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β-D-GLUCOSIDASES OF *Sclerotium rolfsii*. SUBSTRATE SPECIFICITY AND MODE OF ACTION*

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ABSTRACT

The substrate specificity and mode of action of the four pure β-D-glucosidase enzymes (EC 3.2.1.21) from *Sclerotium rolfsii* were studied and their contribution to cellulolysis is discussed. The enzymes are specific for substrates having the β-D configuration. The specificity of the enzymes is not restricted to the β-D-(1→4) linkage, as all four β-D-glucosidases hydrolyzed substrates having β-D-(1→6)-, -(1→3) and -(1→2) linkages. The enzymes require strictly a D-*gluco* configuration for activity. The β-D-glucosidases had no action on highly ordered cellulose, such as Avicel, but slowly hydrolyzed disordered cellulose (phosphoric acid-swollen Avicel) and carboxymethylcellulose, and rapidly cellodextrins, removing D-glucose residues from the nonreducing end. The pure enzymes behaved rather as exo-β-D-glucan glucohydrolase. The K_m values of all four β-D-glucosidases decreased with increase in the chain length of cellodextrins. Cellopentaose was the preferred substrate for all four enzymes. The major route of D-glucose formation from cellulose by hydrolysis with *S. rolfsii* β-D-glucosidases proceeds through higher-molecular-weight cellodextrins.

INTRODUCTION

In the preceding paper[1], the purification, characterization, and study of some of the physicochemical properties of four β-D-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21; BG-I–IV) enzymes from *Sclerotrium rolfsii*, a potential source of cellulase enzymes for saccharification of cellulosic materials[2–6], have been described. The purified BG-III β-D-glucosidase hydrolyzes cellobiose at a rate faster than those of the β-D-glucosidase preparations reported from other sources[7,8]. Cellobiose inhibits the action of cellobiohydrolase and endo-D-glucanase[9–11]. Thus, cellobiase plays a central role in the metabolism of cellulose. The present paper presents data on substrate specificity and mode of action of the β-D-glucosidases of *S. rolfsii*, components of cellulase complex, and their contribu-

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tion to cellulolysis. The data obtained suggest that higher-molecular-weight cellodextrins, and probably insoluble shorter oligosaccharides (produced by the action of endo-D-glucanases on cellulose), and not cellobiose (produced by cellobiohydrolase and endo-D-glucanases) as is generally believed, are the major route of formation of D-glucose from cellulose by *S. rolfsii*.

EXPERIMENTAL

Materials and methods. — Cellotetraitol and *p*-nitrophenyl β-D-cellobioside were kindly supplied by Dr. K. Wakabayashi, Shinshu University, Japan. Cellodextrins (Glc_{3-6}), cellobiitol, cellopentaitol, laminarabiose, sophorose, and pustulan were kindly supplied by Dr. E. T. Reese (U.S. Army Laboratories, U.S.A.), cellodextrins (Glc_{3-5}) also by Prof. D. A. I. Goring (Pulp and Paper Institute, Canada). The following materials were purchased from the suppliers indicated: "Glox" D-glucose reagent from AB, Kabi Diagnostica, Stockholm; melibiose, α,α-trehalose dihydrate, salicin, esculin hydrate, arbutin, phloridzin, methyl α- and β-D-glucopyranoside, phenyl α- and β-D-glucopyranoside, *p*-nitrophenyl α- and β-D-glucopyranoside, *o*-nitrophenyl β-D-glucopyranoside, *p*-nitrophenyl β-D-galactopyranoside, carboxymethylcellulose d.s. 0.7, lichenan and D-xylan (larchwood) from Sigma Chemical Co. (U.S.A.); laminaran and *p*-nitrophenyl β-D-xylopyranoside from Koch–Light (U.K.). Collodion bags were purchased from Sartorius Membran Filter GmbH (Göttingen, West Germany). All other chemicals used were from commercial sources[2,5].

Analytical methods. — Reducing sugars, expressed as D-glucose equivalents, were determined by the dinitrosalicylic acid method[12] or the method of Somogyi[13] and Nelson[14]. D-Glucose was determined by the D-glucose oxidase-peroxidase method[15] using "Glox" reagent, as described previously[5]. Filter-paper chromatography (Whatman filter paper No. 1) was used for qualitative examination of the sugar composition of the products of hydrolysis[5].

