J. Biochem. 131, 87–96 (2002)

Two Glutamic Acids in Chitosanase A from *Matsuebacter chitosanotabidus* 3001 Are the Catalytically Important Residues[1]

Kumiko Shimono,* Kazutaka Shigeru,* Akiho Tsuchiya,* Noriko Itou,* Yukari Ohta,* Katsunori Tanaka,* Tsuyoshi Nakagawa,† Hideyuki Matsuda,* and Makoto Kawamukai*,[2]

**Department of Life Science and Biotechnology, Faculty of Life & Environmental Science, and †Research Institute of Molecular Genetics, Shimane University, Matsue 690-8504*

Received October 9, 2001; accepted November 2, 2001

Chitosanase is the glycolytic enzyme that hydrolyzes the glucosamine GlcN-GlcN bonds of chitosan. To determine the catalytically important residues of chitosanase A (ChoA) from *Matsuebacter chitosanotabidus* 3001, we performed both site-directed and random mutagenesis of *choA*, obtaining 31 mutants. These mutations indicated that Glu-121 and Glu-141 were catalytically important residues, as mutation at these sites to Ala or Asp drastically decreased the enzymatic activity to 0.1–0.3% of that of the wild type enzyme. Glu-141 mutations remarkably decreased kinetic constant k_{cat} for hydrolysis of chitosan, meanwhile Glu-121 mutations decreased the activities to undeterminable levels, precluding parameter analysis. No hydrolysis of $(GlcN)_6$ was observed with the purified Glu-121 mutant and extremely slow hydrolysis with the Glu-141 mutant. We also found that Asp-139, Asp-148, Arg-150, Gly-151, Asp-164, and Gly-280 were important residues for enzymatic activities, although they are not directly involved in catalysis. In addition, mutation of any of the six cysteine residues of ChoA abrogated the enzymatic activity, and Cys-136 and Cys-231 were found to form a disulfide bond. In support of the significance of the disulfide bond of ChoA, chitosanase activity was impaired on incubation with a reducing agent. Thus, ChoA from *M. chitosanotabidus* 3001 uses two glutamic acid residues as putative catalytic residues and has at least one disulfide bond.

Key words: chitosanase, *Matsuebacter chitosanotabidus*, mutation.

Chitosan is a polysaccharide consisting of β-1,4-linked D-glucosamine residues, which can be partially replaced with *N*-acetyl-D-glucosamine ones. Chitooligosaccharides produced through enzymatic hydrolysis of chitosan are used as food additives, pharmaceuticals, and elicitors in plant cell cultures. Hydrolysis of the glycosidic bonds of chitosan is catalyzed by chitosanase [EC 3.2.1.132]. It has been proposed that chitosanases can hydrolyze GlcN-GlcN, GlcN-GlcNAc, and GlcNAc-GlcN but not GlcNAc-GlcNAc bonds (*1, 2*). The ability to hydrolyze 100% deacetylated chitin is an important criterion for classification of an enzyme as a chitosanase.

Chitosanases are produced by many organisms, including actinomycetes (*3, 4*), fungi (*5–9*), plants (*10, 11*), and bacteria (*12–18*). In plants, chitosanases are believed to be used in defense against pathogenic fungi (*19*). In bacteria, chitosanases are important for maintenance of the ecological balance. The bacterial enzymes have been used to determine the chitosan hydrolysis mechanism at both the biochemical and molecular levels. Relative to the considerable knowledge on the primary structures and functions of chitinases and lysozymes, however, chitosanases are still poorly understood.

To date, chitosanase genes have been isolated from *Streptomyces* sp. N174 (*2*), *Bacillus circulans* MH-K1 (*20*), *Nocardioides* sp. N106 (*21*), *Burkholderia gladioli* strain CHB101 (*22*), *Bacillus ehimensis* EAG1 (*23*), *Fusarium solani* f sp. *phaseoli* (*24*), chlorella virus PBCV-1 (*25*), *M. chitosanotabidus* 3001 (*26*), and *Bacillus* sp. strain CK4 (*27*). The first five chitosanases exhibit primary structural similarities but the others do not. The three-dimensional structures have only been determined for the chitosanases from *Streptomyces* sp. N174 (*28*) and *Bacillus circulans* MH-K1 (*29*), which exhibit a high degree of amino acid homology. The three-dimensional structure of *Streptomyces* sp. N174 chitosanase has been shown to closely resemble that of bacteriophage T4 lysozyme (*30*).

