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Identification of a Catalytic Residue of *Clostridium paraputrificum* *N*-Acetyl-β-D-glucosaminidase Nag3A by Site-Directed Mutagenesis

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***Clostridium paraputrificum* M-21 β-*N*-acetylglucosaminidase 3A (Nag3A) is an enzyme classified in family 3 of the glycoside hydrolases. To identify catalytic residues of this enzyme, mutations were introduced into highly conserved Glu and Asp residues. Replacement of Asp175 with Ala abolished the catalytic activity without change in the circular dichroism spectrum, strongly suggesting that this residue is a catalytic residue, a nucleophile/base or a proton donor. Since the K_m values of mutant enzymes D119N, D229N, D229A and D274N increased 17 to 41 times as compared with that of wild-type enzyme, Asp119, Asp229, and Asp274 appear to be involved in substrate recognition and binding. Taking previous studies into consideration, we presume that Asp303 is the catalytic nucleophile and Asp175 is the proton donor of *C. paraputrificum* Nag3A.**

Key words: β-*N*-acetylglucosaminidase; *Clostridium paraputrificum*; catalytic residue; site-directed mutagenesis

Chitin is the second most abundant polysaccharide found in nature. It consists of variable-length linear chains of β-1,4-linked polymers of *N*-acetylglucosamine hydrogen bonded into an ordered insoluble crystalline structure. The enormous amounts of chitin produced annually in the biosphere are degraded by chitinases. Chitinases are ubiquitous in nature, being found in eukaryotes, prokaryotes, archaea, and viruses. They consist of a group of hydrolytic enzymes that are able to break down polymeric chitin to chitin oligosaccharides, diacetylchitobiose [$(GlcNAc)_2$], and *N*-acetylglucosamine (GlcNAc).

Clostridium paraputrificum M-21 was isolated and characterized as a chitin-degrading hydrogen-producing anaerobe in this laboratory.[1,2] It produces a significant amount of hydrogen gas, approximately 2 mol H_2/mol GlcNAc. Its hydrogen gas production was enhanced to 1.7-fold of that of the wild type by overexpression of a hydrogenase gene.[3] Therefore, the bacterium has application possibilities in hydrogen production. Two chitinase genes, *chiA*[4] and *chiB*,[5] and two β-*N*-acetylglucosaminidase (Nag) genes, *nag3A*[6] and *nag84A*[7] of *C. paraputrificum* M-21, were cloned from a gene library and expressed in *Escherichia coli*, and their translated products were characterized. Both chitinases produced $(GlcNAc)_2$ as the major end product from chitin and chitooligosaccharides. Although Nag84A hydrolyzed only chitooligosaccharides to produce GlcNAc, Nag3A produced GlcNAc from either chitin or chitooligosaccharides and showed particularly high activity on chitobiose.[6] It was also found that *C. paraputrificum* M-21 secreted a 72-kD endo-chitinase Chi18C other than ChiA and ChiB when cultivated on ball-milled chitin as a carbon source (unpublished result). Chi18C catalyzes the hydrolysis of chitin at random sites along the polymer to produce chitooligosaccharides. The sequential hydrolysis conducted by *chi18C*, *chiA*, *chiB*, *nag84A*, and *nag3A* gene products must govern the breakdown of chitin and the growth of *C. paraputrificum* M-21 when chitin was used as the sole carbon source. Among these enzymes, Nag3A may be a key enzyme for the final step of sequential hydrolysis, since it prefers chitobiose to larger saccharides as the substrate.

Nag3A is an enzyme classified in family 3 of the glycoside hydrolases (http://afmb.cnrs-mrs.fr/CAZY/index.html).[8] Twelve Nags are found in this family, and four of them have been characterized as to their enzyme properties.[6,9–11] From the alignment of the catalytic domains of the 12 Nags (Fig. 1), it can be seen that two Glu residues and six Asp residues are highly conserved among them and are candidates for catalytic amino acid residues. Barley β-glucan glucanohydrolase ExoI in family 3 is composed of 605 amino acid residues and is the only enzyme of which crystalline structure has been reported.[12,13] Structural analysis of ExoI indicated that this enzyme is a globular protein

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Abbreviations: Nag, β-*N*-acetylglucosaminidase; GlcNAc, *N*-acetylglucosamine; 4-MU-(GlcNAc), 4-methylumbelliferyl β-D-*N*-acetylglucosamine; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; Tris, Tris(hydroxymethyl)aminomethane