Enzyme assays. — Carboxymethylcellulase (saccharifying and liquifying activities) was determined as described previously[1,2,5]. β-D-Glucosidase activity was measured as the amount of D-glucose released from cellobiose, or *p*-nitrophenol from *p*-nitrophenyl β-D-glucopyranoside. For cellobiase-activity determination, the suitably diluted enzyme (0.1 mL) was added to 50mM citrate buffer, pH 4.5 (0.9 mL) containing 16 mg of cellobiose/mL (which is the saturating concentration for the enzyme at 65°), and the mixture incubated for 30 min at 65° or at the indicated temperature. The reaction was stopped by heating in a boiling-water bath for 5 min. With *p*-nitrophenyl β-D-glucopyranoside as substrate, the assay mixture contained the glycoside (0.9 mL, 3 mg/mL) in 50mM citrate buffer, pH 4.2, and the suitably diluted enzyme (0.1 mL). After incubation for 30 min at 68° (or at indicated temperature) 2% sodium carbonate (1 mL) was added, and the amount of *p*-nitrophenol released was calculated from the absorbancy index[16] of 18.5 $cm^2/\mu mol$

for nitrophenol at 410 nm. D-Glucose was estimated by the D-glucose oxidase–peroxidase method[15] to assay β-D-glucosidase activity towards cellodextrins and other substrates.

TABLE I

HYDROLYSIS OF GLUCOSIDES BY β-D-GLUCOSIDASES (I–IV) OF *S. rolfsii*[a]

Substrate	*Components*[b]	*Linkage of D-glycosyl group*	*D-Glucose (μg) liberated by*			
			BG-I	*BG-II*	*BG-III*	*BG-IV*
Cellobiose	Glc→Glc	β-(1→4)	55	98	105	52
Gentiobiose	Glc→Glc	β-(1→6)	51	96	70	48
Laminarabiose	Glc→Glc	β-(1→3)	65	140	140	60
Sophorose	Glc→Glc	β-(1→2)	43	80	110	53
Sucrose	Glc→Fru	α-(1→2)	7	12	17	8
Lactose	Gal→Glc	β-(1→4)	0	0	0	0
Maltose	Glc→Glc	α-(1→4)	0	0	0	0
Melibiose	Gal→Glc	α-(1→6)	0	0	0	0
Trehalose	Glc→Glc	α-(1→1)	0	0	0	0
Arbutin	Hydroquinone→Glc	β	10	17	14	7
Salicin	Salicyl→Glc	β	15	26	26	14
Phloridzin	Phloretin→Glc	β	1	1	2	1
Esculin hydrate	6,7-Dihydroxy coumarin-6-yl→Glc	β	3	4	3	2
Methyl α-D-glucopyranoside		α	0	0	0	0
Phenyl α-D-glucopyranoside		α	0	0	0	0
p-Nitrophenyl α-D-glucopyranoside[b]		α	0	0	0	0
Methyl β-D-glucopyranoside		β	3	6	4	2
Phenyl β-D-glucopyranoside		β	38	65	55	34
o-Nitrophenyl β-D-glucopyranoside[b]		β	52	70	70	42
p-Nitrophenyl β-D-glucopyranoside[b]		β	65	62	70	55
p-Nitrophenyl β-D-cellobioside		β	[c]	[c]	110	[c]
p-Nitrophenyl β-D-galactopyranoside[b]		β	0	0	0	0
p-Nitrophenyl β-D-xylopyranoside[b]		β	0	0	0	0

[a]The reaction mixture contained 20mM substrate (0.5 mL), 50mM citrate buffer, pH 4.5, (0.4 mL) and enzyme (0.1 mL, 0.3 μg). The amount of D-glucose liberated within 30 min at 65° was determined by the D-glucose oxidase–peroxidase procedure[15]. [b]*o*- or *p*-Nitrophenol liberated was measured. [c]Not determined.

RESULTS

The purified β-D-glucosidases from *S. rolfsii* used in the present experiments were pure by the various criteria tested[1] and were free of endo-D-glucanase activity as determined viscosimetrically[17]. The optimum pH for activity for the four enzymes was 4.2 with *p*-nitrophenyl β-D-glucopyranoside and 4.5 with cellobiose as substrate. The optimum temperature of the four enzymes was 68° with *p*-nitrophenyl β-D-glucopyranoside and 65° with cellodextrins (Glc_{2-6}).

