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Structure and Function of Exo-β-glucosaminidase from *Amycolatopsis orientalis*

Tamo Fukamizo[1)] and Ryszard Brzezinski[2)]

1) Department of Advanced Bioscience, Kinki University
2) Centre d'Étude et de Valorisation de la Diversité Microbienne, Département de Biologie, Faculté des Sciences,Université de Sherbrooke

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We cloned and sequenced the gene encoding exo-β-glucosaminidase (GlcNase) from *Amycolatopsis orientalis*, and found that the gene has an open reading frame of 1032 residues with a calculated molecular mass of 110,557. The GlcNase has been classified as a member of family GH-2. Sequence alignments identified a group of GlcNase-related protein sequences forming a distinct subclass of family GH-2. When mono-*N*-acetylated chitotetraose [$(GlcN)_3$-GlcNAc] was hydrolyzed by the enzyme, the GlcN unit was produced from the nonreducing end together with the transglycosylation products. ^{1}H-NMR spectroscopy revealed that the enzyme is a retaining glycoside hydrolase. The rate of hydrolysis of the disaccharide, GlcN-GlcNAc, was somewhat lower than that of $(GlcN)_2$, suggesting that the *N*-acetyl group of the sugar residue located at (+1) site partly interferes with the catalytic reaction. Based on the time-course of the enzymatic hydrolysis of the completely deacetylated chitotetraose [$(GlcN)_4$], we obtained the values of binding free energy changes of +7.0, -2.9, -1.8, -0.9, -1.0 and -0.5 kcal/mol corresponding, respectively, to subsites (-2) (-1) (+1) (+2) (+3) (+4). Synergism resulting from mixing the *A. orientalis* GlcNase with *Streptomyces* sp. N174 endochitosanase was also observed when chitosan polysaccharide was used as the substrate. To identify the catalytic residue, mutations were introduced into the putative catalytic residues resulting in five mutated enzymes (D469A, D469E, E541D, E541Q and S468N/D469E) which were successfully produced. The four single mutants were devoid of enzymatic activity, indicating that Asp469 and Glu541 are essential for catalysis as predicted from sequence alignment of enzymes

belonging to GH-2 family.

Key words: *Amycolatopsis orientalis*, exo-β-glucosaminidase, chitosanase, subsites, chitooligosaccharides

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