ELSEVIER

Carbohydrate Research 337 (2002) 629–634

CARBOHYDRATE RESEARCH

www.elsevier.com/locate/carres

Note

Glycon specificity profiling of α-glucosidases using monodeoxy and mono-*O*-methyl derivatives of *p*-nitrophenyl α-D-glucopyranoside

Toshiyuki Nishio,[a,*] Wataru Hakamata,[b] Atsuo Kimura,[c] Seiya Chiba,[c] Akira Takatsuki,[b] Ryu Kawachi,[a] Tadatake Oku[a]

[a]*Laboratory of Bio-organic Chemistry, Department of Biological Chemistry, College of Bioresource Sciences, Nihon University, 1866 Kameino, Fujisawa-shi, Kanagawa 252-8510, Japan*

[b]*Animal and Cellular Systems Laboratory, Institute of Physical and Chemical Research (RIKEN), 2-1 Hirosawa, Wako-shi, Saitama 351-0198, Japan*

[c]*Division of Applied Bioscience, Graduate School of Agriculture, Hokkaido University, Nishi-9, Kita-9, Kita-ku, Sapporo, Hokkaido 060-8589, Japan*

Received 5 September 2001; received in revised form 18 January 2002; accepted 20 January 2002

Abstract

Hydrolysis of probe substrates, eight possible monodeoxy and mono-*O*-methyl analogs of *p*-nitrophenyl α-D-glucopyranoside (*p*NP α-D-Glc), modified at the C-2, C-3, C-4, and C-6 positions, was studied as part of investigations into the glycon specificities of seven α-glucosidases (EC 3.2.1.20) isolated from *Saccharomyces cerevisiae*, *Bacillus stearothermophilus*, honeybee (two enzymes), sugar beet, flint corn, and *Aspergillus niger*. The glucosidases from sugar beet, flint corn, and *A. niger* were found to hydrolyze the 2-deoxy analogs with substantially higher activities than against *p*NP α-D-Glc. Moreover, the flint corn and *A. niger* enzymes showed hydrolyzing activities, although low, for the 3-deoxy analog. The other four α-glucosidases did not exhibit any activities for either the 2- or the 3-deoxy analogs. None of the seven enzymes exhibited any activities toward the 4-deoxy, 6-deoxy, or any of the methoxy analogs. The hydrolysis results, with the deoxy substrate analogs, demonstrated that α-glucosidases having remarkably different glycon specificities exist in nature. Further insight into the hydrolysis of deoxyglycosides was obtained by determining the kinetic parameters (k_{cat} and K_m) for the reactions of sugar beet, flint corn, and *A. niger* enzymes.

Keywords: α-Glucosidase; Substrate specificity; Glycon specificity profiling; *p*-Nitrophenyl α-D-glucoside; Monodeoxy glucoside; Mono-*O*-methyl glucoside

1. Introduction

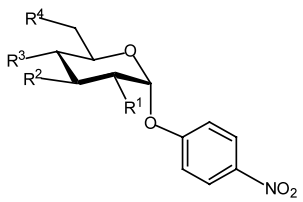
Exo-glycosidases are a group of carbohydrate hydrolases that release monosaccharides from synthetic glycosides and from the nonreducing ends of oligosaccharides and polysaccharides. The different types of glycosidases are classified on the basis of specificities for the glycon structure of their substrates, and their substrate specificities are expressed in their relative activities against substrates having various aglycons. It has been observed that hydrogen bonding between the active site of the enzyme and the hydroxyl groups of the glycosidic substrate is important in the formation of the enzyme–substrate (ES) complex. Studies have reported that various exo-type carbohydrate hydrolases, specifically α-glucosidase (EC 3.2.1.20) from barley malt,[1] β-glucosidases (EC 3.2.1.21) from almond,[2] molds *Aspergillus wetti*,[3] *A. oryzae*,[4] *A. niger*,[5] and bacteria *Agrobacterium faecalis*,[6] β-galactosidases (EC 3.2.1.23) from the bacterium *Escherichia coli*[7] and lamb small intestine,[8] and glucoamylase (EC 3.2.1.3) from *A. niger*[9–13] exhibit significant hydrolytic activities against specific deoxy analogs of their corresponding glycosidic substrates. We have also reported the hydrolytic activities of α-mannosidases (EC 3.2.1.24) from jack bean and almond,[14,15] α-glucosidase from rice,[15,16] and α-galactosidases (EC 3.2.1.22) from green coffee bean, molds *Mortierella vinasea*, and *A. niger*[15,17] against the

* Corresponding author. Fax: +81-466-843951.
E-mail address: nishio@brs.nihon-u.ac.jp (T. Nishio).