Action of β-D-glucosidases on various D-*glucosides.* — Table I summarizes the results obtained when various D-glucosides were subjected to enzymic hydrolysis. The enzymes can tolerate a wide variety of aglycons, provided the D-glucosyl residue of the substrate has the β configuration. However, the rate of hydrolysis depended on the nature of the aglycon moiety. Replacement of a methyl by a phenyl or a nitrophenyl group increased 10 to 20 times the rate of hydrolysis. The β-D-glucosidases hydrolyzed salicin at ~1/3–1/4 the rate of cellobiose. Arbutin was cleaved at a slightly lower rate than salicin. Barely any activity was detected with phloridzin and esculin hydrate. Garibaldi and Gibbins[18] reported that phloridzin is the preferred substrate for a partially purified β-D-glucosidase from *Erwinica herbicola* Y 46. The *S. rolfsii* enzymes possessed strict specificity for the D-*glycero* configuration at C-4 as both *p*-nitrophenyl β-D-galactopyranoside and lactose were not hydrolyzed, whereas the purified β-D-glucosidase from *Clostridium thermocellum* hydrolyzed these two β-D-galactosides[19]. All disaccharides containing a β-D-glucopyranosyl group were good substrates for the *S. rolfsii* enzymes. The specificity of

TABLE II

ACTION OF *S. rolfsii* β-D-GLUCOSIDASES (I–IV) ON VARIOUS β-D-GLUCANS[a]

β-D-*Glucan*	*Linkage*	β-D-*Glucosidase*							
		I		*II*		*III*		*IV*	
		Glox	*S–N*	*Glox*	*S–N*	*Glox*	*S–N*	*Glox*	*S–N*
Laminaran	β-(1→3)	196	220	398	400	392	380	194	210
Lichenan	β-(1→3) and β-(1→4)	23	20	44	32	80	65	41	35
Pustulan	β-(1→6)	53	[c]	134	[c]	127	[c]	44	[c]
Carboxymethyl cellulose[b]	β-(1→4)	18	16	23	20	40	34	16	12
Avicel	β-(1→4)	0	0	0	0	0	0	0	0
Phosphoric acid-treated Avicel	β-(1→4)	17	15	34	28	50	46	8	5
G-37[d]	β-(1→4)	9	7	16	20	31	25	6	4

[a]The reaction mixture contained 1% of D-glucan (0.5 mL), 50mM citrate buffer, pH 4.5 (0.4 mL), and enzyme (0.1 mL, 1 μg) for 24 h at 50°. The D-glucose (μg/mL) was measured in the supernatant solution by the D-glucose oxidase–peroxidase (Glox)[15] and Nelson–Somogyi[13,14] (SN) procedures. [b]D.S. 0.7. [c]Not determined. [d]G-37 = cellooligosaccharide, degree of polymerization 37.

the enzymes was not restricted to the β-D-(1→4) linkage, as all the four enzymes hydrolyzed positional isomers of cellobiose, *viz.*, sophorose [β-D-(1→2)], laminarabiose [β-D-(1→3)], and gentiobiose [β-D-(1→6) linkage]. None of the compounds having an α-D-glucopyranosyl group was hydrolyzed. The enzymes also did not hydrolyze D-xylosides. Wolter *et al.*[20] isolated, from *Poria placenta*, an enzyme that acts on α- and β-D-glucopyranosides, α- and β-D-galactopyranosides, β-D-xylopyranosides, carboxymethylcellulose, D-gluco-D-mannan, and D-xylan as substrates. The β-D-glucosidases from *Lenzites trabea*[16] and *Stachybotrys atra*[21] hydrolyzed both D-glucosides and D-xylosides.

Action of β-D-glucosidases on various β-D-glucans. — None of the four β-D-glucosidase enzymes acted on highly ordered substrates, such as Avicel, but the enzymes slowly hydrolyzed partially disordered substrate such as phosphoric acid–swollen cellulose and carboxymethylcellulose (Table II). However, no measurable decrease in viscosity of a carboxymethylcellulose solution was observed in the presence of the enzymes up to 12 h. Laminaran [β-D-(1→3) linkage] was hydrolyzed at a faster rate than pustulan [β-D-(1→6) linkage] by all the four enzymes. Lichenan [β-D-(1→3) (30%) and β-D-(1→4) (70%) linkages] was hydrolyzed at a rate lower than that of pustulan, except by BG-IV which hydrolyzed pustulan and lichenan at about the same rate. Analysis of the reaction products by paper chromatography showed that D-glucose was the only product in each case, and this was confirmed by quantitative analysis by the D-glucose oxidase–peroxidase procedure[15]; no cellobiose was detected. The values of D-glucose determined in the β-D-glucan hydrolyzates by the D-glucose oxidase–peroxidase or the Somogyi–Nelson[13,14] method (which gives reducing sugar values) were the same, indicating that D-glucose was the only product formed. This result suggests a successive cleavage of D-glucosyl groups from the nonreducing ends of the β-D-glucans.