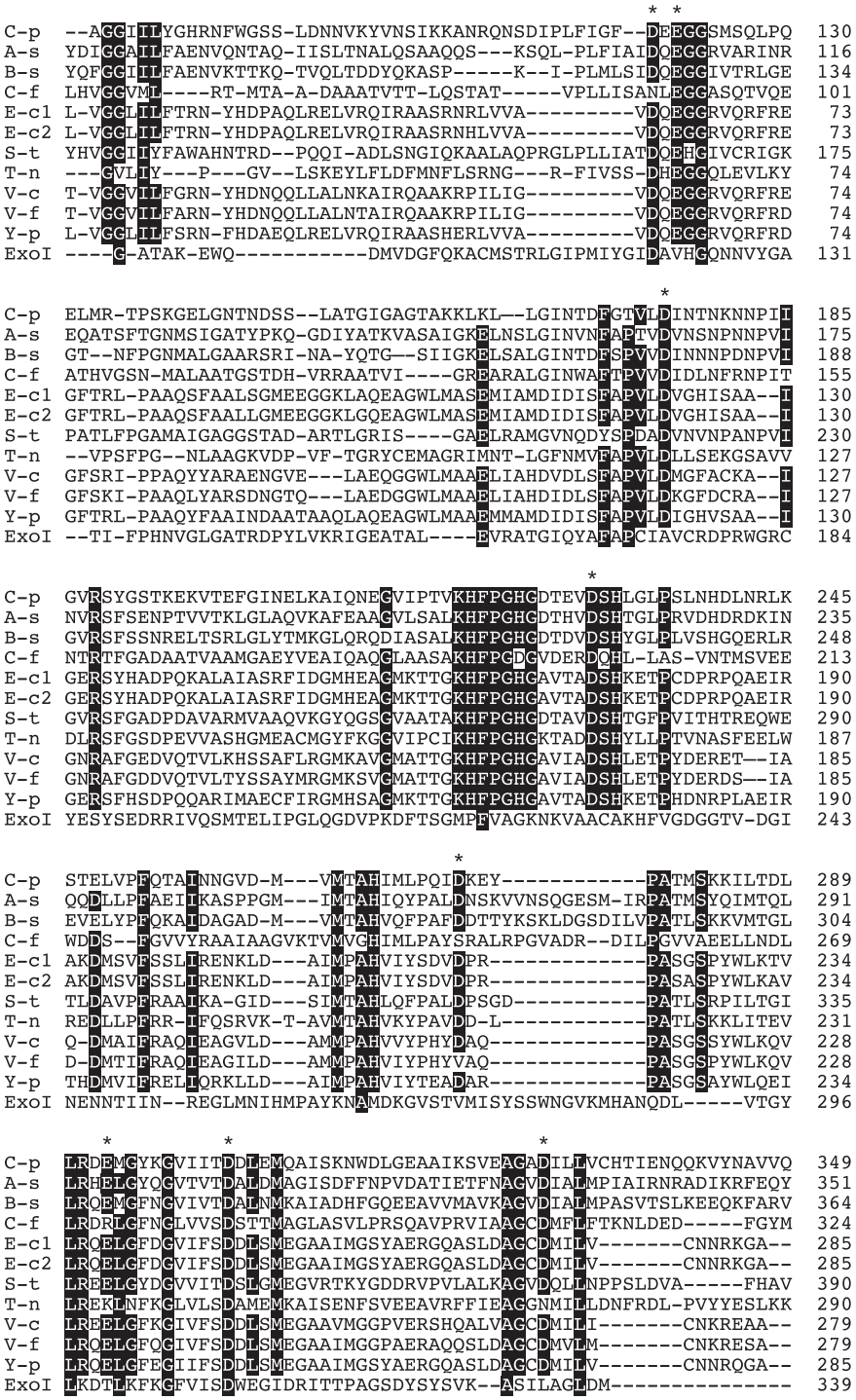

Fig. 1. Multiple Alignment of the Catalytic Domains of Family-3 Nags.

Amino acids conserved in at least 9 of 11 sequences are highlighted; - gap left to improve alignment. Asterisks indicate the amino acids selected for site-directed mutagenesis. Numbers at the end of each line refer to amino acid residue positions. All sequences except ExoI are numbered from Met-1 of the peptide and ExoI sequence is numbered from the Asp residue of the mature form without signal peptide. C-p, *C. paraputrificum* Nag3A; A-s, *Alteromonas* sp. Cht60; B-s, *Bacillus subtilis* YbbD; C-f, *Cellulomonas fimi* Nag3A; E-c1, *E. coli* CFT073 NagZm; E-c2, *E. coli* K-12 NagZ; S-t, *Streptomyces thermoviolaceus* NagII; T-n, *Thermotoga neapolitana* CbsA; V-c, *Vibrio cholerae* NagZ; V-f, *Vibrio furnissii* ExoII; Y-p, *Yersinia pestis* NagZ; ExoI, Barley β-D-glucan exohydrolase isoenzyme ExoI.

