23 β-Glucosidases from Filamentous Fungi: Properties, Structure, and Applications

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I. INTRODUCTION

The recurrent crisis in the Persian Gulf illustrates the overdependence of the world on readily accessible and inexpensive sources of oil, and also shows the importance of developing alternative renewable sources of liquid fuels. Lignocellulosic materials, being inexpensive and abundant, represent one of the most promising raw materials for the production of such fuels. However, despite of numerous research efforts in different parts of the world, the biodegradation of lignocellulose (the main component of which is cellulose) is still not economically competitive, especially because of the high costs of the enzymes involved.[1]

Cellulose hydrolysis is performed in nature by the concerted action of a set of enzymes collectively called "cellulases." These enzymes are the endoglucanases (EC 3.2.1.4), which split internal β-glycosidic bonds, the exoglucanases (EC 3.2.1.91) that hydrolyze cellobiose residues from the nonreducing end of the cellulose fibers, and the β-glucosidases. Strictly speaking, β-glucosidase is not a cellulase, because it does not attack cellulose directly. However, it is considered important for the cellulolytic process, because it hydrolyzes soluble cellodextrins and cellobiose to glucose.

β-Glucosidase (or β-D-glucoside glucohydrolase, EC 3.2.1.21) hydrolyzes glycosidic bonds between two glucose residues or between a glucose residue and an aglycon, that can be an alkyl or aryl residue (Figure 23.1). As will be seen in more detail below, the specificity of β-glucosidase toward different substrates varies depending on the enzyme source. This enzyme is widely distributed in nature and can be found in animals,[2] plants,[3] bacteria,[4] fungi,[5] and yeasts.[6]

As indicated above, β-glucosidases, particularly those from microorganisms, are important in cellulose saccharification. Microorganisms that are poor producers of β-glucosidase do not degrade cellulose efficiently. Cellobiose, which is generated by the action of the endo- and exoglucanase, acts as inhibitor of those enzymes and has to be removed by β-glucosidase.[7] Due to its potential biotechnological importance, the β-glucosidase from numerous sources, particularly bacteria and fungi, have been purified and characterized. The work on β-glucosidases, until 1982, has been reviewed by Woodward and Wiseman;[8] later, Leclerc and co-authors[9] have surveyed the enzymes from yeast, and Stutzenberger[10] has reviewed thermostable β-glucosidases from mesophilic and thermophilic fungi. More recently, Bhatia and colleagues[11] have discussed microbial β-glucosidases particularly cloning, mode of action, and applications.

This review will focus on the fungal enzymes; special emphasis will be on the properties and structure of the enzymes and their possible biotechnological applications.

II. METHODS FOR ASSAY OF β-GLUCOSIDASE

Several methods, both sensitive and easy to use, have been developed for the determination of β-glucosidase activity. The most common are those using alkyl- or arylglucosides as substrates, which upon hydrolysis, release either colored or fluorescent products. The most utilized substrate is *p*-nitrophenyl-β-D-glucopyranoside (PNPG), which releases *p*-nitrophenol.[12] Some authors replace this substrate with the *ortho* isomer; however, it has been found that several fungal enzymes, such as the

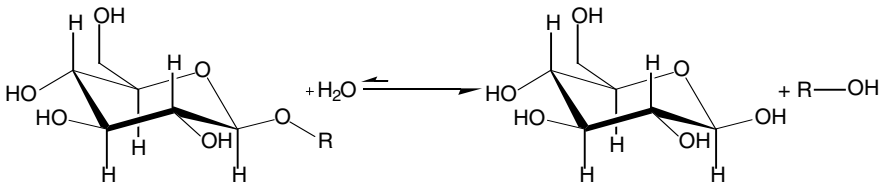

FIGURE 23.1 Reaction catalyzed by β-glucosidase. R can be glucose or an alkyl or aryl residue.

β-glucosidases from *Trichoderma koningii*[13] and *T. reesei*,[14] hydrolyze this isomer more slowly. Other substrates used are methyl-β-D-glucopyranoside, the natural glycosides salicin, esculin, and amygdalin, and the disaccharide cellobiose[12] (Figure 23.2).

If the criterion for assay is activity in cellulose biodegradation, cellobiose should be the substrate of choice.[15] Activity toward cellobiose is measured determining free glucose by the glucose oxidase–peroxidase method.[16] Unfortunately, this method can be influenced by inhibitory products from wood decomposition present in

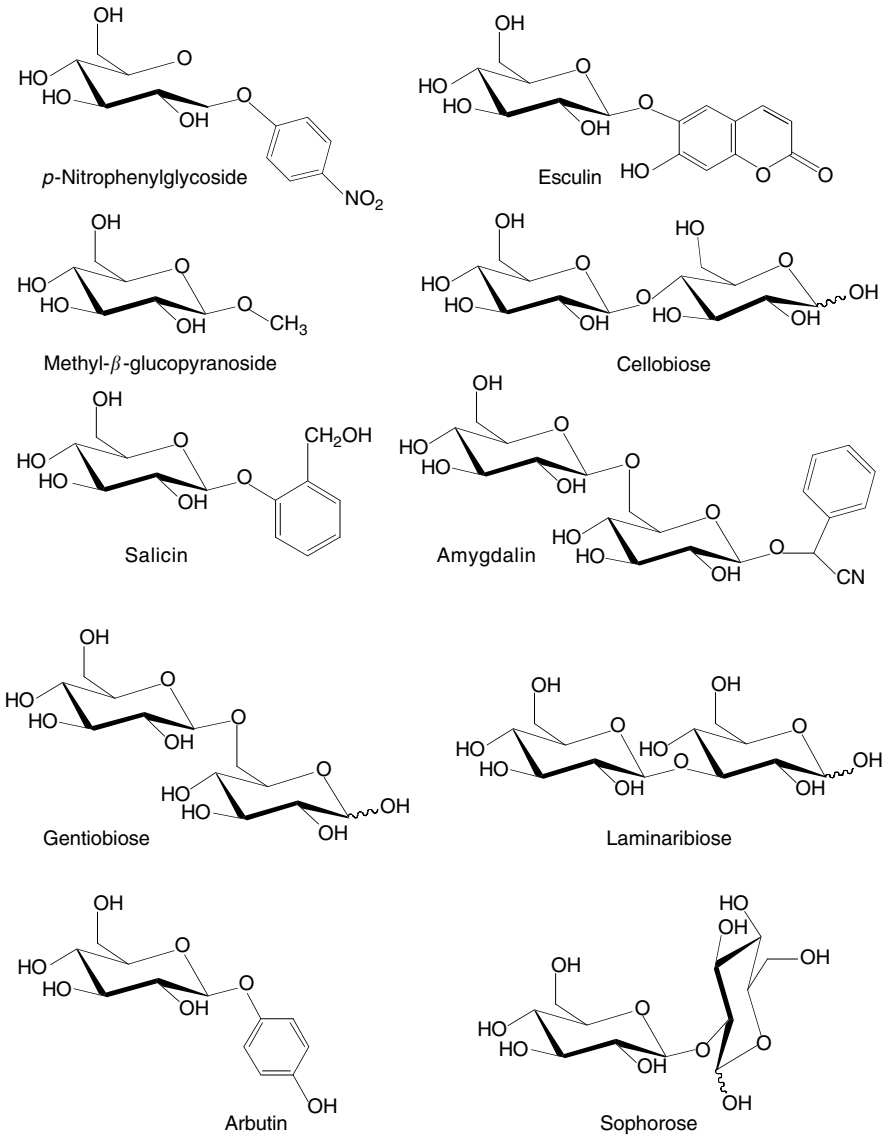

FIGURE 23.2 Substrates of β-glucosidase.

culture filtrates.[17] Glucose detection can also be accomplished by means of the coupled hexokinase/glucose-6- phosphate dehydrogenase assay.[18]

β-Glucosidase products can also be analyzed by high-performance liquid chromatography (HPLC);[19] the use of pulse amperometric detection is particularly sensitive.[20] This analysis is not affected by wood decomposition products.[21] For routine work, thin-layer chromatography (TLC)[22] and, more recently, high-performance thin-layer chromatography (HPTLC) have been used.[23] These techniques allow also the identification of transglycosylation products. Transglycosylating activity is common to many fungal β-glucosidases.[24–26]

Jackson[27] has described a procedure of high resolution for the separation of reducing saccharides, previously derivatized with the fluorophore 8 aminonaphtalene-1,3,6 trisulfonic acid (ANTS), by means of polyacrylamide gel electrophoresis. Amounts as small as 0.2 pmol of ANTS-labeled saccharides can be detected. This method should be useful in the analysis of β-glucosidase-generated products.

Fungal β-glucosidases are often produced in multiple enzyme forms that differ in some physicochemical properties.[14–15,26,28–37] Zymogram techniques are particularly useful in isozyme detection; they are based on gel electrophoresis or isoelectrofocusing, followed by visualization of the activities *in situ* in the gel. Two frequently used substrates are 6-bromo-2-naphtyl-β-D-glucoside and 4-methylumbelliferyl-β-D-glucoside;[38] 5-bromo-4-chloro-3-indolyl β-D-glucopyranoside, an alternative substrate, yields insoluble indigo upon hydrolysis.[39] PNPG and ONPG have also been used, although their reaction products diffuse quickly, and are less sensitive.

III. SUBSTRATE SPECIFICITY

Since fungal β-glucosidases are responsible for the hydrolysis of cellobiose in the saccharification of cellulose, these enzymes have also been called cellobiases. This name, however, is misleading, since it suggests a high substrate specificity for cellobiose. This is not the case, since β-glucosidases are also capable of hydrolyzing other β-1, 4 oligosaccharides with chain lengths of up to eight glucose units (cellooctaose).[19,40–42] β-Glucosidases, however, can be distinguished from the exoglucohydrolases (EC 3.2.1.74); the latter act more rapidly on longer cellooligosaccharides, while the speed of hydrolysis by β-glucosidases slows down with the increasing degree of polymerization (DP) of the substrate.[40,43] Besides, D-glucono-1,5-δ-lactone, a potent inhibitor of β-glucosidases,[43,44] does not inhibit the exoglucohydrolases.[43] Endo- and exoglucanases are also not significantly inhibited by this compound.[44]

β-Glucosidases show a great diversity in substrate specificity, and no definite patterns have been recognized so that every β-glucosidase has to be studied separately. However, the fungal enzymes can be broadly classified in two groups, based on their relative activities toward cellobiose and P(O)NPG:

(1) *Aryl-β-glucosidases*: these enzymes show higher relative activities toward P(O)NPG than cellobiose, or even show no activity toward this last substrate
(2) *Cellobiases*: enzymes that show higher activity toward cellobiose.