Hydrolysis of cellodextrins. — The concerted action of exo- and endo-D-glucanases on cellulose produces cellooligosaccharides, cellodextrins, and cellobiose. Some of the β-D-glucosidases described earlier hydrolyze cellobiose as well as higher oligomers, but vary considerably in their kinetic behavior. The pure β-D-glucosidase enzymes from *S. rolfsii* hydrolyzed cellodextrins, and *p*-nitrophenyl β-D-glucopyranoside and β-D-cellobioside. The initial rates of hydrolysis of cellodextrins up to cellohexaose with time indicated cellopentaose as the preferred substrate for all the four β-D-glucosidase enzymes from *S. rolfsii* (Fig. 1). The initial rate of hydrolysis of *p*-nitrophenyl β-D-cellobioside was greater than that of *p*-nitrophenyl β-D-glucopyranoside (Table I).

Kinetics. — The four enzymes have different K_m values for *p*-nitrophenyl β-D-glucopyranoside and for cellodextrins (Table III). This and the different rates of hydrolysis of cellodextrins with the four enzymes suggest that the four β-D-glucosidase enzymes from *S. rolfsii* are isoenzymes and not the results of purification.

The K_m values of the four β-D-glucosidases decreased with increase in the chain length of the cellodextrins. The lower K_m values for higher-molecular-weight

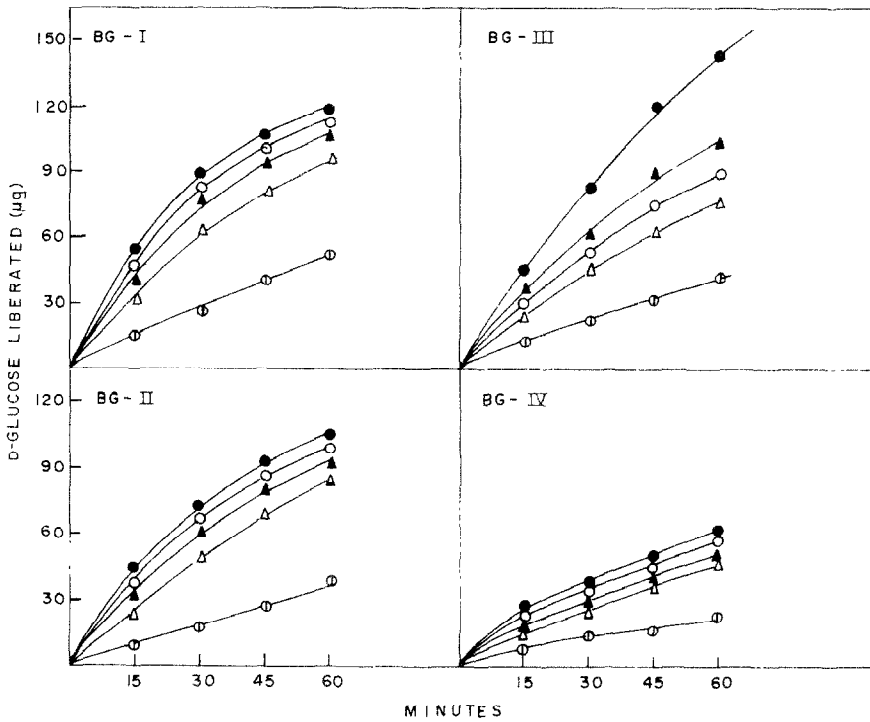

Fig 1 Hydrolysis of cellodextrins (cellohexaose–cellobiose, Glc_6–Glc_2) by *S. rolfsii* β-D-glucosidases, (BGI–IV) The standard assay was used: Enzyme BGI (0 6 μg), BG-II (0.3 μg), BG-IV (0 3 μg), or BG-III (0 1 μg), 0.5mM cellodextrin, pH 4.5, 50°, BSA (0.5 mg/mL), and final volume (1 mL). D-Glucose was estimated by the D-glucose oxidase–peroxidase procedure[15] (□) Glc_2, (△) Glc_3, (○) Glc_4, (●) Glc_5, and (▲) Glc_6

TABLE III

MICHAELIS CONSTANTS (mM) FOR *S. rolfsii* β-D-GLUCOSIDASES I–IV[a]