M. chitosanotabidus 3001, a chitosanase producer, belongs to the *Proteobacterium* β-subclass and was originally isolated from soil in the Japanese city Matsue. The biochemical properties of purified *M. chitosanotabidus* 3001 chitosanase A (ChoA) have been characterized and the gene has been cloned (*26*). The immature enzyme is composed of 391 amino acids and bears an 80 amino acid signal polypeptide at its N-terminus. Mature ChoA is thus composed of 311 amino acids with a calculated molecular weight is 33,613. As shown by a study on a ChoA-GFP fusion protein (*26*), the signal sequence is necessary for the secretion of ChoA.

The catalytic residues of the chitosanases from *Streptomyces* sp. N174 and *Bacillus circulans* MH-K1 have been

[1] This work is supported by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

[2] To whom correspondence should be addressed. Phone: +81-852-32-6587, Fax: +81-852-32-6092, E-mail: kawamuka@life.shimane-u.ac.jp

Abbreviations: GlcN, glucosamine; GlcNAc, *N*-acetylglucosamine; ChoA, chitosanase A from *Matsuebacter chitosanotabidus* 3001.

determined to be a glutamic acid (Glu) and an aspartic acid (Asp) (*31*). However, the catalytic residues of other chitosanases have not been determined yet. To determine which residues of ChoA from *M. chitosanotabidus* 3001 are important in catalysis, we performed site-directed and random mutagenesis. Upon analysis of the catalytic abilities of the resulting mutants, we found that the catalytically important residues of ChoA are two glutamic acids, Glu-121 and Glu-141. Other residues were also found to indirectly affect catalysis and will be discussed.

MATERIALS AND METHODS

Materials—Restriction enzymes and other DNA-modifying enzymes were purchased from TaKaRa Biomedicals (Kyoto) and New England Biolabs. Chitosan 8B (degree of acetylation, 20%) was purchased from Funakoshi. Chitosan was obtained from San-in Kensetsu (Shimane). Chitosan-oligosaccharides $(GlcN)_{1-6}$ were purchased from Seikagaku (Tokyo). Ampicillin and 2-mercaptoethanol were purchased from Wako Pure Chemical Industries (Osaka). Isopropyl-β-D-thiogalactopyranoside (IPTG) was purchased from Nacalai Tesque (Kyoto).

Strains, Plasmids, and DNA Manipulation—*Escherichia coli* strains DH10B, DH5α and JM109 were used for the general construction of plasmids (*32*). Plasmids pBluescript SK–, KS+, pQE31, and pFLAG·ATS were used as vectors (Stratagene, Qiagen, and Eastman Kodak). Plasmids pCHOR1821 and pCHOR1821ΔSmaI contain deletion clones of the *choA* gene (*26*) and were used as positive controls. Extraction of plasmid DNA and transformation of *E. coli* were performed as previously described (*32*), and standard methods were used for the genetic manipulation of *E. coli* (*32*).