Penicillium herquei possesses two isoforms of β-glucosidase called G1 and G2;[28] these should be considered aryl-β-glucosidases, since their relative activities

toward cellobiose are 91.3 and 40.4% as compared with PNPG. Similarly, the relative activity of the enzyme from *P. oxalicum* toward cellobiose is about 81%.[45] The β-glucosidase 3 from *Aspergillus aculeatus* shows only a relative activity toward cellobiose of 6.7%, an extreme case among fungal β-glucosidases with cellobiase activity.[46] Interestingly, the other two isoenzymes from this fungus (β-glucosidases 1 and 2) show relative activities toward cellobiose of 400 to 500%, so they should be considered as cellobiases.[46] However, these enzymes are also very active toward cellooligosaccharides with a degree of polymerization (DP) of 3 to 6, and their relative activities toward these substrates is higher than for cellobiose. These enzymes even show activity toward an insoluble cellooligosaccharide of DP=20 which equals that toward PNPG; for this reason they are also called cellooligoglucosidases.[46] On the other hand, β-glucosidase A purified from *A. niger*[47] and a β-glucosidase from *Stereum sanguinolentum*[48] are good examples of the cellobiase class.

With respect to substrate affinities (K_M), β-glucosidases also show great differences. Again, using their affinities toward P(O)NPG and cellobiose as references, they can be classified operationally into three groups:

(1) β-Glucosidases with affinities (K_M) similar for both substrates
(2) β-Glucosidases which show higher affinity (lower K_M) for cellobiose
(3) β-Glucosidases with higher affinities for P(O)NPG

Table 23.1 shows the K_M values for a set of fungal β-glucosidases for P(O)NPG and cellobiose. Values for P(O)NPG range from 0.055 m*M* (*Stachibotrys atra*[116]) to 34 m*M* (β-glucosidase II from *Phytophtora infestans*[107]) and for cellobiose from 0.031 (*Neocallimastix frontalis*[20]) to 340 m*M* (β-glucosidase II from *P. infestans)*.[107] The majority of the enzymes listed can be classified in the first group. Few enzymes show significantly higher affinities for cellobiase, such as the enzyme from *N. frontalis*[97] and *Piromyces* sp.[107] More common is the finding of lower K_M values for P(O)NPG; examples are the β-glucosidases from *A. oryzae,*[68] *Monila* sp.[92] and *Talaromyces emersonii* β-glucosidase III.[25,116]

Besides hydrolyzing cellooligosaccharides of different lengths, β-glucosidases most often show activity toward both natural (plant) or synthetic aryl-glucosides, with a variety of aglycons. The relative activities toward these glycosides vary from enzyme to enzyme. A β-glucosidase purified from *A. niger* shows relative activities (cellobiose = 100%) toward the disaccharides laminaribiose (β1-3), gentiobiose (β1-6), sophorose (β1-2), and salicin (salicyl-glucose) of 92, 63, 48, 22, and 7%, respectively.[56] The G1 enzyme from *P. herquei* shows relative activities (PNPG = 100%) of 82.7 and 70.3% toward gentiobiose and salicin, while those of the G2 isoenzyme are 8.7 and 54.5%, respectively.[28] This evidence indicates that differences are not only found between enzymes from different species but also between isoenzymes from the same microorganism.

Although in general β-glucosidases show a stronger activity toward oligosaccharides with (β1→4) linkages, several examples are found in the literature, where a higher activity is described toward glucans with β(1→2) and β(1→3) linkages. Enzymes with such properties are found in *A. fumigatus*[32] and *T. koningii,*[13] which are more active toward laminaribiose and sophorose than cellobiose. Such enzymes

TABLE 23.1
Physical Properties, Carbohydrate Content and K_M Values for Fungal β-Glucosidases

Organism	M_W ($\times 10^3$)		pI	Carbohydrate	K_M (m*M*)		Ref.
	Native	SDS–PAGE		Content (%)	PNPG (ONPG)	Cello-biose	
Acremonium persicinum	140	128	4.3	1.5	0.3	0.91	49
Aspergillus aculeatus							35
β-Glucosidase 1		133	4.7	23			
β-Glucosidase 2		132	4.3	22			
β-Glucosidase 3		136	3.6	15			
Aspergillus fumigatus							
β-Glucosidase$_{small}$	40,8		6.3	Glycoprotein			32
β-Glucosidase$_{large}$	340	170		Glycoprotein	0.88	0.84	33
Aspergillus japonicus	>240				(1.17)	5.7	50
Aspergillus kawashii							51
EX1		145		Glycoprotein			
EX2		130		Glycoprotein			
CB-1		120		Glycoprotein			
Aspergillus nidulans							
P-I	125	125	4.4		0.842	0.997	30
P-II	50	50	4.0		0.465	0.796	
β-Glucosidase I	240				0.53 (0.55)	1.17	52
β-Glucosidase II	78				0.22 (0.32)	0.89	
Aspergillus niger							
form I	117.4	121.9	4.2	17	0.47		29
form II	117.4	121.9	4.2	7.7	0.36		
β-Glucosidase A		118			0.43	0.50	47
β-Glucosidase B		109			0.11	0.27	
	325				0.82	1.33	53
β-Glucosidase I	96		4.6	17.5	0.616	0.446	37
β-Glucosidase II	96		3.8	17		0.201	
	230				0.75 (2.18)	1.23	54
					0.67	2.04	55
	137		3.8	12.5		0.85	42,56
Cellobiase A		88		8.8		0.90	57
Cellobiase B		80		9.4		1.63	
Cellobiase C		71		7.2		1.0	
	330	110			1.11		58
Novozym 188			5.0		1.03	5.63	59
					0.5		60
					0.63		61
β-Glucosidase I	105	49	3.2		21.7		62
β-Glucosidase II	360	120	4.0		2.2	15.4	63
	240	120	4.0		0.9	2.3	146

(*Continued*)

TABLE 23.1 (Continued)

Organism	M_W ($\times 10^3$)		pI	Carbohydrate	K_M (m*M*)		Ref.
	Native	SDS–PAGE		Content (%)	PNPG (ONPG)	Cellobiose	
Aspergillus ornatus					0.76		64
Aspergillus oryzae	40	43	4.2		0.55	7	65
Aspergillus phoenicis						0.75	66
Aspergillus roseus							
β-Glucosidase A	110	96		Glycoprotein	1.33	4.7	67
β-Glucosidase B	68	78		Glycoprotein	0.5	2	
Aspergillus sojae	250	118	3.8	23.8	0.14		68
Aspergillus terreus				65	0.78	0.40	69
Aspergillus tubingensis							70
I		131	4.2		0.76		
II		126	3.9		0.35		
III		54	3.7		3.2		
IV		54	3.6		6.2		
Aspergillus wentii							
A3	170		3.8				71
B2	145						72
					1.9	2.7	73
Botryodiplodia theobromae							
	331.6				0.33	1.0	74
Botrytis cinerea	350	88		Glycoprotein	0.06 (0.16)		75
	380	170					76,77
Chaetomium thermophilum var. *coprophilum* (deglycosylated)	40	43	7.7	73	0.76	3.13	78
Chaeomium trilaterale	240	118	4.86		5.1	1.9	79
Chalara paradoxa	170	167			0.52	0.58	80
Cladosporium resinae	98	98	4.2		0.07	2.3	81
Curvularia sp.					0.2		82
Evernia prunastri	311	60, 70	3.12		0.635	0.244	83
Fungal strain Y94	240	120	5.02			2.3	84
Fusarium oxysporum	110	110	3.8		0.093	1.07	41
Fusarium solani	400		3.15				85
Humicola grisea	156	82			0.316		86
	55	55		35	0.12	3.24	87
Humicola insolens		250	4.23		0.53	1.52	88
	45	45	8.1	10			89
Humicola lanuginosa	135	110		9	0.5	0.44	90
Lenzites trabea	320				0.41	1.4	91

(*Continued*)

TABLE 23.1 (Continued)

Organism	M_W ($\times 10^3$)		pI	Carbohydrate	K_M (m*M*)		Ref.
	Native	SDS–PAGE		Content (%)	PNPG (ONPG)	Cello-biose	
Macrophomina phaseolina							
β-glucosidase 1	323.6				(1.25)	1.6	34
β-glucosidase 2	213				(0.125)	0.9	
Monilia sp.	46.5	46.7	8.87	Glycoprotein	0.075	5.7	92
Mucor miehei							
G-a	250		4.3	23.4	0.58	1.04	93
G-b	220		4.3	13.0	0.58	1.04	
Myceliophtora thermophila					4.5		94
Nectria catalinensis					0.25		95
Neocallimastix frontalis		125	7.1		2.5		96
		85	6.95		0.67	0.053	97
	153	165	3.7			0.031	20
Orpinomyces sp.	87	85.4	3.95	8.5	0.35	0.25	98
Penicillium funiculosum					0.4	2.1	99
β-Glucosidase 1	230	120	4.55	10.7	0.23	0.2	100
β-Glucosidase 2	18				1.08		
Penicillium herquei							
G1		125	5.02	12.7	0.83	0.6	28
G2		122	5.24	16.1	0.63	1.3	
Penicillium oxalicum	133.5	110	4.0	Glycoprotein	0.37	10	45
Penicillium purpurogenum							
	90	90	4.2;6.0		0.085	0.8	101
	185	185		8	0.57	1.56	102
Phanerochaete chrysosporium							
Intracellular	410				0.11		103
Extracellular	90				0.16	0.53	
		46		4.64	5.3		104
		114			0.096	2.3	105
Phytophtora infectans							106
β-Glucosidase I	160–230			3.3	1.1	280	
β-Glucosidase II	32			4.7	34	340	
Piromyces sp.	46	44	4.15		1.53	0.05	107
	75.8				1.0		108
Pisolithus tinctorius	450	150	3.8		0.87		109