Substrate	*β-D-Glucosidases*				
	I	*II*	*III*	*IV*	*III*[b]
p-Nitrophenyl β-D-glucopyranoside	1.07	1.38	0.89	0.79	0 51
p-Nitrophenyl β-D-cellobioside	[c]	[c]	0 38	[c]	0 35
Cellobiose	3.65	3 07	5.84	4 15	3.65
Cellotriose	1 00	1 23	1 98	0 70	1 13
Cellotetraose	0.50	0.85	0.83	0 50	0 75
Cellopentaose	0.49	0.40	0 76	0.55	0 60
Cellohexaose	0 62	0 37	0 37	0 67	0.50

[a]Kinetic studies were done with the standard assay systems, at 65° and pH 4.5, and varying the substrate concentration. K_m values were calculated from Lineweaver–Burk plots, which were linear in all cases
[b]Assay carried out at 65° and pH 4.5, in the presence of BSA (0.5 mg/mL) [c]Not determined.

cellodextrins support the preferred order of attack, except for cellohexaose. When the rates of hydrolysis of reduced cellodextrins by BG-III enzyme were compared with those of the corresponding unreduced cellodextrins, it was observed that cellobiitol was almost completely resistant to enzymic hydrolysis. In contrast, cellotetraitol and -pentaitol were hydrolyzed to almost the same extents as those for the corresponding unreduced cellodextrins, *i.e.,* as the hydrolysis of the reduced oligomer proceeded and reached the cellobiitol stage, the influence of the reduced group of the D-glucitol residue became pronounced. Cello-triitol and -hexaitol could not be tested as these were not available. Of the reduced cellodextrins, cellopentaitol was the most favored substrate. The mode of hydrolysis of unreduced and reduced cellodextrins revealed that the linkage split was, in each case, that of the nonreducing group.

Effect of temperature on K_m *and* K_i. — It was reported earlier[1] that D-glucose causes a competitive inhibition of cellobiose hydrolysis by BG-III enzyme with a K_i value of 0.55mM. The experiment was conducted for 30 min at 65° in the absence of bovine serum albumin (BSA). It has now been observed that when inhibition of cellobiase by D-glucose is carried out at 65°, but in the presence of BSA (0.5 mg/mL), the inhibition is noncompetitive and the K_i value, calculated from Lineweaver–Burk and Dixon plots, is 0.75mM. When the BG-III β-D-glucosidase was heated for 30 min at 65° in the absence of BSA, it lost 60–65° of its activity, but only 10–12% in the presence of BSA. It was also observed that the K_m value for cellobiose, when determined at 65° in the presence of BSA, decreased from 5.84 to 3.65mM when determined in the absence of BSA (Table III). It is probable that denaturation of the enzyme in the absence of BSA caused abberations in the earlier kinetic studies.

For all four enzymes, the inhibition by D-glucose was more marked with cellobiose (50–67%) as substrate and decreased with the chain length of cellodextrins (29–40%). Thus, D-glucose accumulation during cellulolysis would suppress more the hydrolysis of cellobiose than that of other cellodextrins.

Products of hydrolysis. — The time course of the formation of hydrolysis products from cellodextrins, phosphoric acid-treated Avicel, and laminaran was followed by analyzing the digest by paper chromatography at 2, 10, 60, and 480 min, and 24 h. The first identifiable product of hydrolysis from all these substrates was D-glucose. Enzymic removal of D-glucose residues from cellodextrins exclusively from the ends of the chain would produce no other products as observed for the hydrolysis of cello-pentaose (Fig. 2) and -hexaose (not shown) at various incubation times. From cellopentaose, cellotetraose and D-glucose were detected at 2 min; cello-tetraose, -triose, and D-glucose at 10 min; and cello-tetraose, -triose, -biose and D-glucose at 60 min. There was a progressive increase in the proportion of D-glucose with time. The enzyme ultimately hydrolyzed all the cellodextrins, cello-biose to -hexaose, completely to D-glucose. D-Glucose was also the only product detected by paper chromatography of hydrolyzates, by BG-III, of phosphoric acid-treated Avicel and laminaran. Thus, the enzymes may be characterized as