Site-Directed Mutagenesis—*choA* mutants were constructed using an LA PCR™ *in vitro* Mutagenesis kit supplied by TaKaRa Biomedicals. Site-directed mutations were introduced into four glutamic acid (E), four aspartic acids (D), one arginine (R), and four cysteines (C) residues with a mutagenesis kit. The oligonucleotides were purchased from Kurabo and are listed in Table I. Three pBS KS+ plasmids containing different deletion sequences of *choA* and cloned into different sites were used as templates for mutagenesis: pBS KS+-U935HB (935–2552 bp, cloned into the *Hin*dIII/*Bam*HI site), pBS KS+-R1821BE (1–1821 bp, cloned into the *Bam*HI/*Eco*RI site), and pBS KS+ cho Sm/B (1–1821 bp, cloned into the *Sma*I/*Bam*HI site). pBS KS+-U935HB was used to construct mutants D148A (GAC→GCC), D148E (GAC→GAG), D148N (GAC→AAC), R150A (CGC→GCC), R150K (CGC→AAA), D152N (GAC→AAC), D164G (GAC→GGC), D164E (GAC →GAA), and D164N (GAC→AAC). pBS KS+-R1821BE was used to construct mutants C307G (TGC→GGC), C315G (TGC→GGC), and C377G (TGC→GGC). pBS KS+ cho Sm/B was used to construct mutants E121A (GAG→GCG), E121D (GAG→GAC), E129A (GAG→GCG), C136G (TGC→GGC), E137A (GAA→GCA), D139A (GAC→GCC), E141A (GAG→GCG), E141D (GAG→GAC), and D152A (GAC→GCC). The plasmids carrying the mutated *choA* genes [pBS KS+-*choA* (mt)] were digested with restriction enzymes (*Sal*I/*Bam*HI, *Bam*HI/*Eco*RI, or *Sma*I/*Bam*HI). The segments were then purified from agarose electrophoresis gels and subcloned into pCHOR1821ΔSmaI. The mutated sites were verified by sequencing with an ABI PRISM™ 377 DNA Sequencer. *E. coli* DH10B was then transformed with the resulting pCHOR1821ΔSmaI (mt) plasmids. As a control *E. coli* DH10B was transformed with plasmid pCHOR1821ΔSmaI, which lacks the pCHO 3′ terminal region downstream of 1821 bp and also has a deletion in the 5′ terminal region between 262–367 bp.

TABLE I. **Synthetic oligonucleotide primers used for site-directed mutagenesis.** Double underlines indicate mutated sites.

Mutation	Oligonucleotide sequence (5′-3′)
E121A	AC GCC TAC CCC GCG AAC GGC ACG AC
E121D	AC GCC TAC CCC GAC AAC GGC ACG AC
E129A	CC AAC TAC CAG GCG GTC GGC CCC TG
E137A	GG CGC TAT TGC GCA GTC GAC TAC GA
D139A	AT TGC GAA GTC GCC TAC GAG GCG GC
E141A	AA GTC GAC TAC GCG GCG GCG CAA GG
E141D	AA GTC GAC TAC GAC GCG GCG CAA GG
D148A	GCC GCG GTA GGC GGA GAT CCC
D148N	CCG CG GTA GTT GGA GAT CCC T
D148E	C GCC GCG GTA CTC GGA GAT CC
R150A	A GGT GTC GCC GGC GTA GTC GGA
R150K	A GGT GTC GCC TTT GTA GTC GGA
D152A	AC TAC CGC GGC GCC ACC TTC GGT CG
D152N	CCG AA GGT GTT GCC GCG GTA G
D164G	A GTC GGG GAA GCC GCC CAC CGT
D164E	A GTC GGG GAA TTC GCC CAC CGT
D164N	TCG GGG GAA GTT GCC CAC CGT G
C136G	CCC TGG CGC TAT GGC GAA GTC GAC TAC
C307G	AGG CT GCC GCC GTT GCC GGC C
C315G	GTG GC GAA GCC GGT CTC GGC G
C377G	CTG A CGT CGG GCC TTT CCC GCT G

Random Mutagenesis—Ten micrograms of purified plasmid pCHOR1821 was mixed with 500 μl of a hydroxylamine solution (1 M hydroxylamine HCl, 0.45 M NaOH, pH 7.0, stored on ice) in a microfuge tube and then incubated at 37°C for 20 h. The reaction was stopped by adding 10 μl of 5 M NaCl, 50 μl of 1 mg/ml BSA, and 1 ml of 100% ethanol, after which the plasmid was kept at –80°C for 10 min. The plasmid was then precipitated in a microfuge and resuspended in 100 μl of TE solution [25 mM Tris-HCl (pH 8.0), 10 mM EDTA]. Ten microliters of 3 M sodium acetate (pH 7.0) and 250 μl of 100% ethanol were then added. After 10 min at –80°C the sample was re-precipitated by centrifugation. The pellet was allowed to air-dry and then resuspended in 100 μl of TE (pH 8.0). This DNA was used for the transformation of *E. coli* DH10B. Single colonies were inoculated into 96-well plates containing LB medium supplemented with 50 μg/ml ampicillin and then incubated at 37°C without agitation.

