Strain No.	Hydrolysis (%) *							
		1 Hr				4 Hr			
		CB-C	CB-E	CT-E	M	CB-C	CB-E	CT-E	M
α-Glucosidase									
Pestalotiopsis westerdijkii	381	0	0	0	80	0	0	0	87
Sclerotium rolfsii	7740	0	0	0	84	0	0	0	89
β-Glucosidase									
Emulsin, almond		61	58	14	6	85	89	29	5
P. westerdijkii	381	72	80	78	6	93	89	89	15

* Substrate 2.5 mg per ml in M/20 citrate pH 4.5. Followed by glucose oxidase 1 hr at 30 C shaken.

CB-C = cellobiose, chemically prepared; CT-E = cellotriose, enzyme prepared; CB-E = cellobiose, enzymatically prepared; and M = maltose, commercial grade.

be used to obtain intermediates. The advantage of enzymes lies in their specificity for linkage type, in the mild conditions of hydrolysis, and in the ability to accumulate intermediates. We are not promoting the use of enzymes as a complete substitute for acid hydrolysis, but there are many instances in which acid treatment is too severe. The acetyl groups, for instance, may be split from chitin during acid hydrolysis to yield glucosamine. Enzyme hydrolysis gives the correct monomeric unit, *N*-acetylglucosamine. Furthermore, enzymes can be used to detect chitin in a mixed substrate, such as that obtained from mushroom (table 9). The chitinase of *Serratia marcescens* strain QM 1455 acting on this substrate yields only *N*-acetylglucosamine. Dilute acid hydrolysis yields only glucose. It is, thus, possible to say that the substrate is a mixture of chitin and glucan, and that it is probably not a single heteropolysaccharide.

B. Oligosaccharides and glycosides. The β-glucosidic linkage has, for a long time, been identified by its susceptibility to hydrolysis by almond emulsin. We have now found fungal enzymes that can be used similarly. *Pestalotiopsis westerdijkii* strain QM 381, grown on cellulose, produces β-glucosidase in the absence of α-glucosidase. Interestingly, the same fungus, when grown on starch, produces the opposite system, that is, α-glucosidase in the absence of β-glucosidase. Either this fungus or *Sclerotium rolfsii* strain QM 7740, can be used to produce enzymes for the detection of α-glucosidic linkages. For conclusive evidence the substrate should be subjected separately to the action of α-glucosidase, and also to the action of a β-glucosidase. In all such experiments, it should be recognized that glucosides differ considerably in their susceptibility to enzyme action.

Almond emulsin, as is well known, varies greatly in its effect on different β-glucosides. For instance, cellobiose is hydrolyzed 20 times as fast as cellobiitol. Its activity also decreases rapidly as chain length increases (table 11). The β-glucosidase of *P. westerdijkii*, on the other hand, has more nearly equal activity on the various β-glucosides we have tested (salicin, disaccharides, cellobiitol) and is less specific as to chain length. The fungus enzyme, therefore, may be more valuable than almond emulsin in establishing linkage type in an unknown glucoside.

Table 11 shows how this procedure can be used to determine linkage type. Our unknowns may be considered to be two sugars resulting from the enzymatic hydrolysis of cellulose. The rate of their movement on chromatograms suggested that they might be cellobiose and cellotriose. The known compounds are chemically prepared cellobiose (β-glucosidic link), and maltose (α-glucosidic link). All four compounds were subjected to the action of each enzyme. The unknowns and cellobiose were hydrolyzed by the β-glucosidases, but not by the α-glucosidases. They have, therefore, β-glucosidic linkages.

An alternate procedure for the detection of linkages depends upon the use of gluconolactone as an inhibitor. It has the advantage that it does not require separate α- and β-glucosidases. One preparation containing both enzymes can be used. The procedure is based on our finding that it takes about 200 times as much gluconolactone to inhibit α- as to inhibit β-glucosidase (table 12). In our test we selected a concentration of gluconolactone sufficient to give over 50 per cent inhibition of the β-glucosidase with little or no effect on the α-glucosidase (table 13). If the addition of the lactone inhibits the hydrolysis, the substrate is β-linked; if the hydrolysis is not inhibited, the substrate must be α-linked.

C. Principles. In general, there are several principles involved in the use of enzymes for identification.

1. A polysaccharide can be identified by the enzyme that hydrolyzes it. Cellulose is anything hydrolyzed by cellulase. The difficulties in obtaining pure enzyme have been elaborated. Having pure enzyme, it is not necessary to have pure polysaccharide.

2. The products of complete hydrolysis, the monomeric units, can be identified by their movement on paper chromatograms. The degree of certainty of identification is increased markedly by the use of two or more chromatograms using different solvent mixtures as irrigants. Known controls are required.

3. Dimers (and trimers) of the same monomer move at rates which are different for different linkages. Thus,

TABLE 12

Ratio gluconolactone/substrate (R I/S) for 50 per cent inhibition of enzyme

Enzyme Source	QM Strain No.	β-Glucosides		α-Glucoside
		Cellobiose	Gentiobiose	Maltose
Aspergillus phoenicis	1005	0.014	0.04	2.5
Aspergillus luchuensis	873	0.005	0.01	1.0
Pestalotiopsis westerdijkii	381	0.06	0.3+	10.0+
Lipase*		0.012	0.022	4.0+

* General Biochemicals, Inc., Chagrin Falls, Ohio.

TABLE 13

Use of gluconolactone inhibition to characterize linkage

Enzyme and Source	QM Strain No.	Hydrolysis (per cent)*			
		Cellobiose (β-1,4)		Maltose (α-1,4)	
		Alone	+ Lactone	Alone	+Lactone
α-, β-Glucosidase mixture					
Aspergillus phoenicis	1005	20	6	22	22
Aspergillus luchuensis	873	54	13	76	74

* Substrate 2.5 mg per ml in M/20 citrate pH 4.5, 40 C, 1 hr. Gluconolactone 0.1 mg per ml in reaction mixture.

in the irrigant we use, β-1,3-diglucose moves faster than β-1,4 which in turn moves faster than β-1,6.

4. Spray reagents are selective for sugar types and, in some instances, for linkage. Details on these are generally available. Glucose oxidase can be used to determine glucose quantitatively and specifically in a mixture of reducing sugars.

5. The linkage types, α and β, can be determined by means of specific glycosidases.

In this paper we have tried to show how enzymes may be obtained and used in isolation and identification problems. The enzyme systems are those with which we are personally familiar, and the examples have been drawn largely from our own work. Space does not permit further reference to the numerous and often elegant experiments of others along this same line.

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