(Short Communication)

A Glycosidase with β-D-Glucosidase and β-D-Fucosidase from Pollen of *Typha latifolia*

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(Received Sep. 22 1999, Accepted Oct. 28 1999)

Both activities of β-D-glucosidase and β-D-fucosidase associated with the cell wall of *Typha* pollen markedly increased during the cultivation on an agar medium. Both enzymes were solubilized with 0.5M NaCl from the cell debris of pollen cultivated, and then purified by three chromatographic steps. Throughout the steps, both activities were detected at the same position as a single peak. The purified preparation gave a single protein band with a molecular mass of 58kDa on SDS-PAGE. The results indicate that the enzyme is a glycosidase with β-D-glucosidase and β-D-fucosidase. The optimum pH was about 4.5. The enzyme acted on *p*-nitrophenyl (PNP)-β-D-fucoside and PNP-β-D-glucoside with the activity ratio of 2.5 : 1 but showed a negligibly low activ-ity toward PNP-β-D-galactoside. *Km* values for PNP-β-D-glucoside and PNP-β-D-fucoside were 0.67mM and 0.36mM, respectively. The enzyme hydrolyzed laminaribiose and salicin but slightly acted on gentiobiose and cellobiose.

Key words : Glycosidase ; β-D-glucosidase and β-D-fucosidase ; *Typha* pollen

Various glycosidases which hydrolyze glycosidic bonds in oligosaccharides and glycosides are widely distributed in living organisms.

Mature pollen of *Typha latifolia* germinates within about 40min followed by a rapid pollen tube elongation on an agar medium. The pollen tube develops with the synthesis of new cell wall. During studies on the enzymes related to the sugar metabolism of the pollen, we found that both activities of β-D-glucosidase and β-D-fucosidase associated with the cell wall markedly increased during the development. The enzyme with both activities was solubilized with 0.5M NaCl, like cell wall-bound glycosidases, invertase [1] from cycad pollen and β-*N*-acetylhexosaminidase [2] from pollen of *Pinus thunbergyii*. This report deals with the changes in both activities during the cultivation of *Typha* pollen, and the purification and some properties of the enzyme with both activities.

Mature pollen grains of *Typha latifolia* were collected, dried at room temperature for 4 days and stored at −20℃. The standard assay condition for β-D-glycosidase activity was as follows : the reaction mixture contained 0.5ml of 4mM PNP-β-D-glucoside (Nakarai Tesque) or PNP-β-D-fucoside (Sigma), 0.48ml of McIlvaine buffer (pH4.5), and 20μl of appropriately diluted enzyme. The mixture

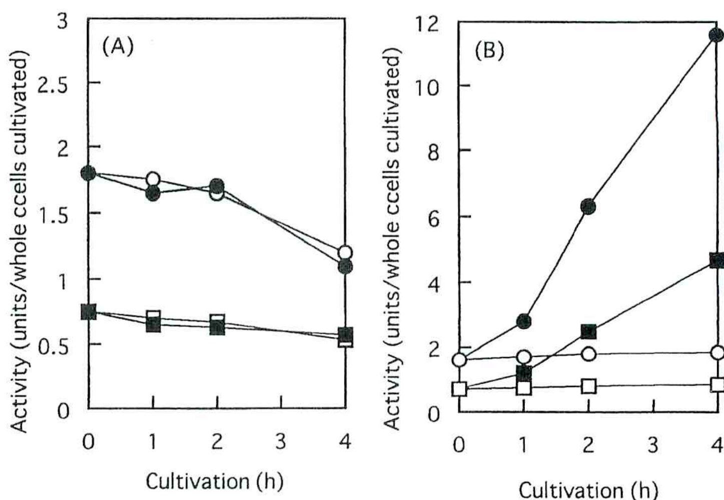

Fig. 1. Changes in activities of β-D-glucosidase and β-D-fucosidase during cultivation of *Typha* pollen. Experimental conditions are described in the text. (A), Changes of activities in the soluble fraction ; (B) Changes of activities in the insoluble fraction. Activities during the cultivation without cycloheximide : (■), β-D-glucosidase ; (●), β-D-fucosidase. Activities during the cultivation with 0.1mM cycloheximide : (□), β-D-glucosidase ; (○), β-D-fucosidase.

was incubated at 37℃ for 10min, and the reaction was stopped by the addition of 1.5ml of 0.1M NaOH. The amount of *p*-nitrophenol released was measured at 400nm. One unit of enzyme activity was defined as the amount of enzyme which released 1 μmol of *p*-nitrophenol per min. Protein was measured by a modification [3] of Lowry's method with bovine serum albumin as a standard. SDS-PAGE was done by the method of Laemmli [4].