0008-6215/02/$ - see front matter
PII: S0008-6215(02)00026-5

four monodeoxy and four mono-*O*-methyl analogs of the corresponding *p*-nitrophenyl (*p*NP) α-D-glycopyranosides. Significant hydrolytic activities were exhibited by α-mannosidases toward the 6-deoxy substrate analog, *p*NP α-D-rhamnopyranoside, and α-glucosidase and α-galactosidases showed high activities toward their 2-deoxy substrates, *p*NP 2-deoxy-α-D-*arabino*-hexopyranoside and *p*NP 2-deoxy-α-D-*lyxo*-hexopyranoside, respectively. In contrast, the α-glycosidases showed no activity toward the mono-*O*-methyl derivatives, probably because of the hydrophobic character and steric hindrance of the methoxy group. The observation that some deoxy substrates are hydrolyzed by certain exo-type carbohydrate hydrolases suggests that specific hydroxyl groups of sugar substrates play a critical role in the hydrolysis reaction.

To gain further insight into the substrate-recognizing mechanism in glycosidases, we have here investigated the glycon specificities of various α-glucosidases (α-D-glucoside glucohydrolase) against partially modified substrates. Several α-glucosidases, widely distributed in microorganisms, plants, insects, and animal tissues have been purified and their amino acid sequences determined. Based on the conserved regions of the amino acid sequences, these enzymes are divided into two groups, the α-glucosidase families I and II.[18] Additionally, based on the amino acid sequence similarities, these α-glucosidases are also classified as carbohydrate hydrolases of family 13 or 31.[19,20] The families I and II α-glucosidases belong to the families 13 and 31 of carbohydrate hydrolases. The enzyme described here, rice α-glucosidase, which hydrolyzes the 2-deoxy glucoside, is classified in family II (or family 31). Herein, we report the hydrolytic activities of four enzymes classified in family I (or family 13): α-glucosidases from yeast *Saccharomyces cerevisiae*, bacteria *Bacillus stearothermophilus*, and honeybee (two enzymes), and three enzymes classified in family II (or family 31), namely the α-glucosidases from sugar beet, flint corn, and *A. niger*. The partially modified hydrolytic substrates are derivatives of *p*NP α-D-glucopyranoside (*p*NP α-D-Glc, **1**), as shown in Fig. 1. The four monodeoxy analogs, modified at C-2, C-3, C-4, and C-6 are respectively, *p*NP 2-deoxy-α-D-*arabino*-hexopyranoside (**2**), *p*NP 3-deoxy-α-D-*ribo*-hexopyranoside (**3**), *p*NP 4-deoxy-α-D-*xylo*-hexopyranoside (**4**), and *p*NP α-D-quinovopyranoside (**5**). The four mono-*O*-methyl derivatives modified at C-2, C-3, C-4, and C-6 are *p*NP 2-*O*-methyl-α-D-glucopyranoside (**6**), *p*NP 3-*O*-methyl-α-D-glucopyranoside (**7**), *p*NP 4-*O*-methyl-α-D-glucopyranoside (**8**), and *p*NP 6-*O*-methyl-α-D-glucopyranoside (**9**). The differences of the glycon specificities among the enzymes are discussed.



1 $R^1 = R^2 = R^3 = R^4 = OH$
2 $R^1 = H, R^2 = R^3 = R^4 = OH$
3 $R^1 = R^3 = R^4 = OH, R^2 = H$
4 $R^1 = R^2 = R^4 = OH, R^3 = H$
5 $R^1 = R^2 = R^3 = OH, R^4 = H$
6 $R^1 = OMe, R^2 = R^3 = R^4 = OH$
7 $R^1 = R^3 = R^4 = OH, R^2 = OMe$
8 $R^1 = R^2 = R^4 = OH, R^3 = OMe$
9 $R^1 = R^2 = R^3 = OH, R^4 = OMe$

Fig. 1. Structures of substrates used in this study.