Measurement of Enzymatic Activity of ChoA Mutants on Colloidal Chitosan Medium—The colloidal chitosan medium consisted of 0.4% colloidal chitosan, 0.05% yeast extract, 0.2% K_2HPO_4, 0.1% KH_2PO_4, 0.07% $MgSO_4 \cdot 7H_2O$, 1.05% NaCl, 0.05% KCl, 0.01% $CaCl_2$, and 1.5% agar. Fifteen microliters of culture medium containing each *E. coli* DH10B transformant was spotted onto the colloidal chitosan medium, followed by incubation for 2–4 days at 37°C. The absence of clearing halos around transformant colonies indicated the presence of mutant enzymes that had lost their enzymatic ability. Plasmids pCHOR1821 and

pCHOR1821ΔSmaI contain deletion clones of the *choA* gene (*26*) and were used as positive controls.

Expression of ChoA Assayed by Western Blotting—SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) on a 12.5% acrylamide gel was performed as described by Laemmli (*33*). To assess ChoA expression, the ChoA proteins on the SDS–polyacrylamide gel were first blotted onto a PVDF membrane (Immobilon-PSQ; pore size, 0.45 μm. IPVH 304FO, Millipore) by electroblotting performed according to the protocol instructions (Nippon Millipore). The membrane-bound conjugate was detected with an ECL™ system according to the manufacturer's recommendations (Amersham Pharmacia Biotech). The rabbit antibody against chitosanase was custom-made by TaKaRa Biomedicals. The secondary antibody was an EIA grade affinity-purified goat anti–rabbit IgG (H+L) horseradish peroxidase conjugate and was purchased from Bio-Rad (USA).

Recombinant FLAG-ChoA fusion proteins (wild type and mutants) were produced by transforming *E. coli* DH5α with pFLAG-ATS-*choA*. In this system, the FLAG tagged ChoA protein was highly produced from the strong *tac* promoter. Bacterial cultures were grown in LB medium supplemented with 50 μg/ml ampicillin at 30°C until the A_{600} reached 0.3–0.5. Subsequently, protein expression was induced with IPTG for 3 h to reach a final concentration of 0.5–1 mM. Cells were harvested by centrifugation (950 ×*g*, 10 min), and the cell pellets were washed twice with 10 mM Tris-Maleate (Tris and maleic acid; pH adjusted to 6.2 with 1 N NaOH) and 30 mM NaCl buffer, and stored at –80°C until further use. Wild type and mutant FLAG-ChoA fusion proteins, expressed as soluble proteins by *E. coli*, were obtained as cell extracts by ultrasonification of the cell pellets of 10 ml bacterial cultures centrifuged and resuspended in 1 ml of 10 mM Tris-Maleate buffer. After sonification, the samples were centrifuged. The supernatants were used for enzyme assays and Western blotting.

Enzyme Assays and Kinetics—The amount of protein was assessed with a protein assay kit (Bio-Rad) according to the manufacturer's instructions. The chitosanase activity of each cell lysate was determined with colloidal chitosan as a substrate by the modified Schales method (*34*). One unit of chitosanase activity was taken as the amount of enzyme that produced 1 μmol of reducing sugars (expressed as glucosamine equivalents) per minute.