(*Continued*)

TABLE 23.1 (Continued)

Organism	M_W (×10³)		pI	Carbohydrate	K_M (m*M*)		Ref.
	Native	SDS–PAGE		Content (%)	PNPG (ONPG)	Cello-biose	
Pleurotus ostreatus							110
F1		35	7.5				
F2		50	7.3				
F3		66	8.5				
Pyricularia oryzae	240	240	4.15		(1.43)	0.91	111
Schizophilum commune							
β-Glucosidase I		110	3.3	Glycoprotein	0.37	2.1	31
β-Glucosidase II		94	3.3	Glycoprotein	0.49	2.1	
Sclerotium rolfsii							40,112
β-Glucosidase 1	90	95.5	4.1		1.07	3.65	
β-Glucosidase 2	90	95.5	4.55		1.38	3.07	
β-Glucosidase 3	107	106	5.10		0.89	5.84	
β-Glucosidase 4	92	95.5	5.55		0.79	4.15	
Scytalidium lignicola							113
I		74			0.2	2.85	
II		69			0.13	5	
Sporotrichum pulverulentum							5
A1		165	4.8				
A2		172	4.52		0.15	4.5	
B1		165	5.15				
B2		172	4.87		0.21	3.7	
B3		182	4.56				
Sporotrichum thermophile							
β-Glucosidase A	440				(0.5)	Inactive	26
β-Glucosidase B	40				(0.18)	0.28	
	240	110			0.29	0.83	114
Stachibotrys atra							
Constitutive	500				0.070		115
Inducible	69		4.8	14.4	0.055		
Talaromyces emersonii							
β-Glucosidase I	138	138	3.4–4.1	51	0.14	0.58	25,116
β-Glucosidase III	40.7	40.7	3.60	26	1.03	23.7	
β-Glucosidase IV	52.5	52.5	4.4–4.5	12	0.81	1.47	
Termitomyces clypeatus	450	110	4.5	24	0.5	1.25	117
Thermoascus aurantiacus	85	89		33	0.52		118
	325	98		15.1			119
Gl-2		175			1.17		120
Gl-3		157			1.38		
	350	120			0.1137 (0.25)	0.637	121

(*Continued*)

TABLE 23.1 (Continued)

Organism	M_W ($\times 10^3$)		pI	Carbohydrate	K_M (m*M*)		Ref.
	Native	SDS–PAGE		Content (%)	PNPG (ONPG)	Cello-biose	
Thermomonospora curvata							
Intracellular	66				5.6	30.3	122
Extracellular	66				1	0.7	
Thermomyces lanuginosus	200	105			0.075	123	
Trametes gibbosa	640				1.48	124	
Trichoderma sp.	178	109	4.8	75	0.096	0.8	125
Trichoderma harzianum	76	75	8.7		0.20 (0.80)		126
Trichoderma koningii							
β-Glucosidase 1	39.8		5.53	No carbohydrate	0.37	1.18	13
β-Glucosidase 2	39.8		5.85	2	0.85	0.86	
Trichoderma reesei	74.6		8.5	0.7	0.1	1.25	127
		78.5	8.3–8.4				39
		70	8.4	35	0.3	0.5	128
β-Glucosidase 1		71	8.7	0.1	0.18	2.1	14
β-Glucosidase 2		114	4.8	9	0.135	11.1	
	98		4.4			3.3	129
					3.5	1.9	130
Trichoderma viride	47	47	5.74	No carbohydrate	0.28	1.5	131
						5.6	132
					0.5	2.5	133
Xylaria regalis		85			1.72		134

may not be involved in cellulose saccharification and may have other biological functions. One can envision their participation, as part of the glucanase complex, in such processes as the utilization of endogenous carbohydrate reserves during conidiogenesis and conidial germination as well as in morphogenesis.[141]

Despite the large variability in relative activities toward cellooligosaccharides, β-1,2/1,3 β-glucans and aryl-glucosides, these enzymes are specific for the β-anomeric configuration. An exception may be the β-glucosidase from *Thermomyces lanuginosus,* which shows significant α-glucosidase activity.[123]

IV. EFFECT OF TEMPERATURE AND pH ON ACTIVITY AND STABILITY

The majority of the fungal β-glucosidases show optimum pH over the range 4.0 to 5.5 (Table 23.2). However, enzymes with optimum pH from as low as 2.5 up to 8.0 have been found; this allows a choice of enzymes for a variety of applications.

TABLE 23.2
Optimum pH and Temperature of Fungal β-Glucosidases

Organism	Optimum pH	Optimal Temperature (°C)	Ref.
Acremonium persicinum	5.5		49
Aspergillus aculeatus			46
β-Glucosidase 1	4–4.5	55	
β-Glucosidase 2	4–4.5	60	
β-Glucosidase 3	3	65	
Aspergillus fumigatus			
β-Glucosidase$_{small}$	5.0		32
β-Gglucosidase$_{large}$	4.0–5.0		33
Aspergillus japonicus	5.0	65	50
Aspergillus kawashii			51
EX1, EX2, EX3	4.5–5	60	
Aspergillus nidulans			
P-1	5.0	50	30
P-2	5.5	60	
β-Glucosidase I	5.0	55–60	52
β-Glucosidase II	5.5	55–60	
Aspergillus niger	4.6	60–65	53
β-Glucosidase A	4	60	47
β-Glucosidase B	3	70	
β-Glucosidase I	5.1		37
β-Glucosidase II	4.1		
	4.6	65	54
	5.0–5.5	60	55
Cellobiase A	4.5	55	57
Cellobiase B	4.5	55	
Cellobiase C	4.5	60	
	4.6–5.3	70	58
Novozym 188	4.5	60–70	59
	4		60
	4.5	60	61
β-Glucosidase I	5.0	55	62
β-Glucosidase II	4.5	60	63
	4.5	55	146
Aspergillus ornatus	4.6	60	64
Aspergillus oryzae	5.0	50	65
Aspergillus phoenicis	4.3		66
Aspergillus roseus			
β-Glucosidase A	4–4.5		67
β-Glucosidase B	4.8–5.2		

(*Continued*)

TABLE 23.2 (Continued)

Organism	Optimum pH	Optimal Temperature (°C)	Ref.
Aspergillus sojae	5.0	60	
Aspergillus terreus	4.8		69
Aspergillus tubingensis			70
I	4.6	65	
II	4.0	65	
III	5.0	60	
IV	5.0	60	
Aspergillus wentii	3.5–5.5	55–65	73
Aureobasidium	4	80	136
Botryodiplodia theobromae	5.0		74
Botrytis cinerea	6.5	50	75
	3.0	60	76,77
Chaetomium thermophilum var. coprophilum	5.5	60	78
Chaeomium trilaterale	4.2		79
Chalara paradoxa	4.0–5.0	45	80
Cladosporium resinae	4.5	50	81
Curvularia sp.	4	70	82
Evernia prunastri	4	50–60	83
Fungal strain Y94	4.8	60	84
Fusarium oxysporum	5-6	60	41
Humicola grisea	4–4.5	60	86
	6.0	50–60	87
Humicola insolens	5.0	50	88
			89
Humicola lanuginosa	4.5	60	90
Lenzites trabea	4.5	75	91
Macrophomina phaseolina			
β-Glucosidase 1	5.5	55	34
β-Glucosidase 2	4.8–6	65	
Monilia sp.	4–5	50	92
Mucor miehei			
G-a	8.0	60	93
G-b			
Myceliophtora thermophila	4.8	60	94
Nectria catalinensis	5.3	45	95
Neocallimastix frontalis	5.5–7.2	45	96
	6	50	20

(*Continued*)

TABLE 23.2 (Continued)

Organism	Optimum pH	Optimal Temperature (°C)	Ref.
Orpinomyces sp.	6.2	50	98
Penicillium funiculosum			
β-Glucosidase 1	4	70	100
Penicillium herquei			
G1	4–4.5	60	28
G2	4–4.5	60	
Penicillium oxalicum	5.5	55	45
Penicillium purpurogenum	3.5	60	101
	4.5	60–65	102
Phanerochaete chrysosporium			
Intracellular	7	45	103
Extracellular	5.5	45	
	5	60	104
	4–5.2		105
Phytophtora infestans			106
β-Glucosidase I	5.5	48	
β-Glucosidase II	5.25	30	
Piromyces sp.	6	55	107
	6	47	108
Pisolithus tinctorius	4	65	109
Pyricularia oryzae	5.5	55	111
Schizophilum commune			
β-Glucosidase 1	5.8		31
β-Glucosidase 2	5.1		
	5.4	52	135
Sclerotium rolfsii			112
β-Glucosidase 1	4.2–4.5	65–68	
β-Glucosidase 2	4.2–4.5	65–68	
β-Glucosidase 3	4.2–4.5	65–68	
β-Glucosidase 4	4.2–4.5	65–68	
Scytalidium lignicola			113
I	5.0	55	
II	4.8	55	
Sporotrichium thermophile			26
β-Glucosidase A	5.6	50	
β-Glucosidase B	6.2–6.3	50	
	5.2–5.4	65	114
Stachibotrys atra			115
Constitutive	4.7–5		
Inducible	6.7		

(*Continued*)

TABLE 23.2 (Continued)

Organism	Optimum pH	Optimal Temperature (°C)	Ref.
Talaromyces emersonii			
β-Glucosidase 1	4.1	70	116
β-Glucosidase 3	2,5	70	
β-Glucosidase 4	5.7	35	
Termitomyces clypeatus	5.0	65	117
Thermoascus aurantiacus	4.5–5	70	118
	4.2	71	119
Gl-2	4.5	75–80	120
Gl-3	4	75–80	
	4.5	80	121
Thermomyces lanuginosus	6	65	123
Trametes gibbosa	3.5	40	124
Trichoderma sp.	4.0	75	125
Trichoderma harzianum	5.0	45	126
Trichoderma reesei	4.5–5		127
	4.5	70	128
β-Glucosidase 1	4.6	65–70	14
β-Glucosidase 2	4	60	
	6.5		129
Trichoderma viride	4.0–4.5	65	133
Xylaria regalis	5.0	50	134

The optimum temperature ranges from 35 to 80°C. As can be seen from Table 23.2, most of the enzymes have an optimum at or above 55°C. The exception corresponds to the intracellular enzyme from *T. emersonii*[116] with optimal activity at 35°C.