2. Results and discussion

The hydrolytic activities of the α-glucosidases from the various sources against **1** and the monodeoxy (**2**, **3**, **4**, and **5**) and mono-*O*-methyl (**6**, **7**, **8**, and **9**) substrate analogs were investigated under the conditions listed in Table 1. Since different reaction conditions were used for each enzyme, it is important to note that our findings allow for only rough comparisons among the activities of the enzymes and other α-glucosidases. The investigations show that the four enzymes belonging to family I (or family 13), from *S. cerevisiae*, *B. stearothermophilus*, and honeybee (honeybee I and III), did not hydrolyze the deoxy analogs. In contrast, the three enzymes, from sugar beet, flint corn, and *A. niger*, showed hydrolytic activities against one or two deoxy analogs. As observed for the rice enzyme,[16] the sugar beet α-glucosidase hydrolyzed only the 2-deoxy analog **2**, however with substantially higher activity than against **1**. The enzymes from flint corn and *A. niger* showed activities, not only toward **2** (substantially higher than for **1** in both cases), but also toward the 3-deoxy analog **3** (although still lower than for **1** in each case). None of the seven enzymes exhibited activities against the 4- and 6-deoxy analogs, **4** and **5**. Since every glycon hydroxyl group of **1** was shown to be necessary for the hydrolytic actions of α-glucosidases from *S. cerevisiae*, *B. stearothermophilus*, and honeybee, while OH-2 does not appear to be essential for the catalytic activities of the enzymes from rice, sugar beet, flint corn, and *A. niger*, the deoxy substrate analogs demonstrate that α-glucosidases with remarkably different glycon specificities exist in nature. The enzymes from *S. cerevisiae*, *B. stearothermophilus*, and honeybee belong to family 13, whereas the enzymes of rice, sugar beet, and *A. niger* belong to family 31.[19,20] Based on its amino acid sequence, we have classified flint corn α-glucosidases as a member of family 31 (unpublished data). The hydrolysis results for the deoxy substrate indicate that a correlation exists between the glycon specificities and the amino acid sequences of these eight α-glucosidases.

Table 1
Relative rates of hydrolysis on monodeoxy and mono-*O*-methyl derivatives of *p*NP α-D-glucopyranoside by each α-glucosidase

Enzyme source	Relative rate of hydrolysis (%) [a]							
	Compound							
	2	**3**	**4**	**5**	**6**	**7**	**8**	**9**
S. cerevisiae	–	–	–	–	–	–	–	–
B. stearothermophilus	–	–	–	–	–	–	–	–
Honeybee I	–	–	–	–	–	–	–	–
Honeybee II	–	–	–	–	–	–	–	–
Rice [b]	175	–	–	–	–	–	–	–
Sugar beet	244	–	–	–	–	–	–	–
Flint corn	231	3.7	–	–	–	–	–	–
A. niger	259	11.9	–	–	–	–	–	–

Hydrolytic activity of each α-glucosidase was assayed under the following conditions: *S. cerevisiae* and *B. stearothermophilus*, in 50 mM sodium phosphate buffer (pH 7.0) at 37 °C; honeybee I, in 50 mM sodium acetate buffer (pH 5.0) at 35 °C; honeybee II, in 50 mM sodium acetate buffer (pH 5.5) at 35 °C; sugar beet and flint corn, in 50 mM sodium acetate buffer containing 0.05% Triton X-100 (pH 4.5) at 37 °C; *A. niger*, in 50 mM sodium acetate buffer (pH 4.0) at 37 °C. After the reaction was stopped by addition of 0.3 M Na_2CO_3 solution, the amount of *p*-nitrophenol in each mixture was measured spectrophotometrically at 405 nm.

[a] Relative rate of hydrolysis was expressed by comparison with the amount of *p*-nitrophenol that was released from *p*NP α-D-glucoside (**1**) for 30 min by each α-glucosidase, which was taken as 100%.