Five hundred milligrams of the pollen grains were cultivated at 30℃ on a 1.5% agar medium with or without 0.1mM cycloheximide, a protein synthesis inhibitor, in a Petri dish of 20cm in diameter. Then the pollen was collected in 10ml of 10mM Tris-HCl buffer (pH7.5, T-buffer) at indicated times in Fig. 1. The suspensions were individually homogenized with a teflon-glass homogenizer at 2000rpm for 10min, and centrifuged at 20,000 × g for 10min. Each precipitate was suspended in 10ml of T-buffer, centrifuged again and resuspended in 10ml of T-buffer for use as an insoluble fraction for enzyme assay. The supernatants obtained by the centrifugations were combined individually and used as soluble fractions. Fig. 1 shows the changes in activities of β-D-glucosidase and β-D-fucosidase during the cultivation of the pollen. Both activities in the soluble fraction were kept at the same level for 2 h-cultivation and then gradually decreased, irrespective of addition of cycloheximide (A). On the contrary, both activities in the insoluble fraction individually increased by about 6-fold during 4 h-cultivation without cycloheximide, and also the ratio of β-D-glucosidase activity to β-D-fucosidase activity was kept at about 0.4. However, increase in the activities remained negligibly small during the cultivation with cycloheximide, which resulted in strong inhibition of the pollen tube elongation (B). The results suggest that there is a relationship between the increase in activities and pollen tube elongation.

Both enzymes of β-D-glucosidase and β-D-fucosidase of the *Typha* pollen were purified as follows: twenty grams of the pollen grains cultivated for 4 h on agar media without cycloheximide were

collected in 140ml of T-buffer. Ten-ml each of the suspension was homogenized with a teflon-glass homogenizer at 2000rpm for 10min, and the combined homogenate was centrifuged at 20,000 × g for 10min. After the precipitate was suspended in 140ml of T-buffer containing 0.5% Triton X-100, the suspension was stirred for 1 h at 4 ℃ to solubilize the lipids, and centrifuged at 20,000 × g for 10min. Both activities were detected in the precipitate but not in the supernatant. The precipitate was suspended in 140ml of T-buffer and centrifuged at 20,000 × g for 10min. The precipitate washed was resuspended in 140ml of T-buffer containing 0.5M NaCl, stood for overnight at 4 ℃, and centrifuged at 20,000 × g for 10min. About 70% of each activity in the precipitate was solubilized by NaCl, suggesting that both enzymes are ionically bound to the cell wall. The supernatant was dialyzed against T-buffer, and applied to a column (2.5 × 25cm) of DEAE Bio-Gel Agarose equilibrated with the same buffer. After the column was washed with 120ml of T-buffer, proteins were eluted with a linear gradient of 0 to 0.6M NaCl in 440ml of T-buffer. Active fractions eluted with about 0.25M NaCl were pooled, concentrated to about 3 ml under reduced pressure, and put on a column (2 × 80cm) of Sephadex G-75 equilibrated with T-buffer containing 0.5M NaCl. Elution was done with the same buffer. The active fractions were pooled, concentrated to about 3 ml, and put on a column (2 × 80cm) of Cellulofine GCL-1000*sf* equilibrated with T-buffer containing 0.5 M NaCl. Elution was done with the same buffer. Table shows that the enzyme was purified about 100-fold with a yield of 14.5% as β-D-glucosidase. Throughout the chromatographic steps, both activities of β-D-glucosidase and β-D-fucosidase were detected at the same position as a single peak. The ratio of activity of the former to that of the latter was also kept at about 0.4. The purified enzyme gave a single protein band with a molecular mass of 58kDa on SDS-PAGE (Fig. 2). The results show that the enzyme is a glycosidase with β-D-glucosidase and β-D-fucosidase. The optimum pH of the enzyme was 4.5 on both activities. The optimum temperature for both activities was 50℃. The enzyme was stable at pH between 6.0 and 11.0 at 4 ℃ for 24 hr, and up to 50℃ on heating for 10min at pH 7.0. No effects of Mn^{2+}, Zn^{2+}, Ca^{2+}, Mg^{2+} or Cu^{2+} at 1mM on both activities were observed. D-Glucose, D-mannose and D-xylose at 10mM inhibited the β-D-glucosidase activity by about 20% but increased the β-D-fucosidase activity by about 60%. β-D-Fucosidase activity in β-D-glycosidase [5] from *Bifidobacterium breve* clb with a transferase activity for β-D-fucosyl group was also activated by the addition of these sugars. D-Fucose at 10mM slightly inhibited the β-D-fucosidase activity of the pollen enzyme. D-Galactose was not effective. Glucono-1, 5-lactone is a