The extracellular fungal β-glucosidases from mesophilic fungi are thermostable up to 60°C, while those of thermophilic fungi are stable to even higher temperatures.[10] A purified β-glucosidase from the mesophile *T. reesei* QM 9414[128] shows a high stability at temperatures of 50 to 55°C: after incubation for 7 h at pH 4.5 and 50°C no significant activity loss (less than 3%) was detected. A ($t_{½}$) of 5h was measured for this enzyme at 60°C. Similarly, the β-glucosidase purified from *P. oxalicum* has half-lives of 225 and 45 min at 50 and 60°C, respectively.[45] However, a very different situation is encountered with the intracellular enzyme from *T. reesei* QM 9123: 1 h at 40°C produces an inactivation of 95%, and total inactivation is observed when incubated at 60°C for the same period of time.[129] On the other hand, the thermophilic fungus *Thermoascus aurantiacus* possesses a β-glucosidase that is totally stable for 1 h at 70°C and retains 70% of its activity after 1h at 75°.[118] Curiously, the thermophilic fungus *Humicola lanuginosa* possesses a highly thermolabile β-glucosidase, much more labile than many enzymes from mesophilic origin.[90] This enzyme is stable at

30°C for 1 h, but at 60 and 70°C the enzyme is totally inactivated after incubation for a similar time period.

An important condition to achieve maximal stability is to keep the enzymes in a medium of appropriate pH. This statement can be illustrated with the enzymes from the fungi *A. japonicus* and *Macrophomina phaseolina*.[50,34] The former produces a β-glucosidase that shows no appreciable loss of activity after 40 min at 55°C, either at pH 5.8 or 7.0. This situation, however, changes drastically at 65°C. When the enzyme is incubated for 40 min at 65°C and pH 5.0 there is no appreciable loss of activity, while at the same temperature but at pH 7.0, a loss of activity of nearly 80% is observed. A similar phenomenon occurs with β-glucosidase I from *M. phaseolina* at 60°C; it is stable at pH 5.0 but loses 70% of its activity after 20 min at pH 7.0.

Cellulases, including β-glucosidases, are mostly glycoproteins. It has been shown that the carbohydrate moiety may significantly affect the thermostability of these enzymes. In the β-glucosidases of *Mucor miehei* [93] and β-glucosidases I, III, and IV from *T. emersonii,*[116] the enzymes with a higher carbohydrate content are more stable. The half-lives of the latter enzymes at 70°C and pH 5.0 are 410, 175, and 2 min, respectively. On the other hand, it has been observed in *A. aculeatus* F 50 and *P. herquei*[64,28] that the isoenzyme with lower carbohydrate content presents higher stability. For this reason, the role of the carbohydrates in thermostability is not conclusive. A comparison of the enzyme stability between the native, glycosylated enzyme, and a nonglycosylated form produced by genetic engineering or glycosidase treatment may help clarify this issue.

V. EFFECT OF INHIBITORS

Some cellulolytic fungi produce oxidoreductases, such as glucose oxidase, which are capable of oxidizing glucose, cellobiose, and other cellodextrins to the corresponding lactones.[137,138] Glucono-δ-lactone is produced in large amounts by grape-attacking fungi and can reach concentrations up to 2 g/L in wine.[65] This compound (Figure 23.3) is a potent competitive inhibitor of many β-glucosidases; values of K_i ranging from 0.0083 μ*M* to 12.5 m*M* have been reported (see Table 23.3). However, some β-glucosidases show mixed inhibition by gluconolactone.[51,53,54,64,101] Inhibition by this compound has been explained by steric similarities between the lactone and the enzyme-bound substrate.[51,52] It may be important in the regulation of cellulose hydrolysis *in vivo*; therefore, this topic merits further investigation.

Some β-glucosidases are inhibited by high substrate concentration.[54,55] Besides, glucose, the product of the enzymic reaction, is a competitive inhibitor of many fungal β-glucosidases; K_i values for glucose between 17 μ*M* and 1360 m*M* have been reported (Table 23.3). However, some β-glucosidases are noncompetitively inhibited,[57] thus, like gluconolactone, glucose can inhibit by different mechanisms. The degree of glucose inhibition may be an important factor in choosing a β-glucosidase for the industrial saccharification of cellulose.

When P(O)NPG is used as substrate, cellobiose may act as competitive inhibitor (see Reference 117 and unpublished observations by the authors). This result indicates that at least some β-glucosidases use the same active site for these two substrates.

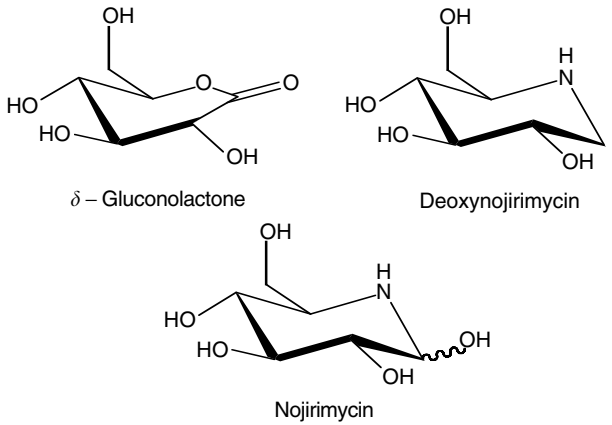

FIGURE 23.3 Inhibitors of β-glucosidase.

TABLE 23.3
Inhibition of β-Glucosidase

Organism	Substrate	Inhibitor	Inhibition type	$K_i(K_i')$ (μM)	Ref.
Acremonium persicinum	PNPG	Glucose	Competitive	350	49
	PNPG	Glulact	Competitive	5	
Aspergillus japonicus	ONPG	Glulact	Competitive	40	50
	ONPG	Nojirim	Competitive	1.8	
Aspergillus nidulans					
P-1	PNPG	Glulact	Not specified	3,480	30
	PNPG	Glucose	Not specified	9,170	
P-2	PNPG	Glulact	Not specified	850	
	PNPG	Glucose	Not specified	5,480	
β-Glucosidase I	Cellobiose	Glucose	Competitive	630	51
	Cellobiose	Glulact	Mixed	100 (950)	
β-Glucosidase II	PNPG	Glucose	Competitive	2,340	
	PNPG	Glulact	Mixed	110 (4860)	
Aspergillus niger					
β-Glucosidase A	PNPG	Glucose	Competitive	2,500	47
	PNPG	Gluclact	Competitive	150	
	PNPG	Denojir	Competitive	2	
β-Glucosidase B	PNPG	Glucose	Competitive	400	
	PNPG	Gluclact	Competitive	5	
	PNPG	Denojir	Competitive	1	
	PNPG	Glucose	Competitive	2,890	53
	PNPG	Gluclact	Mixed	700 (14,200)	
	PNPG	Glucose	Competitive	3,220	54
	PNPG	Gluclact	Mixed	240	

(*Continued*)

TABLE 23.3 (Continued)

Organism	Substrate	Inhibitor	Inhibition type	$K_i(K_i')$ (μM)	Ref.
Novozym 188	PNPG	Glucose	Part competitive	3,000	59
β-Glucosidase I	PNPG	Glucose	Competitive	543,000	62
β-Glucosidase II	PNPG	Glucose	Competitive	5,700	63
Aspergillus ornatus	PNPG	Glucose	Competitive	5,130	64
	PNPG	Gluclact	Mixed	240 (13 700)	
Aspergillus oryzae					
	PNPG	Glucose	Competitive	$1{,}360 \times 10^3$	65
	PNPG	Gluclact	Competitive	12,500	
BGI	PNPG	Glucose	Competitive	3,500	139
BGII	PNPG	Glucose	Competitive	953,000	
Aspergillus phoenicis	Cellobiose	Nojirim	Not specified	0.64	66
Aspergillus terreus	PNPG	Glucose	Competitive	3,500	69
Aspergillus tubingensis					70
I	PNPG	Glucose	Not specified	5,800	
II	PNPG	Glucose	Not specified	1,300	
III	PNPG	Glucose	Not specified	470,000	
IV	PNPG	Glucose	Not specified	600,000	
Aspergillus wentii	Cellobiose	Glucose	Competitive	14,000	73
Botrytis cinerea	PNPG	Glucose	Competitive	5,500	75
	PNPG	Glulact	Competitive	20	
Chaetomium trilaterale	PNPG	Glulact	Competitive	534	140
	PNPG	Nojirim	Competitive	19.5	
Chalara paradoxa	PNPG	Glucose	Competitive	11,020	80
Cladosporium resinae	PNPG	Glucose	Competitive	22,000	81
Evernia prunasti	PNPG	Glucose	Competitive	1,260	83
Fungal strain Y 94	cellobiose	Glucose	Competitive	1,350	84
Lenzites trabea	PNPG	Glucose	Noncompetitive	2,700	91
Macrophomina phaseolina					
βG1	ONPG	Glulact	Competitve	17	34
	ONPG	Nojirim	Competitve	0.5	
βG2	ONPG	Glulact	Competitive	30	
	ONPG	Nojirim	Competitve	1.6	
Monilia sp.	PNPG	Glucose	Competitive	670	92
Neocallimastix frontalis	Mumbgl	Glulact	Not specified	5.57	2
	Mumbgl	Glucose	Not specified	3,600	
	PNPG	Glucose	Competitive	10,500	96
	PNPG	Glulact	Competitive	240	
	PNPG	Glucose	Competitive	4,800	97
	PNPG	Glulact	Competitive	62	

(*Continued*)

TABLE 23.3 (Continued)