composed of two domains: the first 357 residues constitute an $(\alpha/\beta)_8$ TIM-barrel domain, and the second domain is arranged in a six-stranded β sandwich with three α helices on either side of the sheet. Furthermore, Asp285 in the first domain and Glu491 in the second domain were identified as the catalytic nucleophile and proton donor respectively.[12–14] On the other hand, *C. paraputrificum* Nag3A consists of 413 amino acid

residues, suggesting that it is a single-domain protein devoid of a second domain. Therefore, although a possible nucleophile identified in ExoI is conserved as Asp303 in *C. paraputrificum* Nag3A, no possible proton donor residue is found in Nag3A. To identify catalytic residues of *C. paraputrificum* Nag3A, we constructed a number of mutant Nag3A proteins with substitutions at possible catalytic residues by site-directed mutagenesis and characterized their enzyme properties.

Materials and Methods

Bacterial strains, plasmids, and culture conditions. pQE-30T, a derivative of pQE-30 (Qiagen, Hilden, Germany), which contains a short stretch of nucleotides encoding a thrombin recognition sequence (LVPRGS) between the six-His affinity tag–coding region and the multiple cloning sites, was used as the expression vector.[15] *E. coli* strain XL1-Blue was used as the host for construction and isolation of recombinant plasmids. *E. coli* M15 (pREP4) (Qiagen) was used as the host for expression of the recombinant proteins from plasmids derived from pQE-30T. All *E. coli* strains were grown at 37 °C on LB medium supplemented with appropriate antibiotics.

Construction of site-directed mutants and heterologous gene expression in E. coli. Heterologous expression of the *nag3A* gene (DDBJ accession no. AB078776) using the pQE-30T vector and an *N*-terminal fusion to a six-His-tag (plasmid pNAG3A) were described elsewhere.[6] The site-directed mutants of several codons were constructed using the overlap extension method[16] with pNAG3A as a template. The mutants constructed and the oligonucleotides used for site-directed mutagenesis are listed in Table 1. The PCR products were digested with *Bam*HI and *Sal*I and ligated to the pQE-30T vector digested with the same enzymes. After transformation of *E. coli* XL1-Blue cells, the resulting plasmid constructs were prepared and sequenced to verify the presence of the desired mutations. *E. coli* M15 cells were transformed with the original pNAG3A and the mutant plasmids for gene expression. Expression of the *nag3A* gene and its derivatives was induced by the addition of isopropyl-β-D-thiogalactopyranoside (final concentration, 1 mM) to the cultures growing in LB medium at 37 °C at an $OD_{600} = 0.6$–0.8, and incubated with vigorous shaking for 4 h after induction.

Purification of recombinant Nags. The wild-type and mutant Nags produced by the recombinant *E. coli* M15 were purified with a HiTrap chelating HP column (1 ml; Amersham Pharmacia Biotech, Tokyo) according to the supplier's protocol. Active fractions were collected and desalted by dialysis against 20 mM Tris–HCl buffer (pH 7.5). The enzyme was treated with thrombin protease (10 units per mg of each Nag) and subjected to purification on a Ni-NTA spin column. Proteins that did not bind to the column were collected and desalted by dialysis as described above, and further purified using a MonoQ HR5/5 column (0.5 × 5 cm; Amersham Pharmacia Biotech) equilibrated with 50 mM Tris–HCl buffer (pH 7.5).

Table 1. Mutants and Synthetic Oligonucleotides Used for Amplification and Mutagenesis of the *nag3A* Gene in This Study

Oligonucleotide		Sequence (5′→3′)
N- and C-terminal flanking oligonucleotides		
NAG-For		ggatccactattttaacagggaaaaatg
NAG-Rev		gtcgacctatttatacattgcttcaactac
Mutation-introducing oligonucleotides		
D119N	fwd	attgggttt**AaC**gaagaagga
	rev	tccttcttc**GtT**aaacccaat
E121Q	fwd	tttgatgaa**Caa**ggaggaagt
	rev	acttcctcc**ttG**ttcatcaaa
E121A	fwd	tttgatgaa**gCa**ggaggaagt
	rev	acttcctcc**tGc**ttcatcaaa
D175N	fwd	ggtactgttttta**AaC**attaatact
	rev	agtattaat**GtT**taaaacagtacc
D175A	fwd	ggtactgttttta**gCt**attaatact
	rev	agtattaat**aGc**taaaacagtacc
D229N	fwd	acggaggtg**AaC**tctcattt
	rev	aaatgaga**GtT**cacctccgt
D229A	fwd	acggaggtg**gCt**tctcattt
	rev	aaatgaga**aGc**cacctccgt
D274N	fwd	ccacaaatt**AaC**aaagagtac
	rev	gtactctttt**GtT**aatttgtgg
E293Q	fwd	ttacgtga**tCa**aatgggttata
	rev	tataacccatt**tGa**tcacgtaa
D303N	fwd	attattact**AaC**gatcttgaaa
	rev	tttcaagatc**GtT**agtaataat
D339N	fwd	gcaggtgca**AaC**attttatta
	rev	taataaaat**GtT**tgcacctgc

The positions underlined are the restriction recognition sites, the mutated codons are shown in bold, and the capital letters indicate the displaced bases from the wild type.