[b] Data with rice α-glucosidase was taken from Ref. 16.

–, activity was too low to be measured.

Using the methoxy derivatives (**6**, **7**, **8**, and **9**) as substrates, none of the seven enzymes exhibited any hydrolytic activity, similarly observed for rice α-glucosidase.[15] For these monomethylated glucosides, it may be postulated that the hydrophobic character and steric hindrance of the methoxy group prevented their binding to the enzymes.

To further examine the details of the hydrolytic activities of sugar beet, flint corn, and *A. niger* α-glucosidases against the deoxy substrates, kinetic studies on the hydrolysis of **1**, **2**, and **3** were carried out (Table 2). From the reaction velocities measured at different substrate concentrations in each reaction mixture, the k_{cat} and K_m values for each enzyme–substrate (ES) combination were calculated using the Lineweaver–Burk plot of $1/v$ versus $1/S$. The K_m values obtained using the enzymes from sugar beet, flint corn, and *A. niger* against substrate **1** were 1.04, 0.88, and 0.59, respectively, and against substrate **2** were 5.70, 7.38, and 6.09, respectively. The K_m values of the flint corn and *A. niger* enzymes against substrate **3** were 9.98 and 10.2. In comparison to substrate **1**, the higher K_m values shown by each enzyme toward substrates **2** or **3** are probably attributable to a partial loss of hydrogen bonding, which is necessary in the formation of the ES complex. Since the k_{cat} or k_{cat}/K_m values of each enzyme for **2** were higher than those for **1**, it can be postulated that the C-2 hydroxyl group of **1** contributes to the stability of its glycosidic bond, thereby depressing formation of the carbonium–oxonium ion of the reaction intermediate by its inductive effect. Conversely, the lack of an OH-2 group in the deoxy analog **2** destabilizes its glycosidic bond. These higher k_{cat} and k_{cat}/K_m values from the hydrolysis of **2** may thus result from the instability of the glycosidic bond in **2**.

It has been reported that the OH-2 group of aryl β-D-glucopyranoside substrates play an important role in the catalytic reactions of some β-glucosidases.[2–6] X-ray analysis and protein mutation studies of the β-glucosidase of the bacterium *Cellulomonas fimi* indicated that the glycon OH-2 contributes to transition-state stabilization of a covalent glycosyl–enzyme intermediate.[21,22] Moreover, a similar mechanism involving OH-2 of the glycon has also been mentioned in studies involving yeast α-glucosidase.[23] In barley malt α-glucosidase, every hydroxyl groups of the nonreducing end and OH-3 of the reducing end of maltose were confirmed to be essential for the formation of hydrogen bonds between the enzyme and the substrate in the transition-state complex.[13] Our present studies indicate that the glycon OH-2 of *p*NP α-D-glucopyranoside is not involved in stabilizing the intermediate in the cases of family II (family 31) α-glucosidases from sugar beet, flint corn, and *A. niger*, or that from rice. Further studies, including protein-engineering techniques, should be carried out to elucidate details of glycon specificities of α-glucosidases from different sources.

Table 2
Kinetic parameters of the family II α-glucosidases for the hydrolysis of *p*NP α-D-glucopyranoside and its monodeoxy derivatives

Enzyme source	Rice [a]			Sugar beet			Flint corn			*A. niger*		
Compound	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m
1	43.8	2.62	16.7	0.071	1.04	0.068	2.00	0.88	2.27	3.44	0.59	5.83
2	237	6.66	35.6	0.64	5.70	0.11	17.0	7.38	2.30	96.9	6.09	15.9
3							0.44	9.98	0.044	4.23	10.2	0.41

Hydrolytic activity of each α-glucosidase was assayed at substrate concentration between 0.5 and 2.5 mM under the following conditions: sugar beet and flint corn, in 50 mM sodium acetate buffer containing 0.05% Triton X-100 (pH 4.5) at 37 °C; *A. niger* in 50 mM sodium acetate buffer (pH 4.0) at 37 °C. After the reaction was stopped in a fixed time interval by addition of 0.3 M Na_2CO_3 solution, the amount of *p*-nitrophenol in each mixture was measured spectrophotometrically at 405 nm. The values k_{cat} and K_m were calculated from reciprocal plots of the reaction curves and molar concentrations of the enzymes.