Organism	Substrate	Inhibitor	Inhibition type	$K_i(K_i')$ (μM)	Ref.
Orpinomyces sp.	PNPG	Glucose	Competitive	8 750	98
	PNPG	Glulact	Competitive	16.8	
	Cellobiose	Glulact	Competitive	2,570	
Penicillium funiculosum					
βG1	PNPG	Glucose	Competitive	920	100
	PNPG	Glulact	Competitive	67	
	Cellobiose	Glulact	Not specified	32	
βG2	PNPG	Glulact	Not specified	70	
	PNPG	Glucose	Competitive	1,700	99
	PNPG	Glulact	Competitive	8	
	Cellobiose	Glucose	Competitive	1,000	
Penicillium oxalicum	PNPG	Glulact	Not indicated	300	45
	PNPG	Glucose	Noninhibited up to 10 mM		
Penicillium purpurogenum	PNPG	Glucose	Competitive	1400	101
	PNPG	Glulact	Mixed	67	
Phanerochaete chrysosporium	PNPG	Glucose	Competitve	500	103
	PNPG	Glulact	Competitive	35	104
	PNPG	Denojir	Competitive	68.7	
	PNPG	Glucose	Competitive	270	105
	PNPG	Glulact	Competitive	4	
Piromyces sp.	PNPG	Glulact	Competitive	30	107
	PNPG	Glulact	Competitive	22	108
Pyricularia orizae	Salicin	Glulact	Competitive	7.4	111
Schizophyllum commune					
βG I	Cellobiose	Glucose	Competitive	1,000	31
	PNPG	Glucose	Competitive	3,700	
	Cellobiose	Glulact	Competitive	4	
βG II	Cellobiose	Glucose	Competitive	9,100	
	PNPG	Glucose	Competitive	7,700	
	Cellobiose	Glulact	Competitive	4.5	
	PNPG	Glucose	Competitive	17	135
Sclerotium rolfsii βG3	Cellobiose	Glucose	Competitive	550	112
	Cellobiose	Glulact	Competitive	10	
	Cellobiose	nojirim	Competitive	1	
Scytalidium lignicola					113
I	PNPG	Glucose	Noncompetitive	5,000	
II	PNPG	Glucose	Noncompetitive	7,500	
Sporotrichum pulverulentum					5
A	PNPG	Glulact	Competitive	0.35	
B	PNPG	Glulact	Competitive	1.5	
Stachybotrys atra	PNPG	Glulac	50% at 45 μM		115
	PNPG	Glucose	50% at 13 μM		

(*Continued*)

TABLE 23.3 (Continued)

Organism	Substrate	Inhibitor	Inhibition type	$K_i(K_i')$ (μM)	Ref.
Talaromyces emersonii					
β-glucosidase 1	PNPG	Glucose	Competitive	710	116
β-glucosidase 4	PNPG	Glucose	Competitive	52,000	
Termitomyces clypeatus	PNPG	Glucose	Competitive	1700	117
Thermoascus aurantiacus	PNPG	Glucose	Competitive	290	121
	PNPG	Glulact	Competitive	0.0083	
Tramites gibbosa	PNPG	Glucose	Competitive	5,200	124
Trichoderma sp.	PNPG	Glucose	Competitive	650	125
	PNPG	Glulact	Competitive	90	
Trichoderma harzianum	PNPG	Glucose	Competitive	1,400	126
	PNPG	Glulact	Competitive	1.8	
Trichoderma koningii					
βG1	ONPG	Glulact	Competitive	1.8	13
	ONPG	Glucose	Competitive	1050	
βG2	ONPG	Glulact	Competitive	1,17	
	ONPG	Glucose	Competitive	660	
Trichoderma reesei	Mumblg	Glucose	Competitive	700	127
βG1	PNPG	Glucose	Competitive	624	14
	PNPG	Glulact	Competitive	3,13	
	PNPG	Denojir	Competitive	1.26	
	Cellobiose	Glulact	Competitive	99.5	
	Cellobiose	Denojir	Competitive	3.93	
βG2	PNPG	Glucose	Competitive	189	
	PNPG	Glulact	Competitive	4.1	
	PNPG	Denojir	Competitive	1.19	
	Cellobiose	Glulact	Competitive	40	
	Cellobiose	Denojir	Competitive	1.72	
	Cellobiose	Glucose	Competitive	500	130
	PNPG	Glucose	Competitive	8,700	
Trichoderma viride	Cellobiose	Glucose	Noncompetitive	22,600 (28,300)	132
	PNPG	Glucose	Competitive	530	133
	Cellobiose	Glucose	Competitive	390	

Abbreviations: mumblg = methylumbelliferyl glucoside; glulact = gluconolactone; nojirim = nojirimycin; denojir = deoxynojirimycin

Other powerful inhibitors of β-glucosidase are nojirimycin and deoxynojirimycin[44] (Figure 23.3), antibiotics produced by strains of *Streptomyces*. These compounds, as expected from their structure similarity to glucose, are competitive inhibitors and have K_i values in the range from 0.64 to 68.7 μM (Table 23.3).

Other β-glucosidase inhibitors include heavy metals such as Hg^{2+}, Cu^{2+}, Pb^{2+} and Co^{2+}, and *p*-chloromercuribenzoate. The following examples can be cited: 0.63 m*M*

$CuSO_4$ and 0.37 m*M* $HgCl_2$ inhibit *N. frontalis* β-glucosidase by 90%,[96] while 8 m*M* $Pb(NO_3)_2$ and 70 μM $CuCl_2$ inhibit β-glucosidase B from *Sporotrichum thermophile* by 50%.[26] Ethanol has been reported to act as inhibitor or activator, depending on the concentrations used: 30% ethanol activated while 80% inhibited the β-glucosidase from *T. aurantiacus,*[121] 10% ethanol activated by 30% while 40% inhibited by similar amount the β-glucosidase I from *A. niger*.[62] This dual effect may reflect different effects of ethanol on enzyme conformation. Some β-glucosidases may accept alcohols as acceptors in transglycosylation reactions.[80] Ethanol inhibition is of particular concern in certain industrial applications such as simultaneous cellulose saccharification and fermentation, because β-glucosidases may be exposed to substantial concentrations of this alcohol.

VI. ENZYME PURIFICATION

β-Glucosidases have been found in fungal culture supernatants, bound to the cell wall and cell membrane[51,142] and in the cytoplasm.[129] Most commonly, these enzymes are secreted to the medium. When purified from the culture supernatant, they are first concentrated by ultrafiltration or ammonium sulfate fractionation.[117] Less frequently used fractionation methods utilize ethanol precipitation[50,61] or bentonite adsorbtion.[61]

To obtain enzymes in their native form, not modified during the purification process, the number of purification steps should be kept to a minimum to avoid proteolytic cleavage among other unwanted modifications. To facilitate the purification, it is advantageous to choose culture conditions where the amount of impurities is minimized.

Purification procedures are under continuous development and several steps are usually necessary, based on different separation principles such as gel filtration, ion-exchange and hydrophobic interaction chromatographies. In addition, some investigators have employed adsorbtion and desorbtion from hydroxylapatite.[143] Affinity chromatography has also yielded good results. Rogalski and co-workers[144] have utilized controlled porosity glass activated by aminopropyltriethoxysilane and oxiranes and linked to salicin or cellobiose. Kmínkova and Kucera[145] have utilized concanavalin A coupled to Sepharose to purify a cellobiase from *T. viride*, taking advantage of its glycoprotein nature. Watanabe and colleagues[146] have used cellobiamine linked to epoxy-activated Eupergit C-30N. Others have successfully utilized isoelectrofocusing[5,101,128] and chromatofocusing.[37,146] These last two methods have allowed the isolation of β-glucosidase isoenzymes with different pI values, which are not easily separated by more conventional techniques.

Another strategy employed in the purification of β-glucosidases is the use of polyacrylamide gel electrophoresis under nondenaturing conditions, obtaining the enzyme by elution from the gel.[30,39]

The degree of purification attained is most commonly determined by SDS polyacrylamide gel electrophoresis. This technique is also used in molecular weight determination (see below). Isoelectrofocusing in polyacrylamide has also been used for purity determination, giving at the same time the pI values (see below).

VII. MOLECULAR MASS AND ISOELECTRIC POINT

Native fungal β-glucosidases show molecular weight (M_w) in the broad range from 40,800 to 640,000 (Table 23.1). SDS gel electrophoresis gives single polypeptide chains from 35,000 through 250,000. Quaternary structures from monomers up to tetramers have been found (Table 23.1).

The molecular weight of the native enzymes has been mostly determined by gel filtration, although on occasions other procedures have been used. The molecular weight of the enzyme from *A. fumigatus* (48,800) was measured by low speed sedimentation equilibrium.[32] Nondenaturing polyacrylamide gel electrophoresis at different concentrations, with proteins of known molecular weight as standards, has also been employed.[147] Using this method, a M_w of 325,000 has been measured for a β-glucosidase from *A. niger*.[53] Caution must be exercised in the interpretation of molecular weight data of glycoproteins, as is the case of most β-glucosidases, since carbohydrate content may affect chromatographic or electrophoretic behavior. The presence of proteases in the culture medium may also cause artefactual results in molecular weight determinations.

Sequencing of β-glucosidases or of their genes or cDNAs allow a very precise determination of molecular mass, and comparison with the values obtained by physical means allow estimation of the degree of posttranslational modification (glycosylation).

Although the majority of the enzymes show pI values in the range of 4 to 6 (Table 23.1), some β-glucosidases are very acidic (pI of 3.1) or basic (pI of 8.87), suggesting significant differences in their amino acid composition.

VIII. CARBOHYDRATE CONTENT

Fungal β-glucosidases are mostly glycoproteins; in some cases the carbohydrate portion of the molecule ranges up to 75% (w/w) (Table 23.1). The most commonly used method for carbohydrate quantification is the phenol–sulfuric acid method using glucose as standard.[148] Qualitatively, the glycoproteins can be visualized in polyacrylamide gels using the periodate–fuchsin stain.[32,149] Another frequently used option, although indirect, is to adsorb the protein to a concanavalin A–agarose column.[45,67,92] This method is based on the presence of α-D-mannopyranosyl and α-glucopyranosyl units in the carbohydrate portion.