Enzyme and protein assays. 4-Methylumbelliferyl β-D-*N*-acetylglucosamine [4-MU-(GlcNAc); Sigma] was dissolved in *N*,*N*′-dimethylformamide at a concentration of 10 mM and stored at −20 °C. Nag activity was measured in 10 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM 4-MU-(GlcNAc) as a substrate according to a modification of the procedure of O'Brien.[17] Reaction mixtures (1 ml) containing 0.5 unit of the respective enzymes were incubated at 37 °C for 10 min, and the amount of 4-methylumbelliferone released from the substrate was spectrofluorometrically measured with an excitation wavelength at 355 nm and an emission wavelength at 465 nm on a Shimadzu spectrofluorometer RF-1500 (Shimadzu, Tokyo). One unit of Nag activity was defined as the amount of enzyme that produces 1 μmol of 4-MU per min. Protein concentration was measured according to the method of Bradford[18] with bovine serum albumin (Sigma) as a standard.

Circular dichroism. Circular dichroism measurements

were performed using a Jasco automatic recording spectropolarimeter Model J-720M (Jasco, Tokyo). The measurements were made in 20 mM Tris–HCl buffer (pH 7.5) in a cylindrical cell with a light path of 1 mm and constant nitrogen flushing. The scan wavelength was from 200 to 250 nm. The protein concentration was about 200 μg/ml. Each spectrum was scanned once at 5 nm/min. Calculations were made according to $[\theta] = \theta^{\circ}M/100\,lc$, where M is the mean residue molecular weight (taking 110.68 for the wild type, D119N, D175N, D229N, D274N, D303N, and D329N, 110.57 for D175A and D229A, and 110.53 for E121A), θ° is the measured ellipticity in degree, l is light pass of the cell in decimeters, and c is the protein concentration in grams per cubic centimeter.[19)]

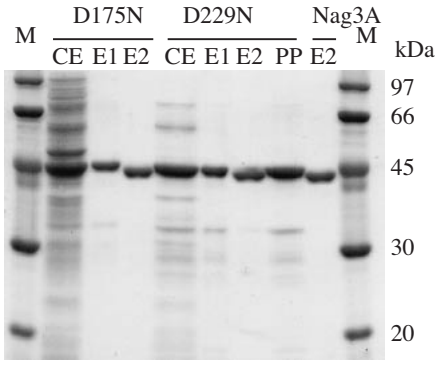

Fig. 2. SDS–PAGE of D175N, D229N, and Wild-Type Nag3A.
CE, crude enzyme; E1, eluate from HiTrap column; E2, eluate from MonoQ column; PP, precipitated protein; M, molecular mass markers (molecular masses shown at the right).

Results

Heterologous gene expression and purification of recombinant wild-type and mutant Nags

Eleven different mutant genes derived from *nag3A* were successfully constructed using the strategy described in "Materials and Methods." Sequencing of the constructs showed that all of them carried the planned mutation. The wild-type and mutant Nag proteins were purified from recombinant *E. coli* M15 cells in a one-step procedure over the HiTrap chelating HP column, and all of the purified preparations appeared to be more than 95% homogeneous as judged by SDS–GAGE. Typical examples of SDS–PAGE profiles are shown in Fig. 2. All the proteins were produced in soluble state in *E. coli* cell lysates, *i.e.*, there were no inclusion bodies formed in any of the mutants. The yield was 30 to 40 mg of each soluble Nag protein per liter of *E. coli* culture, but partial protein precipitation of mutant proteins D229A and D229N occurred during dialysis against 20 mM Tris–HCl buffer (pH 7.5) after purification on the HiTrap chelating HP column. The precipitated proteins showed migration in SDS–PAGE identical to that of the same proteins in soluble form (Fig. 2).