The kinetic constants ranged from 0.06 to 1.5 mg/ml chitosan. Wild type 6× His-ChoA and some mutant 6× His-ChoA proteins (E121A, E121D, D139A, E141A, E141D, D164G, D164E, and D164N) were prepared using a Ni-NTA column, as described below. Wild-type chitosanase was used at a concentration of 100 μg/ml. Mutant proteins were used at concentrations that gave overall hydrolysis levels equivalent to that of the wild-type control. Reaction mixtures containing the wild type, D164E and D164N enzymes were incubated at 30°C for 60 min, those containing D139A enzymes were incubated at 30°C for 120 min, and those containing E121A, E121D, E141A, E141D, and D164G enzymes were incubated at 30°C for 180 min. Liberation of reducing sugars was measured as above. K_m and k_{cat} values were obtained from direct linear plots.

Circular Dichroism Measurement of ChoA Mutants—CD spectra were recorded with a Jasco J-720 spectropolarimeter. The spectra were obtained at a protein concentration of 4.2 μM in phosphate-buffered saline, pH 6.2, at 25°C using a 0.1-cm path length quartz cuvette (Hellma, 121.021-QS). Each spectrum was accumulated from 3 scans between 200 and 250 nm, with a scan rate of 60 nm/min.

Time Course of Enzymatic Degradation of $(GlcN)_6$—The substrate, $(GlcN)_6$, was dissolved in 10 mM phosphate buffer, pH 7.0, to give a 15 mM solution. The purified wild-type or a mutant chitosanase (0.4–1.2 μg) was added to the substrate solution, and then the reaction mixture was incubated at 40°C. After an appropriate reaction time, a portion of the reaction mixture was withdrawn and boiled for 3 min in order to terminate the enzymatic reaction. The reaction products were analyzed by HPLC on a μBondapak NH_2 column (8 × 100 mm, Waters) with acetonitrile:water = 65:35 as the solvent system using a Shimadzu RID-10A differential refractometer at room temperature and a flow rate of 2 ml/min. $(GlcN)_n$ product concentrations were calculated from peak areas in the HPLC profiles using the standard curves obtained for pure saccharide solutions.

Product Analysis for Chitosanase Treated with 2-Mercaptoethanol—The chitosanase activity of wild type 6× His-ChoA, which had been treated with 2-mercaptoethanol, was measured using chitosan oligosaccharide $(GlcN)_5$ as a substrate. 10 mM 2-mercaptoethanol was added to the purified 6× His-ChoA protein in phosphate buffer (pH 7.0), and then the mixture was incubated at 40°C for 20 min. 35 mM iodoacetoamide was added to the reaction mixture to a final concentration of 3.5 mM, followed by incubation at 37°C for 15 min, and then 12.5 μl of 60 mM $(GlcN)_5$ was added. The mixtures were incubated at 40°C for 30 min. The reactions were stopped by boiling for 3 min. As a control, the enzyme that had been boiled for 10 min was used in the same reaction. The products of the enzymatic hydrolysis of substrates were analyzed by HPLC.

Expression and Purification of Cysteine Mutants—For the production of recombinant wild type and mutant 6× His-ChoA fusion proteins, *E. coli* JM109 was transformed with pQE31-*choA*. Bacterial cultures were grown in LB medium supplemented with 50 μg/ml ampicillin at 30°C until the A_{600} reached 0.3–0.5. Protein expression was then induced with IPTG for 3 h, which yielded a final concentration of 1 mM.

Cells were harvested by centrifugation (950 ×*g*, 10 min) and the cell pellets were stored at –80°C until further use. For purification of the wild type and mutant 6× His-ChoA fusion proteins, which are expressed as insoluble proteins by *E. coli*, a denaturing/refolding protocol (Qiagen) was used. The eluants were pooled and dialyzed overnight against 20 mM Tris-HCl (pH 8.0) at 4°C. The dialyzed solutions was concentrated with Centriprep 30 or Microcon 30 (Amicon).