The finding of carbohydrates in purified samples of β-glucosidases does not assure that these carbohydrates are covalently linked to the protein. For example, Witte and Wartenberg[37] have observed that two purified β-glucosidases from *A.niger* possess two types of carbohydrates. One type is weakly bound to the enzymes, and is lost during the purification, so that the carbohydrate content of the enzymes drops from 27 to 17%. Schmid and Wandrey[128] have found that a β-glucosidase from *T. reesei* QM 9414 is not a glycoprotein according to the gel staining method, although the quantitation of carbohydrates shows a percentage of 35%. These results suggest that the carbohydrate is not covalently bound to the enzyme. On the other hand, Messner and co-workers[150] have described the purification of a fungal cell-wall polysaccharide from *T. reesei*,

which operates as an anchor glycan for the β-glucosidases from this organism. This polysaccharide is capable of reassociation with the extracellular enzyme and the enzyme released from the fungal cell wall. An interesting observation is the ability of this polysaccharide to increase the activity of the enzyme toward PNPG *in vitro*.

The importance of the carbohydrate moiety in β-glucosidase function is unknown. Some evidences point toward a relationship between degree of glycosylation and enzyme stability: a thermostable β-glucosidase from *M. miehei*, with a higher carbohydrate content yields a less thermostable form when subjected to digestion by a β-glucosaminidase preparation.[93] But, as has been pointed out before, the cases studied show contradictory results.

IX. ISOENZYME FORMS

A number of fungi have been found that produce multiple forms of β-glucosidase[5,14,25,28–37,70,116] (see Table 23.1). In many cases, the origin of this multiplicity is unclear, although some explanations have been postulated. β-Glucosidase heterogeneity in *S. pulverulentum* has been tentatively attributed to proteolysis.[5] Other possible posttranslational mechanism is glycosylation heterogeneity.[25] Protein–protein or protein–carbohydrate interactions are other possibilities that cannot be ruled out.[151] Two closely related forms of β-glucosidase (M_w 95,700 and 93,800) are secreted by *Schizophyllum commune;* they are postulated to arise from heterogeneity of the mRNA but from a single gene.[152] Alternatively, different forms of an enzyme may be the product of separate genes. Hypotheses concerning the multiplicity of β-glucosidase are put forward in numerous papers, but conclusive results are lacking.

Techniques such as isoelectrofocusing and chromatofocusing have often been successful in separating enzyme forms that could not be resolved by other methods. Witte and Wartenberg[37] have separated from *A. niger*, by means of chromatofocusing, two isoenzymes of similar M_w (96,000) but different pI values (3.8 and 4.6). Hidalgo and co-workers[101] have found, by both chromato- and isoelectrofocusing, two forms of β-glucosidase from *P. purpurogenum* with pI values of 4.2 and 6.0; again, both enzymes show the same molecular weight (90,000). Besides the pI values, many isoenzymes differ from each other in specific activity and molecular weight.

The isoenzymes may also have a different location in the cell, and this location may vary depending on the condition and age of the cultures and nutritional conditions. So, the localization of the β-glucosidase activity depends upon the carbon source as demonstrated in *S. pulverulentum*:[5] with cellobiose as the sole carbon source, only cell-wall-bound enzyme is produced, while the presence of cellulose may be necessary for secretion of the enzyme.

Several forms of β-glucosidases have been found in *T. reesei*, either in culture supernatants or bound to the cell wall and membrane, with pI values ranging from 4.4 to 8.7 (Table 23.1). A well-known fact is that *T. reesei* releases low amounts of β-glucosidase to the culture medium;[66] this has been explained by the observation that a large part of the enzyme remains bound to the cell wall during fungal growth.[141] Kubicek[153] has postulated that the membrane-bound β-glucosidase participates in the formation of sophorose, a potent inducer of cellulases. It has already

been mentioned that the β-glucosidases from *T. reesei* may be involved in cell-wall metabolism during conidiogenesis, and thus may not be a true component of the cellulolytic enzyme system.[141] Inglin and colleagues[129] have isolated an intracellular β-glucosidase, which could have two possible functions: (1) in metabolic control of cellulase induction; and (2) as a proenzyme before transport through the cell membrane to the external milieu.

Analytical isoelectrofocusing of culture supernatants of *T. reesei* QM9414 shows that isoenzymes appear very early in the cultivation, suggesting that they are an inherent property of the fungus.[154] The pattern of isoenzymes may also vary with the age of the culture, as has been found in the wood-staining fungus *Botryodiplodia theobromae*, which produces a series of β-glucosidase forms in the culture filtrates. Enzymes of high M_w (350,000 to 380,000 Da) predominate in young cultures, while smaller forms (45,000 to 47,000) are more abundant in older filtrates.[155]

X. SEQUENCE AND STRUCTURE

Henrissat156 has developed a classification of glycosyl hydrolases based on amino acid sequence similarity. The enzymes are grouped in families, and as of January 2005, 99 families had been recognized. A website, which can be accessed at http://afmb.cnrs-mrs.fr/CAZY and is frequently updated, lists all the families and the enzymes assigned to them and gives direct access to the nucleotide and amino acid sequence databases.

β-Glucosidases are found in families 1, 3, and 9, and enzymes from filamentous fungi are present in families 1 and 3. Both family 1 and 3 glycosyl hydrolases have a catalytic mechanism with retention of the β-anomeric configuration,[157] and in the catalytic process participate a nucleophile/base, and a proton donor (acid–base catalyst).[157] The reaction mechanism and the role of different amino acids in catalysis and substrate binding have recently been discussed in detail by Bhatia and co-workers.[11]

Family 1 includes sequences of β-glucosidases from the filamentous fungi *A. niger, H. grisea, Orpinomyces* sp., *Piromyces* sp. (two sequences: Bgl1A and Cel1C), *T. emersonii, T. reesei* and *T. viride.* These sequences have been aligned in the bottom part of Figure 23.4. The legend to the figure indicates the GenBank accession number and references to publications where available. The conserved motif NEP (residues 430 to 433) includes the glutamate residue implicated as acid–base catalyst,[158] while the I(Y,V)(V,I)TENG motif (residues 893 to 899) contains the glutamate, which acts as nucleophile.[159] Significant similarity can also be observed in other regions of the sequence.

No three-dimensional structure of family 1 fungal β-glucosidase has been described so far. However, the structures of several bacterial and plant enzymes have been published (see References 160 and 161 and references therein). The structure found in all cases is that of a $(\alpha/\beta)_8$ barrel.

The family 3 fungal β-glucosidases include sequences from *Agaricus bisporus, A. aculeatus, A. kawachii, Botryotinia fuckelania, Coccidioides posadasii* (Bgl1 and Bgl2), *Phaeosphaeria avenaria, Phanerochaete chrysosporium* K3 and OGC101, *Piromyces* sp., *Septoria lycopersici, T. emersonii, T. reesei* (Cel3b and Cel3A/Bgl1), and *Volvariella volvacea.* These sequences have been aligned in the top part of

Figure 23.4. The alignment of this family of enzymes has been separated into two groups, which show high internal sequence similarity.

The first group includes the β-glucosidases from *A. bisporus* and *V. volvacea*, both basidiomycetes; these sequences have been found to be very similar to that of the enzyme from the slime mold *Dictyostelium discoideum*, which has also been included in this alignment for comparison purposes. No biochemical data are available for the two fungal enzymes; active-site residues for the *D. discoideum* protein have been recognized in the conserved sequence (T,S)DW (residues 411 to 413), as determined from a tryptic peptide of *A. wentii* β-glucosidase (162, 163) containing this sequence.

The remaining fungal enzymes are aligned in the central part of Figure 23.4. Several conserved motifs can be recognized. Among them are the sequences (T,S)DW and KH (F,Y,L) (residues 320 to 322), which seem to be common to all family 3 β-glucosidases; both sequences have been implicated as contributing to the active-site structure and catalysis. There is evidence supporting a role for the D in the (T,S)DW motif as the catalytic nucleophile.,[164] and it has been suggested that the proton donor may be the H in KH (F,Y,L).[11]

The only structure for a family 3 glycosyl hydrolase described so far is the barley β-glucan exohydrolase.[165] The amino-terminal segment constitutes an $(\alpha/\beta)_8$ barrel domain (such as found in family 1 enzymes), with a second domain arranged in a six-stranded β-sandwich. Two acidic amino acids (Asp^{285} and Glu^{491}) are probable catalytic residues. The first is in the (T,S)DW conserved sequence. Although the highly conserved sequence KH (F,Y,L) is also found in the barley enzyme, no specific function is assigned to the H residue; thus the nature of the proton donor in family 3 enzymes remains controversial.

FIGURE 23.4 Alignment of the sequences of β-glucosidases from filamentous fungi. The alignment has been performed using CLUSTAL W (www.ebi.ac.uk/clustalw) and further analyzed by means of AMAS (http://barton.ebi.ac.uk/servers/amas_server.html). The upper part of the alignment includes family 3 enzymes, two from the fungi *Agaricus bisporus* (AJ 293760) (AGABI) and *Volvariella volvacea* (AF 32973) (VOLVO), and a β-glucosidase from the slime mold *Dictyostelium discoideum* (L21014[166]) (DICDI). The central part of the alignment refers to family 3 enzymes from *Aspergillus aculeatus* (D64088[167]) (ASPAC), *Aspergillus kawachii* (AB003470[168]) (ASPAW), *Botryotinia fuckelania* (AJ0130890) (BOTFU), *Coccidioides posadasii* (U87805) (COCPO), *Coccidioides posadasii* (AF022893[169]) (COCPOBgl2), *Phaeosphaeria avenaria* (AJ276675[170]) (PHAAV), *Phanerochaete chrysosporium* K-3 (AB081121) (PHACHK-3), *Phanerochaete chrysosporium* OGC101 (AF036872[171]) (PHACHOGC101), *Piromyces sp.* (AY72977[164]) (PIRSP), *Septoria lycopersici* (SLU24701[172]) (SEPLY), *Talaromyces emersonii* (AY072918) (TALEM), *Trichoderma reesei* QM6a Cel3B (AY281374[173]) (TRIRECel3B), *Trichoderma reesei* QM9414 Cel3A/Bgl1 (U09580[174]) (TRIREBgl1). The lower part includes family 1 β-glucosidases from *Aspergillus niger* (AF 268911) (ASPNI), *Humicola grisea* (AB 0003109[175]) (HUMGR), *Orpinomyces* sp (AF016864) (ORPSP), *Piromyces* sp. Bgl1a (AJ276438[176]) (PIRSPBgl1A), *Piromyces* sp. Cel1C (AF500784[177]) (PIRSPCel1C), *Talaromyces emersonii* (AF439322) (TALEM_F1), *Trichoderma reesei* Cel1B (AY281377[173]) (TRIRE_F1), *Trichoderma viride* (AY 343988) (TRIVI). White letters on black background represent sequence identities and the gray boxes indicate sequence similarity. Numbers on top indicate the numbering of the amino acid sequence.