[a] Data with rice α-glucosidase was taken from Ref. 16.

3. Experimental

Enzymes and substrates.—Pure grade α-glucosidases of *S. cerevisiae* and *B. stearothermophilus* were obtained from Oriental Yeast Co. and Sigma–Aldrich Co., respectively, and were further purified using ion-exchange column chromatography (DEAE-Toyopearl 650 resin, TOSO Co.) and gel-filtration column chromatography (Toyopearl HW-55F resin, TOSO Co.). α-Glucosidases from honeybee,[24] sugar beet,[25] flint corn,[26] and *A. niger*[27] were purified as reported. Homogeneity of the purified enzyme was confirmed using SDS- and native-polyacrylamide gel electrophoresis. *p*NP α-D-Glc (**1**) was purchased from Tokyo Kasei Ind., and was further purified by recrystallization from EtOH. Syntheses of the four monodeoxy analogs, *p*NP 2-deoxy-α-D-*arabino*-hexopyranoside (**2**),[14] *p*NP 3-deoxy-α-D-*ribo*-hexopyranoside (**3**),[16] *p*NP 4-deoxy-α-D-*xylo*-hexopyranoside (**4**),[17] and *p*NP α-D-quinovopyranoside (**5**),[16] and the four mono-*O*-methyl derivatives,[15] *p*NP 2-*O*-methyl-α-D-glucopyranoside (**6**), *p*NP 3-*O*-methyl-α-D-glucopyranoside (**7**), *p*NP 4-*O*-methyl-α-D-glucopyranoside (**8**), and *p*NP 6-*O*-methyl-α-D-glucopyranoside (**9**) were accomplished according to the procedures as described in the literature. The $^4C_1$ chair conformations of these partially modified glycopyranosides were confirmed based on their ^{1}H NMR spectra in D_2O,[14–17] and supported by computational studies of the low-energy conformations using the MM2 force field by CAChe (Sony Tektronix) (data not shown).

Assay of hydrolytic activity of the enzymes.—The enzymatic hydrolysis of substrates was monitored by the amount of *p*-nitrophenol released by the reaction under appropriate conditions for each enzyme as follows: *S. cerevisiae* and *B. stearothermophilus*, in 50 mM sodium phosphate buffer (pH 7.0) at 37 °C; honeybee I, in 50 mM NaOAc buffer (pH 5.0) at 35 °C; honeybee III, in 50 mM NaOAc buffer (pH 5.5) at 35 °C; sugar beet and flint corn, in 50 mM NaOAc buffer containing 0.05% Triton X-100 (pH 4.5) at 37 °C; *A. niger*, in 50 mM NaOAc buffer (pH 4.0) at 37 °C. After the addition of enzyme to substrate that was present at a concentration of 1.0 mM in a final volume of 0.5 mL, the mixture was incubated. After stopping the reaction by the addition 0.3 M Na_2CO_3 solution (0.5 mL), the amount of *p*-nitrophenol in the mixture was measured spectrophotometrically at 405 nm. Kinetic studies of the enzyme reaction were carried out at substrate concentrations between 0.5 and 2.5 mM, and the values of k_{cat} and K_m were calculated from reciprocal plots of the reaction curves and the molar concentrations of the enzymes. Molecular weights of the enzymes are as follow: sugar beet, 91 kDa; flint corn, 65 kDa; *A. niger*, 125 kDa.[28]

Acknowledgements

The authors express their sincere gratitude to Dr H. Matsui of Hokkaido University for his support. This work was supported in part by a Grant from the Ministry of Education, Science, Sports, and Culture (Japan) to promote advanced scientific research.

