

## Studies on *N*-acetyl- $\beta$ -D-glucosaminidases from *Clostridium paraputrificum* M-21

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The studies were carried out to describe the characterization of *Clostridium paraputrificum* M-21 *N*-acetyl- $\beta$ -D-glucosaminidase genes *nag3A*, *nag84A*, their translated products, and the identification of a catalytic residue of Nag3A by site-directed mutagenesis.

*Clostridium paraputrificum* M-21 is a Gram-positive, spore-forming, anaerobic and chitinolytic bacterium which is known to produce a significant amount of hydrogen gas when chitin and various chitinous wastes such as row shrimp and lobster shells serve as carbon source. To study the role of chitin degradation by *C. paraputrificum* M-21, two  $\beta$ -*N*-acetylglucosaminidases genes, *i.e.* *nag3A* and *nag84A*, were cloned into *Escherichia coli* and characterized along with their translated products.

The first  $\beta$ -*N*-acetylglucosaminidases gene cloned from *C. paraputrificum* M-21 in *E. coli* was *nag3A*. The *nag3A* gene consists of an open reading frame of 1,239bp, encoding 413 amino acids with a deduced molecular weight of 45,531. Nag3A is a single domain enzyme consisting of a family-3 catalytic domain of glycoside hydrolases. Nag3A purified from a recombinant *E. coli* was characterized. The enzyme hydrolyzed chitooligomers such as di-*N*-acetylchitobiose, tri-*N*-acetylchitotriose, tetra-*N*-acetylchitotetraose, penta-*N*-acetylchitopentaose, and hexa-*N*-acetylchitohexaose, ball-milled chitin, and synthetic substrates such as 4-methylumbelliferyl *N*-acetyl  $\beta$ -D-glucosaminide [4-MU-(GlcNAc)], but not *p*-nitrophenyl- $\beta$ -D-glucoside, *p*-nitrophenyl- $\beta$ -D-xyloside, and *p*-nitrophenyl- $\beta$ -D-galactosamine at al., The enzyme was optimally active at 50°C and pH 7.0, and the apparent *K*<sub>m</sub> and *V*<sub>max</sub> values for 4-MU-(GlcNAc) were 7.9  $\mu$ M and 21.8  $\mu$ mol/min/mg of protein, respectively. SDS-PAGE, zymogram, and immunological analyses suggested that the Nag3A was induced by ball-milled chitin.

To identify catalytic residues of enzyme Nag3A, mutations were introduced into highly conserved Glu and Asp residues: eleven different mutant genes derived from *nag3A* were successfully constructed. Substitution of Asp175 with Ala abolished the catalytic activity without change in circular dichroism spectrum, strongly suggesting that this residue is one of catalytic residues, a nucleophile/base or a proton donor. Since *K*<sub>m</sub> values of mutant enzymes D119N, D229N, D229A

and D274N increased 17 to 41 times compared with that of wild-type enzyme, Asp119, Asp229 and Asp274 seem to be involved in substrate recognition and binding. Asp303 is presumed to be the catalytic nucleophile and Asp175 is the proton donor of *C. paraputrificum* Nag3A.

The second  $\beta$ -*N*-acetylglucosaminidases gene, *nag84A*, of *C. paraputrificum* M-21 was also cloned, sequenced and expressed in *E. coli*. The *nag84A* gene consists of 4,647 nucleotides encoding a protein of 1,549 amino acids with a predicted molecular weight of 174,311. Nag84A has a catalytic domain classified in family-84 of the glycoside hydrolases. Nag84A purified from a recombinant *E. coli* was characterized. Although Nag84A exhibited high homology to the hyaluronidase from *Clostridium perfringens*, it did not degrade hyaluronic acid. The enzyme showed substrate specificity similar to that of Nag3A, except that Nag84A was active only on chitooligosaccharides but not on highly polymerized chitin. The enzyme was optimally active at 50°C and pH 6.5, and the apparent  $K_m$  and  $V_{max}$  values for 4-MU-(GlcNAc) were 8.5  $\mu$ M and 1.39  $\mu$ mol/min/mg of protein, respectively. SDS-PAGE, zymogram, and immunological analyses suggested that Nag84A was inducible by ball-milled chitin.