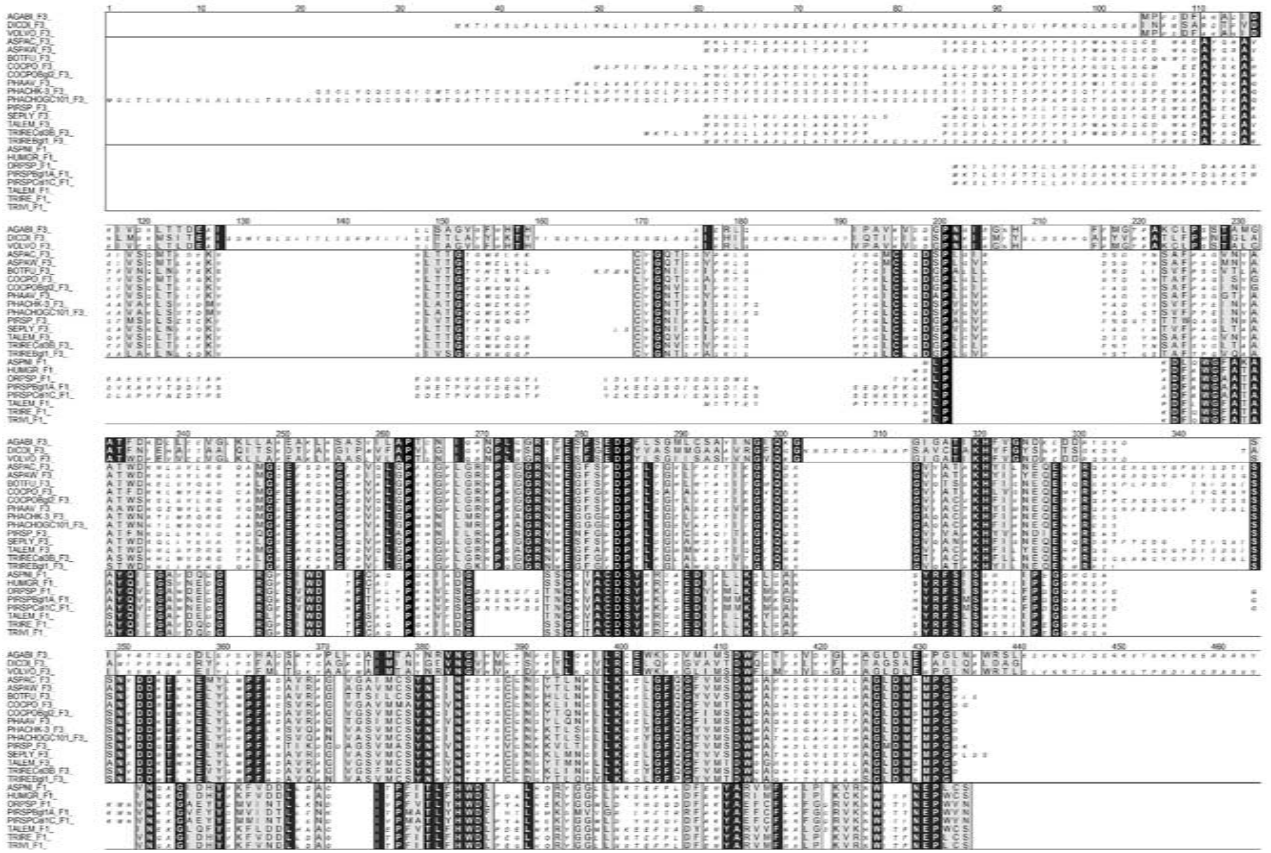

FIGURE 23.4

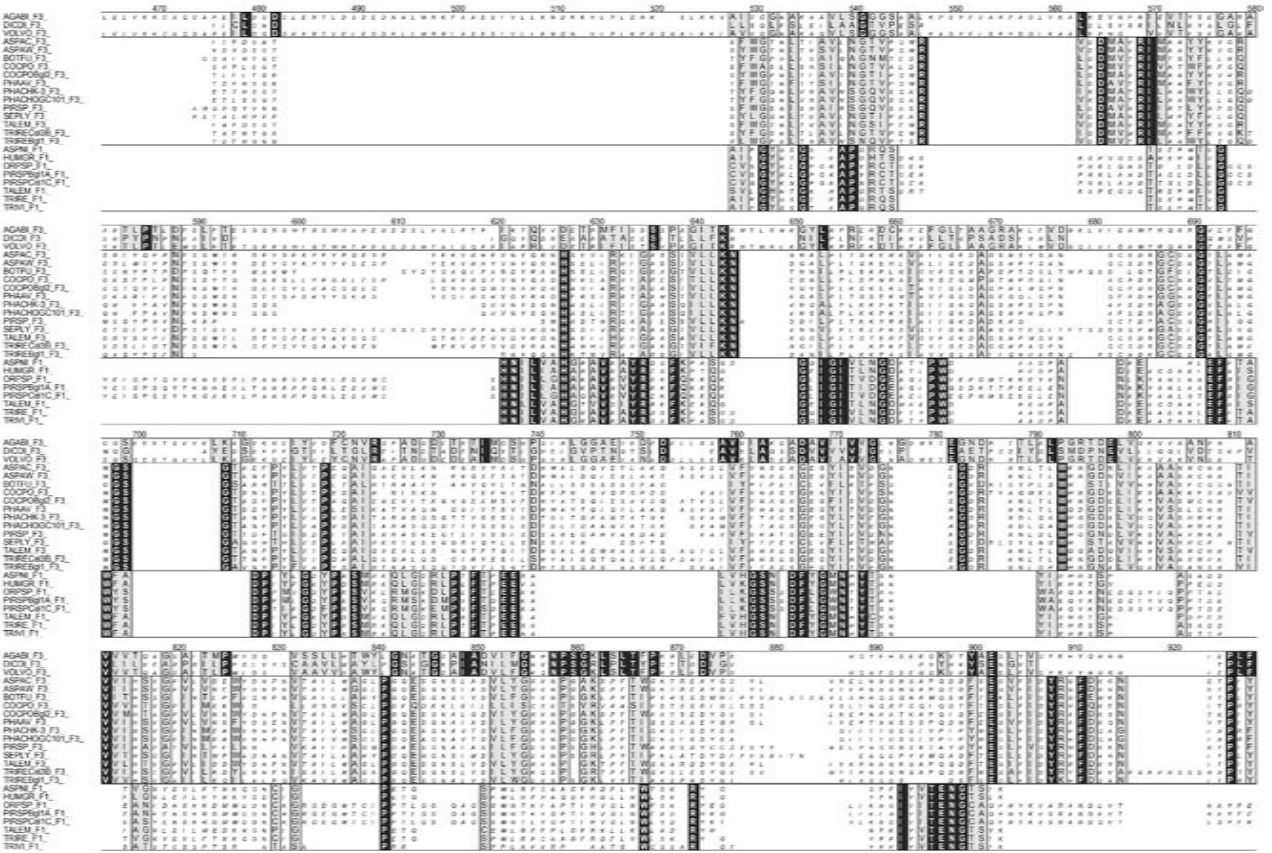

FIGURE 23.4

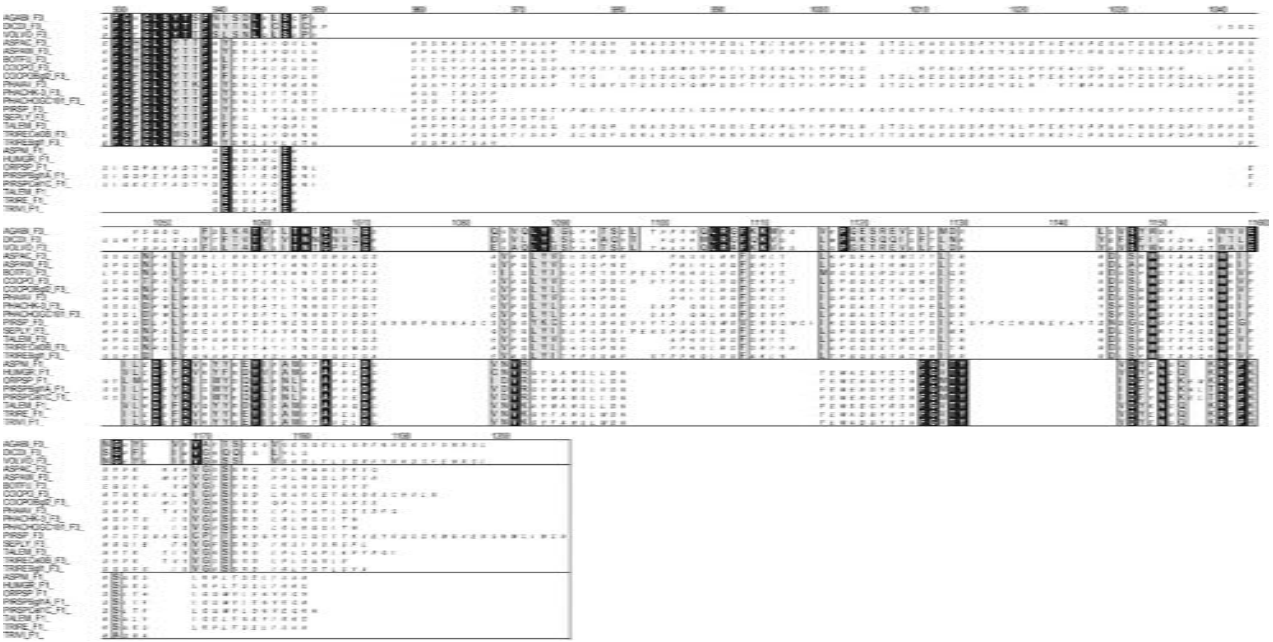

FIGURE 23.4

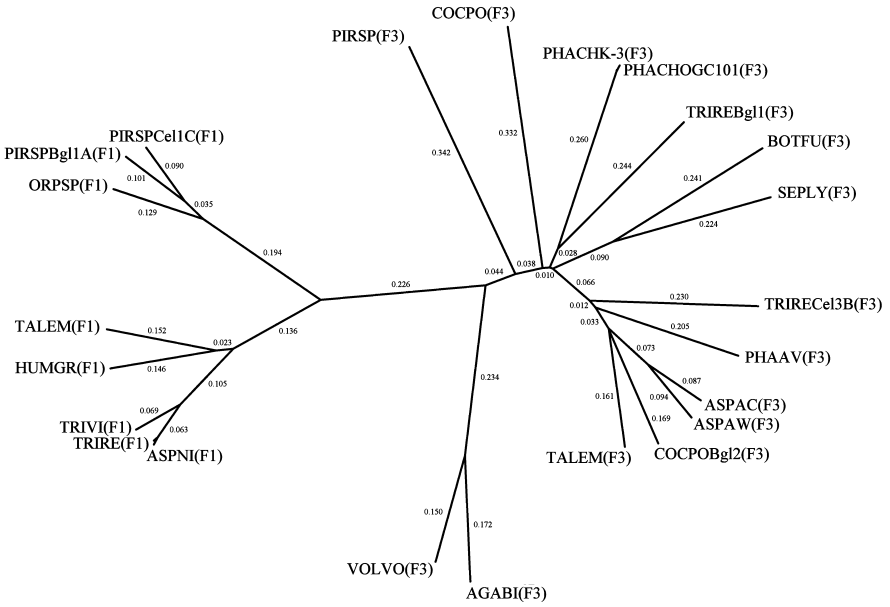

FIGURE 23.5 Phylogenetic tree of the β-glucosidases from filamentous fungi. The tree was constructed using CLUSTAL W and PHYLIP.[178] The numbers indicate the evolutionary distance between the enzymes analyzed in the multiple alignment of Figure 23.4. Those enzymes with the lowest similarity are at an arbitrary distance of 1. The abbreviations used have been defined in Figure 23.4.

Figure 23.5 presents a phylogenetic tree for the β-glucosidases from filamentous fungi. Family 1 enzymes form a clearly separate cluster. Although the enzymes from family 3 are closely related, those from *A. bisporus* and *V. volvacea* form a subgroup that can be differentiated, as evident from the sequence alignment.

XI. BIOTECHNOLOGICAL APPLICATIONS

β-Glucosidases are produced at an industrial scale from cultures of *A. niger*, which is a GRAS (generally regarded as safe) microorganism, that is, its enzymes can be safely used in processing of foods and beverages for human consumption.[60] Spagna and co-workers[61] have developed a simple and inexpensive method for the purification of β-glucosidases from *A. niger,* which is applicable at the industrial level.

An important application of β-glucosidases that is receiving increasing attention is its use in flavor enhancement of fruit juices and wines by liberating flavor compounds from glucosidic precursors. Some fruits (such as apples and grapes) as well as their juices and fermentation products such as wine, in addition to a free fraction of volatile odorous terpenols, contain nonodorous and nonvolatile aroma precursors (i.e., terpenylglycosides). The terpenic residues (such as linalool, nerol, citronellol, and geraniol) (Figure 23.6) are bound to glucose by β-linkages; they are not efficiently liberated by the β-glucosidases from grapes and yeast present during

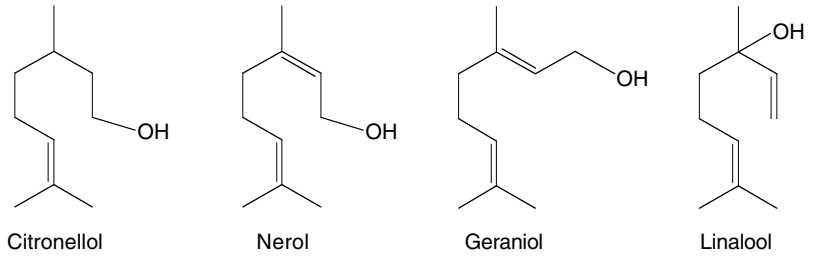

FIGURE 23.6 Terpenols present in some fruits (such as apples and grapes) as well as their juices and fermentation products such as wine.

winemaking,[65] and can be released by the addition of exogenous β-glucosidases.[179] Since the amount of bound monoterpenes is relatively high, there is a great potential in the use of this enzyme for flavor enhancement. Particularly valuable for this purpose are β-glucosidases which are active and stable at pH values 3-4 (the pH of wine and fruit juices) in the presence of ethanol, and that show a broad substrate specificity toward terpenyl glycosides.[179] Enzymes that fulfill these conditions are β-glucosidase HGT from *A. oryzae*[65] and β-glucosidase I from *A. niger*.[62] The flavor of the Japanese alcoholic beverage shochu, obtained by fermentation of sweet potatoes, is improved by β-glucosidase from *A. kawachii*, which is used to liberate nerol and geraniol from their respective glucosides.[180]

β-Glucosidases can also induce a loss of color in red fruit juices and wines by hydrolyzing antocyanins, which are responsible for this color. Antocyanins are mainly β-glucosides of antocyanidins (such as malvidin, peonidin, and cyanidin),[181] which upon hydrolysis yield colorless compounds.[182] It may be important in the processing of red wines to have enzymes available, which can be used for flavor enhancement but which show low decolorizing effect. Le Traon-Masson and Pellerin[47] have isolated two β-glucosidases from an *A. niger* preparation; one of them (β-glucosidase A) is highly active on cellobiose and geranyl β-D-glucoside but degrades malvidin–3-glucoside very slowly. Thus, this enzyme shows good potential for flavor enhancement without degradation of antocyanins. On the other hand, β-glucosidase B is a specific anthocyanase, with low activity on cellobiose and terpenyl glucosides; it may be useful for the specific decolorization of products derived from red fruits.

Citrus fruits contain glucosidic compounds such as prunin and naringin (4′,5,7-trihydroxyflavanone-7-rhamnoglucoside) which impart a bitter taste to their juices. Debittering of citrus juices can be achieved through successive hydrolysis by α-rhamnosidase and β-glucosidase.[183]