Enzyme activity of wild-type and mutant Nags

The wild-type and mutant Nags purified from *E. coli* were analyzed for their specific activities and K_m and V_{max} values. Specific activity was measured in 10 mM phosphate buffer (pH 7.0) containing 100 μM 4-MU-(GlcNAc) as substrate, and the K_m and V_{max} values were determined with the same substrate at a concentration from $1/2\,K_m$ to $2\,K_m$ by Lineweaver-Burk plots. As shown in Table 2, neither D175N nor D175A showed any detectable activity, strongly suggesting that Asp175 residue has an important role, probably as one of the catalytic residues in the catalysis of Nag3A. Mutant enzymes E121Q, E121A, D229N, D229A, D119N, and D303N lost almost all activity (less than 5% left), and E293Q kept about 80% of the activity of the wild-type enzyme. On the other hand, D329N showed a little higher activity than the original activity. When the K_m values of these enzymes were compared, mutants D119N, D229A, D229N, and D274N showed extremely high K_m values, 17–41 times as high as that of the wild type, two mutants in Glu121 showed 50% increases, and the other three mutants kept the K_m value of the wild type.

Table 2. Enzyme Activities, K_m and V_{max} of Wild-Type and Mutant Nags

Nag	K_m (μM)	V_{max} (μmol min^{-1} mg^{-1})	Enzyme activity Specific activity (U/mg)	Relative activity (%)
Wild type	20.3	41.3	41.3	100
D119N	349.2	2.9	2.0	4.8
E121Q	30.3	0.45	0.45	1.1
E121A	30.7	0.42	0.42	1.0
D175A	ND	ND	ND	ND
D175N	ND	ND	ND	ND
D229N	833.5	0.81	0.52	1.3
D229A	823.6	0.80	0.51	1.2
D274N	474.5	7.5	5.4	13.1
E293Q	24.5	32.6	32.6	78.9
D303N	20.8	1.7	1.7	4.1
D329N	21.5	45.1	45.1	109.2

ND, not detectable

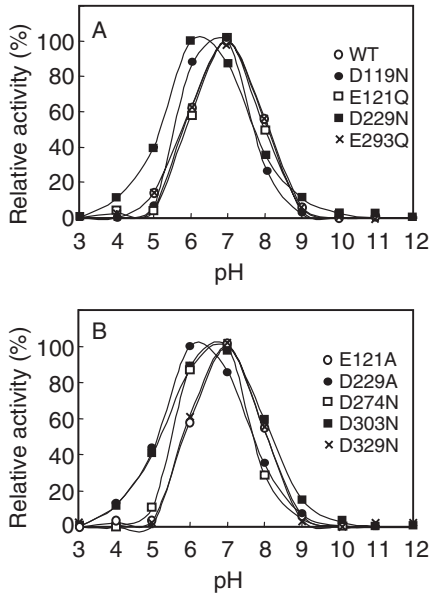

Fig. 3. Optimum pH of Wild-Type and Mutant Nags.

The enzyme activity was measured in Brittion-Robinson's universal buffer solution (40 mM phosphoric acid-40 mM boric acid-40 mM acetic acid; pH was adjusted with NaOH) at various pHs containing 0.1 mM 4-MU-(GlcNAc) as a substrate. Reaction mixtures were incubated at 37 °C for 10 min. The maximum activity was expressed as 100%. Figures are arbitrarily divided into A and B for easier identification of graphic symbols.

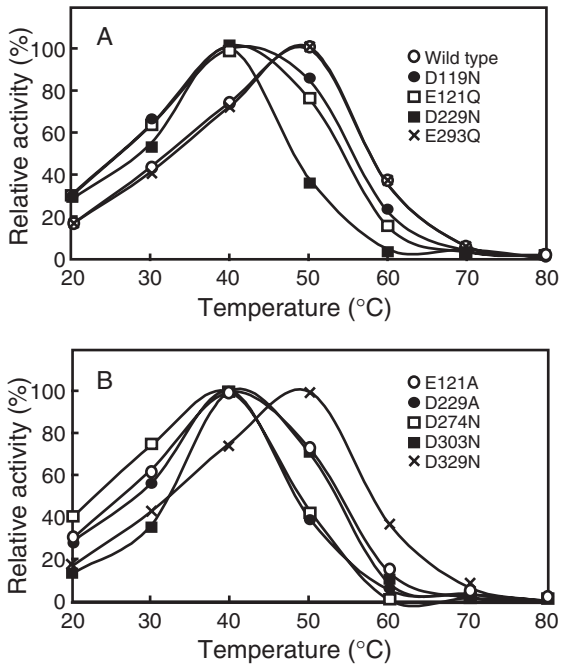

Fig. 4. Optimum Temperature of Wild-Type and Mutant Nags.

The enzyme activity was measured in 10 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM 4-MU-(GlcNAc) as a substrate. Reaction mixtures were incubated at 37 °C for 10 min. The maximum activity was expressed as 100%. Figures were arbitrarily divided into A and B for easier identification of graphic symbols.