Analytical SDS-PAGE of Cysteine Mutants—The purified 6× His-ChoA fusion proteins were subjected to SDS-PAGE, in the presence or absence of 1 μM reductant DTT. The denatured samples were suspended in 8× sample-migration buffer [16% SDS, 80% glycerol, 0.006% BPB, 0.5 M Tris-HCl (pH 6.8)] containing DTT, after which the samples were heated at 100°C for 5 min and then left on ice for 10 min. The native samples were suspended in 8× sample-migration buffer and then left on ice for 10 min. Supernatants of all samples were obtained by centrifugation for 1 min at 15,000×*g* and analyzed by SDS-PAGE on a 10% acrylamide gel. The gels were run at 20 mA until the

tracker dye (bromophenol blue) ran off the gel (approximately 5 h). The gels were stained with Coomassie Brilliant Blue R in 50% methanol and 10% acetic acid, and destained in 25% methanol and 10% acetic acid.

RESULTS

Identification of Putative Catalytic Sites in ChoA by Sequence Homology Analysis—Various mutants of ChoA from *M. chitosanotabidus* 3001 were constructed by site-directed and random mutagenesis for determination of the functional domains of this enzyme. First, however, a putative catalytic center was identified by comparing the sequence of ChoA with those of the chitosanases from *Streptomyces* sp. N174 (*2*), *Bacillus circulans* MH-K1 (*20*), *Nocardioides* sp. N106 (*21*), *Fusarium solani* f. sp. *phaseoli* (*24*), and chlorella virus PBCV-1 (*25*). The homologies of these chitosanases with ChoA were 13.0, 22.6, 22.9, 20.3, and 22.6%, respectively. Despite these low overall homologies, three amino acids (Glu-121, Asp-148, and Arg-150), which include the catalytic region in the chitosanases from *Streptomyces* sp. N174 and *Bacillus circulans* MH-K1,

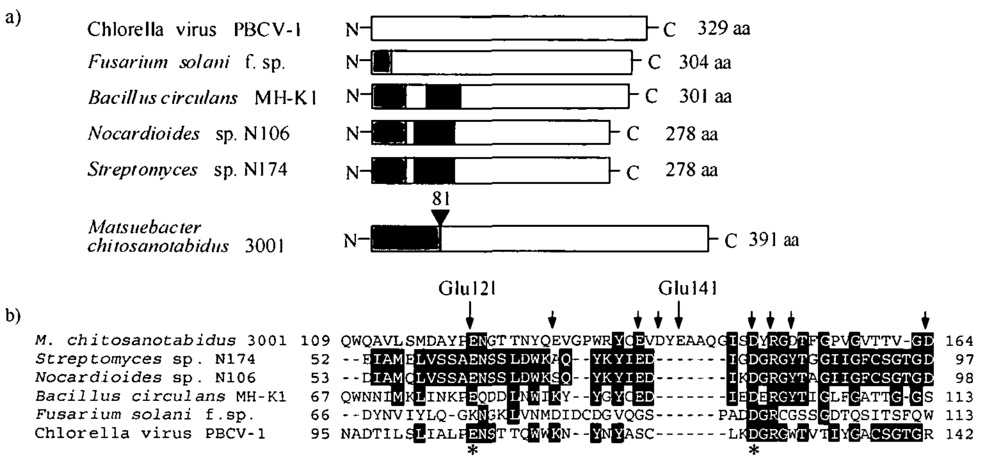

Fig. 1. **Comparison of the known amino acid sequences of chitosanases.** (a) Shaded bars indicate signal regions, and black bars conserved catalytic centers. 81 indicates the site of mature *M. chitosanotabidus* 3001 ChoA. (b) Asterisks (*) indicate the catalytic residues of the *Streptomyces* sp. N174 and *Bacillus circulans* MH-K1 chitosanases. Residues 121, 129, 137, 139, 141, 148, 150, 152, and 164 are the amino acids of *M. chitosanotabidus* 3001 ChoA that we mutagenized by site-directed mutagenesis (→). Filled boxes indicate the residues conserved in *Streptomyces* sp. N174 chitosanase and more than one of the six sequences.

TABLE II. **Mutation sites of ChoA mutants and enzymatic activity on chitosan medium of ChoA mutants expressed by *E. coli* transformants.** Halo formation indicates the size of clearing halos around colonies on the minimum medium containing 0.4% chitosan. Asterisks (*) show mutants produced on random mutagenesis.