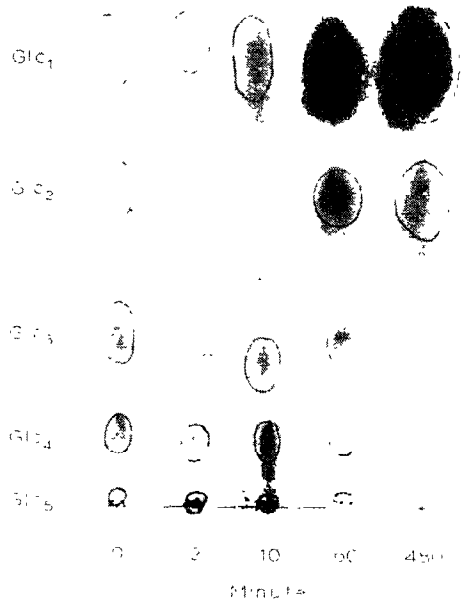

Fig 2 Paper chromatogram of the products of hydrolysis. Hydrolysis was carried out with BG-III (1 μg) and cellopentaose (6 mg) in mM citrate buffer at pH 4 5 and 50°

exo-glucosidases acting by cleavage of D-glucosyl groups from the nonreducing ends of the chain of the substrates.

DISCUSSION

Our understanding of the role played by β-D-glucosidase in the overall metabolism of cellulose is still limited. The reason for this is that few sufficiently pure β-D-glucosidases, free from contaminating components of the cellulase system, have been obtained from active cellulolytic microorganisms. An exo-D-glucanase purified from *Trichoderma viride* culture filtrate was active as a nitrophenyl β-D-glucosidase, which showed considerable hydrolysis of carboxymethylcellulose also[22]. Wood and McCrae[23] have purified a nitrophenyl β-D-glucosidase, from *T. viride*, that contained up to 2% of the culture filtrate's carboxymethylcellulase. The isolation and purification of four pure β-D-glucosidase enzymes from *S. rolfsii*[1], free from other cellulolytic components, has made possible a study of their contribution in the overall process of cellulolysis by *S. rolfsii*.

The β-D-glucosidases of *S. rolfsii* have some features in common with the corresponding purified enzymes from other sources[7,24]. The enzymes of *S. rolfsii*, *Pyricularia oryzae*[25], and *Aspergillus aculeatus*[26] hydrolyze carboxymethylcellulose and phosphoric acid-swollen cellulose slowly. However, the pure β-D-glucosidase of *Clostridium thermocellum* does not hydrolyze carboxymethylcellulose[19]. Like the K_m values of the β-D-glucosidase enzymes of *S. rolfsii*, that of the K_m of the

Trichoderma koningii cellobiase is lower, and the reaction velocity of the enzyme is higher for the larger-molecular-weight cellodextrins (up to cellopentaose) than that for the dimer[7]. However, the reaction velocity of the β-D-glucosidase of *Trichoderma reesei*, characterized by Berghem and Pettersson[27], was lower with oligomers, such as tetraose (the only one reported), than with cellobiose.

The pure β-D-glucosidase enzymes of *S. rolfsii* hydrolyzed cellodextrins rapidly and phosphoric acid-swollen cellulose slowly, removing D-glucose units successively from the nonreducing end of the substrates. The β-D-glucosidases of *S. rolfsii* are, thus, exo-β-D-glucan glucohydrolases. An enzyme having the capacity to attack long-chain polymers, such as phosphoric acid-treated cellulose, cellodextrins, and carboxymethylcellulose may be classified, according to the definition of Resse *et al.*[28], as an exoglucanase. The β-D-glucosidases of *T. koningii*[7], *P. oryzae*[25], and *Aspergillus niger*[29] have been classified as exo-(1→4)-β-D-glucan glucohydrolases, and an exo-D-glucanase, isolated from *Sporotrichum pulverulentum*, had mixed endo-D-glucanase–D-glucosidase activity[30].

The affinity for the substrates may be a key to the efficacy of the β-D-glucosidase. The higher reaction velocities of the four *S. rolfsii* β-D-glucosidase enzymes with higher-molecular-weight cellodextrins as compared to cellobiose, the decrease in their K_m values, and the decrease in their inhibitory effect of D-glucose on the rate of hydrolysis with the increase in the chain length of cellodextrins indicate that higher-molecular-weight cellodextrins, and not cellobiose, are the major route of D-glucose formation from cellulose.

S. rolfsii excretes large amounts of β-D-glucosidase activity in the culture broth[2]. Though the culture broth has also been found to contain cellobiose dehydrogenase, the activity excreted is quite low[31]. Furthermore, the optimum pH is 6.5, whereas that for saccharification and cellobiase activity is 4.5. Cellobiose phosphorylase was not detected in the culture broth. Therefore, the major route of cellobiose metabolism during saccharification seems to be through the β-D-glucosidase degradation and not through oxidation[32] or cellodextrins phosphorylase-degradation[33–35].

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