As was pointed out at the beginning of this chapter, an important potential application is the use of β-glucosidase in the saccharification of cellulose for the production of glucose and eventual fermentation to obtain ethanol.[1] This technology, however, is not as yet commercially feasible, mainly due to the high cost of the enzymes involved. Endoglucanases and exoglucanases by attacking cellulose generate cellobiose, which is an inhibitor of cellulases,[8] and which is not directly fermentable by yeasts. β-Glucosidase facilitates the action of these enzymes by producing easily fermentable glucose. This compound, in turn, inhibits β-glucosidase

(see discussion on inhibitors). An important development has been the production of glucose-tolerant β-glucosidases. Yan and Lin,[62] Günata and co-workers,[65,139] and Decker and co-workers[70] have obtained β-glucosidases with high K_i values for glucose (see Table 23.3) from different strains of *Aspergillus*. The cellulase system of *T. reese*i has been the most studied for cellulose hydrolysis; however, it has insufficient β-glucosidase for this purpose. The addition of external enzyme significantly reduces the time required for saccharification.[66] Current efforts are being made to reduce enzyme costs by a factor of ten in a program directed by the U.S. Department of Energy in conjunction with biotechnology companies such as Genencor and Novozyme. In the framework of this project, a study of the transcriptional regulation of the biomass-degrading enzymes from *T. reesei* has been published.[173]

Enzyme immobilization for the development of continuous systems, and more efficient use of catalysts and other purposes has received a growing interest in the last decade. This is also true for immobilization of β-glucosidase. Roy and co-workers[94] have evaluated the usefulness of immobilizing β-glucosidase from *Myceliophtora thermophila* for its eventual use in the saccharification of cellulose. The enzyme was immobilized using cross-linked polyacrylamide and cyanogen bromide-activated Sepharose gel beads, and it showed marked improvement in operational and storage stability. Aguado and colleagues[184,185] have immobilized a commercial cellulase from *P. funiculosum* containing β-glucosidase on nylon powder. A high-activity retention (67%) was obtained and the immobilization resulted in a remarkable increase in the thermal stability of the enzyme. Martino and co-workers[186,187] have immobilized β-glucosidase purified from a commercial preparation (CYTOLASE. PCL5, obtained from a selected GRAS strain of *A. niger*), obtaining best results when immobilization was carried out by cross-linking on chitosan with glutaraldehyde. The properties of the immobilized enzyme were analyzed to consider its use in improving the aromatic potential of wines[188] and the immobilized preparation was tested in laboratory-scale reactors.[189] Ortega and co-workers[190] have studied the optimization of the entrapment of β-glucosidase from *A. niger* in alginate and polyacrylamide gels and have compared the properties of the bound and free β-glucosidase. Changes in their kinetic parameters and pH–activity profile were observed on binding; therefore, in order to use immobilized enzymes effectively, their properties must be carefully analyzed. The results presented are examples of numerous studies performed at the laboratory level with immobilized enzymes. These results, however, await application at an industrial scale.

Some β-glucosidases, besides hydrolysis, catalyze transglycosylation reactions.[41,108]. This activity can be very useful in the synthesis of oligosaccharides and glycoconjugates. Several fungal enzymes have been studied for this purpose, such as β-glucosidase II from *A. niger*, used for the synthesis of alkyl β-glucosides[191] and cellooligosaccharides[192] from cellobiose. Applications of this property of β-glucosidases have been discussed recently in detail by Bhatia and colleagues.[11]

XII. CONCLUSIONS

β-Glucosidases have been studied from a large variety of fungal sources. They show ample differences in properties such as size, substrate specificity, resistance to

inhibitors, and stability and activity at different pH and temperatures. Thus, these enzymes form an ample toolbox that can be effectively used in numerous and growing biotechnological applications.

ACKNOWLEDGMENTS

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