Mutant	121	129	136	Mutation site																											164	231	307	315	370	377aa	
Wild type	E --	E --	C	E	V	D	Y	E	A	A	Q	G	I	S	D	Y	R	G	D	T	F	G	P	V	G	V	T	T	V	G	D --	C --	C --	C --	C --	C	++++
E121A	A																																				–
E121D	D																																				–
E129A		A																																			++
E137A				A																																	+++
D139A						A																															+
E141A								A																													–
E141D								D																													–
D148A															A																						+
D148E															E																						+++
D148N															N																						+
R150A																	A																				–
*R150H																	H																				–
R150K																	K																				+++
D152A																			A																		+++
D152N																			N																		+
D164G																															G						–
D164E																															E						++
D164N																															N						–
C136G			G																																		±
*C231Y																																Y					–
C307G																																	G				–
C315G																																		G			±
*C340Y																																			Y		–
C377G																																				G	–

could be aligned (Fig. 1). These three amino acids, together with other aspartic acid and glutamic acid residues around this region (Glu-129, Glu-137, Asp-139, Glu-141, Asp-152, and Asp-164) were then changed by site-directed mutagenesis to some other amino acids, as summarized in Table II.

Chitosanase Activity of Mutant ChoA Produced by Site-Directed Mutagenesis—The chitosanase activities of the mutants were first estimated by assessing the clearing halos around colonies of the *E. coli* DH10B transformants grown with 0.4% chitosan (Fig. 2A and Table II). Clearing halos were absent around colonies with the E121A, E121D, E141A, E141D, R150A, R150H, D164G, and D164N mutations, and were reduced to some extent around colonies carrying the E129A, E137A, D139A, D148A, D148N, D152N, and D164E mutations. The intensities of the clearing halos of the other mutants (D148E, R150K, and D152A) resembled that of the wild-type. Expression of mutant proteins was assessed by Western blotting of cell extracts with an anti-chitosanase rabbit serum (representative results are shown in Fig. 2B). Since the activities of the original mutant clones were too low to be measured, all *choA* mutants were recombined with pFLAG-ATS, allowing ChoA tagged with FLAG to be expressed at high levels under the *tac* promoter. The enzymatic properties, namely specific and relative activities, of the crude cell extracts were then determined using chitosan 8B (degree of acetylation, 20%) as a substrate (Table III). The Glu-129, Asp-152, and D148A mutants had nearly normal chitosanase activity. The E137A, D148E, and D148N mutants retained chitosanase 20–35% of the wild type activity level, while the three Asp-164 mutants exhibited 0.1–2.1% of the wild type level. The chitosanase activities of the R150A and R150H mutants was less than 0.5% of the wild type activity level, but R150K retained some activity (3.2%). All mutations at Glu-121 and Glu-141 severely affected the activity.

Kinetic Analysis of Mutant ChoA Chitosanase Activity—The clearing halo and crude enzyme activity results described above indicated that Glu-121, Glu-141, and Asp-164 are important residues for catalysis. Then, we next determined kinetic constants k_{cat} and K_m for the hydrolysis of chitosan 8B by the purified wild type and mutant ChoAs (Table IV). Because the D139A mutant expressed a low level of protein in *E. coli* DH5α (Table III), we also analyzed this mutant for kinetic constants k_{cat} and K_m. The wild type and mutant ChoAs (E121A, E121D, D139A,