Optimum pH and optimum temperature of wild-type and mutant Nags

The optimum pH was measured in Brittion-Robinson's universal buffer solution (40 mM phosphoric acid-40 mM boric acid-40 mM acetic acid; pH was adjusted with NaOH) at various pHs. As shown in Fig. 3, it was found that the optimum pH of mutant D229N and D229A shifted from pH 7.0 to pH 6.0, but there was no significant difference in optimum pHs of the other mutant Nags as compared with the wild-type enzyme. On the other hand, (Fig. 4), changes in their optimum temperatures were remarkable. Only mutants D329N and E293Q kept the optimum temperature of the wild-type enzyme. The optimum temperature for all the other mutant enzymes shifted from 50 °C to 40 °C.

Circular dichroism of wild-type and mutant Nags

Circular dichroism provides a direct method for determining the gross conformational properties of protein. Although there was a small difference in circular dichroism profiles among D175N, E121Q, and wild-type Nag3A, the other mutant proteins showed almost the same profiles as the wild type, as shown in Fig. 5. This means that most of the mutant proteins retain the native conformation of the wild-type enzyme Nag3A.

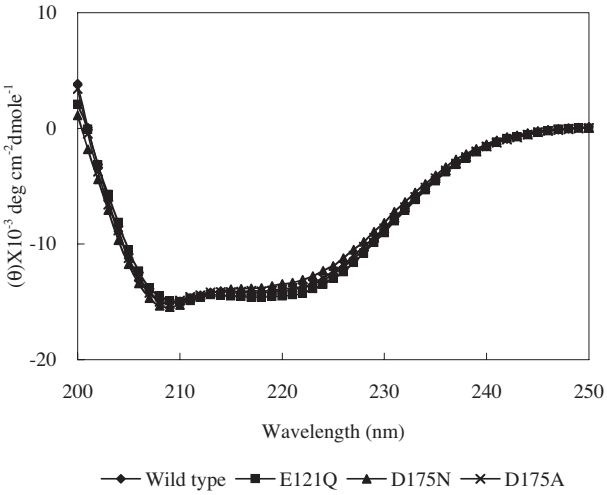

Fig. 5. Far-UV CD Spectra of Wild-Type and Mutant D175N, D175A and E121Q Nags.

Far-UV CD spectra were measured in 20 mM Tris–HCl, pH 7.5. Start wavelength 250 nm, end wavelength 200 nm, response 8 second, step resolution 0.1 nm, scan speed 5 nm/min, number of scan 1, sensitivity 50 mdeg. Mutants other than D175N, D175A and E121Q Nags gave profiles similar to the wild type.

Discussion

Family 3 of the glycoside hydrolases (http://afmb.cnrs-mrs.fr/CAZY/index.html) contains *β*-glucosidase (EC 3.2.1.21); xylan 1,4-*β*-xylosidase (EC 3.2.1.37); *β*-*N*-acetylhexosaminidase (EC 3.2.1.52); glucan 1,3-*β*-glucosidase (EC 3.2.1.58); glucan 1,4-*β*-glucosidase (EC 3.2.1.74); exo-1,3-1,4-glucanase (EC 3.2.1.-); and

α-L-arabinofuranosidase (EC 3.2.1.55). In general, the active sites of glycoside hydrolases are composed of aspartic acid or/and glutamic acid residues: one serves as catalytic nucleophile and another as catalytic proton donor. Barley β-D-glucan exohydrolase isoenzyme ExoI was the first reported structure of a family-3 glycoside hydrolase, which was determined by X-ray crystallography to be a globular protein composed of two domains. In this enzyme, Asp285 in the N-terminal domain and Glu491 in the C-terminal domain are considered to be involved in catalysis as nucleophile and proton donor, respectively.[12–14] Based on this finding, a putative active site of Nags in this family was also deduced to be Asp242 of *Vibrio furnissii* ExoII[10] and Asp303 of *C. paraputrificum* Nag3A,[6] corresponding to Asp285 of ExoI.[12] However, another active site, a putative proton donor, was not expected in single-domain enzymes, including *C. paraputrificum* Nag3A, from the information about barley ExoI, since Nag3A and other single-domain enzymes of family 3 did not contain a second domain. On the other hand, in *V. furnissii* ExoII, a single-domain enzyme composed of 329 amino acid residues, Asp242 was identified as the catalytic nucleophile by use of the mechanism-based reagent 2-acetamido-2-deoxy-5-fluoro-α-L-idopyranosyl fluoride. As described above, Asp242 of *V. furnissii* ExoII corresponds to Asp285 of barley ExoI, identified as the nucleophile.[20] We expected that a second catalytic residue existed in highly conserved Asp or Glu residues of the 430 amino acids of *C. paraputrificum* Nag3A, and we introduced mutations into conserved Asp and Glu residues.