References

1. Frandsen, T. P.; Lok, F.; Mirgorodskaya, E.; Roepstorff, P.; Svensson, B. *Plant Physiol.* **2000**, *123*, 275–286.
2. Lemieux, R. U.; Spohr, U. In *Advances in Carbohydrate Chemistry and Biochemistry*; Horton, D., Ed. How Emil Fisher was Led to the Lock and Key Concept for Enzyme Specificity; Academic Press: New York, 1994; Vol. 50, pp. 1–20.
3. Roeser, K.-R.; Legler, G. *Biochim. Biophys. Acta* **1981**, *657*, 321–333.
4. Mega, T.; Matsushima, Y. *J. Biochem.* **1983**, *94*, 1637–1647.
5. Sigurskjold, B. W.; Duus, B.; Bock, K. *Acta Chem. Scand.* **1991**, *45*, 1032–1041.
6. Namchuk, M. N.; Withers, S. G. *Biochemistry* **1995**, *34*, 16194–16202.
7. McCarter, J. D.; Adam, M. J.; Withers, S. G. *Biochem. J.* **1992**, *286*, 721–727.
8. Rivera-Sagredo, A.; Canada, F. J.; Nieto, O.; Jimenez-Barbero, J.; Martin-Lomas, M. *Eur. J. Biochem.* **1992**, *209*, 415–422.
9. Bock, K.; Pedersen, H. *Acta. Chem. Scand.* **1987**, *B41*, 617–628.
10. Sierks, M. R.; Bock, K.; Refn, S.; Svensson, B. *Biochemistry* **1992**, *31*, 8972–8977.
11. Lemieux, R. U.; Spohr, U.; Bach, M.; Cameron, D. R.; Frandsen, T. P.; Stoffer, B. B.; Svensson, B.; Palcic, M. M. *Can. J. Chem.* **1996**, *74*, 319–335.
12. Frandsen, T. P.; Stoffer, B. B.; Palcic, M. M.; Hof, S.; Svensson, B. *J. Mol. Biol.* **1996**, *263*, 79–89.
13. Sierks, M. R.; Svensson, B. *Biochemistry* **2000**, *39*, 8585–8592.
14. Nishio, T.; Miyake, Y.; Tsujii, H.; Hakamata, W.; Kadokura, K.; Oku, T. *Biosci. Biotechnol. Biochem.* **1996**, *60*, 2038–2042.
15. Hakamata, W.; Nishio, T.; Sato, R.; Mochizuki, T.; Tsuchiya, K.; Yasuda, M.; Oku, T. *J. Carbohydr. Chem.* **2000**, *19*, 359–377.
16. Hakamata, W.; Nishio, T.; Oku, T. *J. Appl. Glycosci.* **1999**, *46*, 459–463.
17. Hakamata, W.; Nishio, T.; Oku, T. *Carbohydr. Res.* **2000**, *324*, 107–115.
18. Chiba, S. *Biosci. Biotechnol. Biochem.* **1997**, *61*, 1233–1239.
19. Henrissat, B. *Biochem. J.* **1991**, *280*, 309–316.
20. Henrissat, B.; Bairoch, A. *Biochem. J.* **1993**, *293*, 781–788.
21. Notenboom, V.; Birsan, C.; Nitz, M.; Rose, D. R.; Warren, R. A. J.; Withers, S. G. *Nature Struct. Biol.* **1998**, *5*, 812–818.
22. Withers, S. G. *Carbohydr. Polym.* **2001**, *44*, 325–337.
23. Tanaka, K. S. E.; Zhu, J.; Huang, X.; Lipari, F.; Bennet, A. J. *Can. J. Chem.* **2000**, *78*, 577–582.

24. Takewaki, S.; Chiba, S.; Kimura, A.; Matsui, H.; Koike, Y. *Agric. Biol. Chem.* **1980**, *44*, 731–740.
25. Chiba, S.; Inomata, S.; Matsui, H.; Shimomura, T. *Agric. Biol. Chem.* **1978**, *42*, 241–245.
26. Chiba, S.; Shimomura, T. *Agric. Biol. Chem.* **1975**, *39*, 1033–1040.
27. Kita, A.; Matsui, H.; Somoto, A.; Kimura, A.; Takata, M.; Chiba, S. *Agric. Biol. Chem.* **1991**, *55*, 2327–2335.
28. Chiba. S. Data on Individual Amylases. In *Handbook of Amylases and Related Enzymes*, 1st ed.; The Amylase Research Society of Japan, Eds.; Pergamon Press: Oxford, 1988; pp. 104–116.