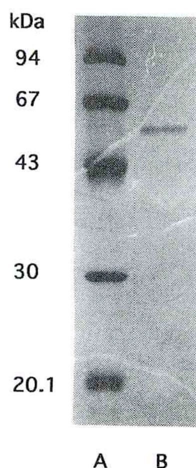

Fig. 2. SDS-PAGE of the purified enzyme. The purified enzyme (5 μg) was electrophoresed at a current of 15mA using 12.5% polyacrylamide slab gel containing 0.1% SDS in 25mM Tris-glycine (pH8.3) containing 0.1% SDS. After electrophoresis, protein on the gel was stained with Coomassie brilliant blue R-250. A, marker proteins ; B, the purified enzyme.

Table 1. Summary of purification of glycosidase with β-D-glucosidase and β-D-fucosidase from pollen of *Typha latifolia*

Step	Protein (mg)	β-D-Glucosidase			The ratio of β-D-glucosidase activity to β-D-fucosidase activity
		Activity (units)	Specific activity (units / mg)	Yield (%)	
NaCl extract	116	143	1.23	100	0.40
DEAE Bio-Gel Agarose	1.60	61.9	38.7	43.3	0.38
Sephadex G-75	0.339	36.9	109	25.8	0.38
Cellulofine GCL-1000*sf*	0.167	20.8	125	14.5	0.39

potent inhibitor for β-D-glucosidase [6]. Both activities of the pollen enzyme were also inhibited 95% by 0.1mM the lactone. The enzyme acted on PNP-β-D-fucoside and PNP-β-D-glucoside with the activity ratio of 2.5 : 1. But activities toward PNP-β-D-galactoside and the other PNP-sugars were lower than 2 %. Glycosidase [7] from *Polygonum tinctorium* has both activities of β-D-fucosidase and β-D-glucosidase with the ratio of 1.5 : 1 although some glycosidases have also β-D-galactosidase activity [5, 8-10]. The pollen enzyme hydrolyzed laminaribiose and salicin but was less active on cellobiose, gentiobiose and laminarin. *Km* values of the enzyme for PNP-β-D-fucoside and PNP-β-D-glucoside were 0.36mM and 0.67mM, respectively, lower than those (2.2mM and 7.1mM) of *P. tinctorium* enzyme [7].

The enzyme from *Typha* pollen is apparently different from β-D-glucosidase [11] from *Pinus* pollen, since the latter enzyme showed no β-D-fucosidase activity. The enzyme was not detected in the *Typha* leaf. Therefore, the enzyme seems to be specifically present in the *Typha* pollen. Nakamura and Suzuki reported the presence of fucose in pollen grain walls from *Tulipa gesneriana* and in pollen tube walls from *Camellia japonica, Camellia sinensis* and *Tulipa gesneriana* [12]. The role of the *Typha* enzyme in the sugar metabolism and the modification of glycoprotein during the pollen tube elongation was now in progress.

Acknowledgements

This work was supported in part by a grant for scientific research from Meijo University.

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β-D-グルコシダーゼと β-D-フコシダーゼを合わせ持つ ガマ花粉のグリコシダーゼ

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ガマ花粉の壁結合型の β-D-グルコシダーゼと β-D-フコシダーゼの活性は培養に伴い著しく上昇した．この酵素は培養した花粉の破砕物から 0.5M の NaCl で可溶化された後，3つのクロマトグラフィを行って精製した．精製過程において，両活性は同位置に検出され，その標品は SDS-PAGE で均一，分子量は 58kDa であった．これらの結果は，この酵素が β-D-グルコシダーゼと β-D-フコシダーゼを合わせ持つグリコシダーゼであることを示す．最適 pH は 4.5 で，PNP-β-D-フコシッドと PNP-β-D-グルコシッドに対する活性の比は 2.5：1 であった．PNP-β-D-ガラクトシッドはほとんど分解しなかった．PNP-β-D-フコシッドと PNP-β-D-グルコシッドに対する本酵素の *Km* 値はそれぞれ 0.36mM と 0.67mM であった．本酵素はラミナリビオースとサリシンには作用したが，ゲンチオビオースとセロビオースにはわずかしか作用しなかった．