Mutation of Asp303 into Asn did not abolish but strongly affected the catalytic activity of Nag3A, *i.e.*, the remaining activity accounted for 4.1% of the original activity of the wild-type enzyme (Table 2). This result is similar to the observation that displacement of Asp52, the catalytic nucleophile, of chicken egg white lysozyme did not result in complete loss of the original activity.[21,22] Taking previous studies about barley ExoI and *V. furnissii* ExoII into consideration, it is most plausible that Asp303 is the catalytic nucleophile of Nag3A.

When mutations were separately introduced into Glu121, Asp175, and Asp229 that were completely conserved among all Nags except barley ExoI, the mutations in all these residues drastically lowered V_{max}, suggesting that one of them was the proton donor. Neither D175N nor D175A showed any catalytic activity, although the mutations in Glu121 did not completely deaden the activity. Therefore, we assume that Asp175 is the first candidate for a proton donor and Glu121 the second candidate. Only a slight difference in circular dichroism profiles was observed between D175N and the wild type, and no difference was detected between the circular dichroism profiles of D175A and the wild type (Fig. 5). This Asp residue is not conserved in barley ExoI (Fig. 1), suggesting that the proton donor of Nag3A is completely different from that of ExoI. Although some mutations affected optimum pH and temperature of the parental enzyme, as shown in Figs. 3 and 4, these effects do not appear to have had a substantial influence on the K_m and V_{max} values of the mutant enzymes shown in Table 2, because the enzyme assay condition, *viz.*, 10 min incubation at pH 7.0 and 37 °C, was not far from optimal conditions for all the enzymes. All the mutant enzymes were optimally active at 40 °C or higher, and D229N and D229A at pH 7.0 retained about 80% of the highest activity at pH 6.0.

Asp119, Asp229, and Asp274 residues must be involved in substrate recognition and binding because mutations in these positions reduced the affinity of Nag3A for the substrate at least 17 times as judged from their K_m values. Asp229 is different from the other highly conserved Asp and Glu residues in that mutation Asp229 into Asn and Ala strongly affected various enzyme properties such as kinetic parameters, optimum temperature, and optimum pH. Furthermore, the mutant proteins D229A and D229N became unstable; *i.e.*, partial precipitation of the enzyme proteins occurred while the elutates from the HiTrap column were dialyzed against 20 mM Tris–HCl buffer (pH 7.5).

Mutations in Asp119, Glu121, Asp229, Asp274, and Asp303 not only affected the strength of catalytic activity but also decreased their optimum temperature from 50 °C to 40 °C, *i.e.*, mutations in these positions strongly affected the structural stability of the enzyme. On the other hand, Asp329 and Glu293, which are less conserved among family-3 Nags, are not important in the catalytic mechanism of Nag3A, because both D329N and E293Q mutants maintained enzymatic properties such as K_m, V_{max}, optimum pH, and optimum temperature quite similar to those of the wild-type enzyme.

In conclusion, it is quite possible that Asp303 is the catalytic nucleophile and Asp175 the proton donor of *C. paraputrificum* Nag3A. Tertiary structure analysis of Nag3A should be done to identify the catalytic residues correctly.

Acknowledgments

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References

1) Evvyernie, D., Yamazaki, S., Morimoto, K., Karita, S., Kimura, T., Sakka, K., and Ohmiya, K., Identification and characterization of *Clostridium paraputrificum* M-21, a chitinolytic, mesophilic and hydrogen-producing bacterium. *J. Biosci. Bioeng.*, **89**, 596–601 (2000).
2) Evvyernie, D., Morimoto, K., Karita, S., Kimura, T., Sakka, K., and Ohmiya, K., Conversion of chitinous wastes to hydrogen gas by *Clostridium paraputrificum* M-21. *J. Biosci. Bioeng.*, **91**, 339–343 (2001).
3) Morimoto, K., Kimura, T., Sakka, K., and Ohmiya, K.,

Overexpression of a hydrogenase gene in *Clostridium paraputrificum* to enhance hydrogen gas production. *FEMS Microbiol. Lett*., **246**, 229–234 (2005).

4) Morimoto, K., Karita, S., Kimura, T., Sakka, K., and Ohmiya, K., Cloning, sequencing, and expression of the gene encoding *Clostridium paraputrificum* chitinase ChiB and analysis of the functions of novel cadherin-like domains and a chitin-binding domain. *J. Bacteriol.*, **179**, 7306–7314 (1997).
5) Morimoto, K., Karita, S., Kimura, T., Sakka, K., and Ohmiya, K., Sequencing, expression, and transcription analysis of the *Clostridium paraputrificum chiA* gene encoding chitinase ChiA. *Appl. Microbiol. Biotechnol.*, **51**, 340–347(1999).
6) Li, H., Morimoto, K., Katagiri, N., Kimura, T., Sakka, K., and Ohmiya, K., A novel *β*-*N*-acetylglucosaminidase of *Clostridium paraputrificum* M-21 with high activity on chitobiose. *Appl. Microbiol. Biotechnol.*, **60**, 420–427 (2002).
7) Li, H., Morimoto, K., Kimura, T., Sakka, K., and Ohmiya, K., A new type of *β*-*N*-acetylglucosaminidase from hydrogen-producing *Clostridium paraputrificum* M-21. *J. Biosci. Bioeng.*, **96**, 268–274 (2003).
8) Coutinho, P. M., and Henrissat, B., The modular structure of cellulases and other carbohydrate-active enzymes: an integrated database approach. In "Genetics, Biochemistry and Ecology of Cellulose Degradation," eds. Ohmiya, K., Hayashi, K., Sakka, K., Kobayashi, Y., Karita, S., and Kimura, T., Uni Publishers, Tokyo, pp. 15–23 (1999).
9) Tsujibo, H., Fujimoto, K., Tanno, H., Miyamoto, K., Imada, C., Okami, Y., and Inamori, Y., Gene sequence, purification and characterization of *N*-acetyl-beta-glucosaminidase from a marine bacterium, Alteromonas sp. strain O-7. *Gene*, **146**, 111–115 (1994).
10) Chitlaru, E., and Roseman, S., Molecular cloning and characterization of a novel beta-*N*-acetyl-D-glucosaminidase from *Vibrio furnissii*. *J. Biol. Chem.*, **271**, 33433–33439 (1996).
11) Cheng, Q., Li, H., Merdek, K., and Park, J. T., Molecular characterization of the *β*-*N*-acetylglucosaminidase of *Escherichia coli* and its role in cell wall recycling. *J. Bacteriol.*, **182**, 4836–4840 (2000).
12) Varghese, J. N., Hrmova, M., and Fincher, G. B., Three-dimensional structure of a barley *β*-D-glucan exohydrolase, a family 3 glycosyl hydrolase. *Structure*, **7**, 179–190 (1999).
13) Hrmova, M., Gori, R. D., Smith, B. J., Vasella, A., Varghese, J. N., and Fincher, G. B., Three-dimensional structure of the barley *β*-D-glucan glucohydrolase in complex with a transition state mimic. *J. Biol. Chem.*, **279**, 4970–4980 (2004).
14) Hrmova, M., Varghese, J. N., Gori, R. D., Smith, B. J., Driguez, H., and Fincher, G. B., Catalytic mechanism and reaction intermediates along the hydrolytic pathway of a plant *β*-D-glucan glucohydrolase. *Structure*, **9**, 1005–1016 (2001).
15) Mursheda, K. A., Hayashi, H., Karita, S., Goto, M., Kimura, T., Sakka, K., and Ohmiya, K., Importance of the carbohydrate-binding module of *Clostridium stercorarium* Xyn10B to xylan hydrolysis. *Biosci. Biotechnol. Biochem.*, **65**, 41–47 (2001).
16) Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R., Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene*, **77**, 51–59 (1989).
17) O'Brien, M., and Colwell, R. R., A rapid test of chitinase activity that uses 4-methylumbelliferyl-*N*-acetyl-*β*-D-glucosaminide. *Appl. Environ. Microbiol.*, **53**, 1718–1720 (1987).
18) Bradford, M. M., A rapid and sensitive method for the quantitation of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248–254 (1976).
19) Umekawa, H., Endo, T., and Hidaka, H., A rapid separation of bovine brain S-100a and S-100b protein and related conformation studies. *Arch. Biochem. Biophys.*, **227**, 147–153 (1983).
20) Vocadlo, D. J., Mayer, C., He, S., and Withers, S. G., Mechanism of action and identification of Asp242 as the catalytic nucleophile of *Vibrio furnisii* *N*-acetyl-*β*-D-glucosaminidase using 2-acetamido-2-deoxy-5-fluoro-*α*-L-idopyranosyl fluoride. *Biochemistry*, **39**, 117–126 (2000).
21) Malcolm, B. A., Rosenberg, S., Corey, M. J., Allen, J. S., Baetselier, A. D., and Kirsch, J. F., Site-directed mutagenesis of the catalytic residues Asp-52 and Glu-35 of chicken egg white lysozyme. *Proc. Natl. Acad. Sci. USA*, **86**, 133–137 (1989).
22) Hashimoto, Y., Yamada, K., Motoshima, H., Omura, T., Yamada, H., Yasukochi, T., Miki, T., Ueda, T., and Imoto, T., A mutation study of catalytic residue Asp 52 in hen egg lysozyme. *J. Biochem.*, **119**, 145–150 (1996).