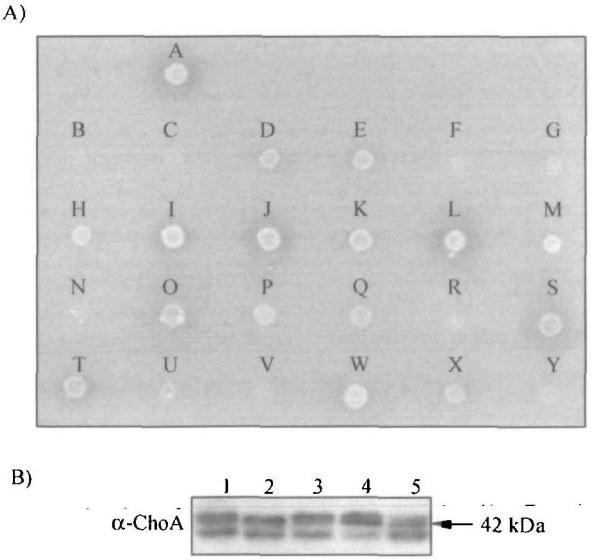

Fig. 2. **Enzymatic activity on chitosan medium of ChoA mutant enzymes expressed by transformed *E. coli*.** (A) *E. coli* carrying plasmid pCHOR1821ΔSmaI (wild type) and ChoA mutants of it generated by site-directed and random mutagenesis. Colonies were grown for 3–4 days at 37°C. A, wild type; B, E121A; C, E121D; D, E129A; E, E137A; F, E141A; G, E141D; H, D139A; I, D148A; J, D148E; K, D148N; L, D152A; M, D152N; N, D164G; O, D164E; P, D164N; Q, R150A; R, R150H; S, R150K; T, C136G; U, C231Y; V, C307G; W, C315G; X, C340Y; and Y, C377. (B) Western blot analysis of ChoA mutant proteins. Lane 1, recombinant wild type; lane 2, E121A; lane 3, E121D; lane 4, E141A; and lane 5, E141D. 5 μg of protein was loaded in each lane.

TABLE III. **Chitosanase activity of crude extracts of *E. coli* cells expressing the wild type and ChoA mutants with a FLAG tag under the *tac* promoter.** Chitosan 8B was used as the substrate. Chitosanase activity was measured as the amount of reducing sugars produced. Asterisks (*) show mutants produced on random mutagenesis. Each experiment was repeated three times. [a]The relative amount of loaded protein was estimated by Western blotting and NIH imaging. [b]Relative activity was calculated after adjustment for the amount of the ChoA protein.

Enzyme	Specific activity (U/mg protein)	Western blot[a]	Relative activity[b] (%)
Wild type	2.62 ± 0.51	1.00	100.00
E121A	0.002 ± 0.001	0.96	0.08
E121D	0.002 ± 0.002	0.90	0.10
E129A	1.94 ± 0.20	0.62	102.18
E137A	0.80 ± 0.18	0.94	32.37
D139A	0.09 ± 0.02	0.06	59.32
E141A	0.009 ± 0.005	0.94	0.35
E141D	0.004 ± 0.004	0.84	0.19
D148A	1.56 ± 0.58	0.72	82.17
D148E	0.26 ± 0.05	0.47	20.94
D148N	0.82 ± 0.10	0.91	34.43
R150A	0.009 ± 0.003	0.54	0.60
*R150H	0.007 ± 0.007	0.73	0.39
R150K	0.063 ± 0.010	0.75	3.20
D152A	1.74 ± 0.41	0.78	85.67
D152N	2.05 ± 0.36	0.95	82.16
D164G	0.001 ± 0.001	0.30	0.13
D164E	0.032 ± 0.021	0.57	2.15
D164N	0.021 ± 0.015	0.50	1.62
C136G	0.001 ± 0.001	0.73	0.05
*C231Y	0.008 ± 0.006	0.11	2.59
C307G	0.008 ± 0.003	0.68	0.47
C315G	0.006 ± 0.004	0.33	0.69
*C340Y	0.012 ± 0.006	0.61	0.75
C377G	0.028 ± 0.009	0.83	1.28

TABLE IV. **Kinetic parameters of the purified wild type and mutant ChoA proteins.** The substrate was chitosan 8B. The reaction mixtures containing the wild type, D164E and D164N enzymes were incubated at 30°C for 60 min, those with the D139A enzymes at 30°C for 120 min, and those with the E141A and E141D enzymes at 30°C for 180 min. [a]Each experiment was repeated three times, and numbers are presented with standard deviation.

Enzyme	Specific activity[a] (U/mg protein)	K_m (mg/ml)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} $(mg/ml)^{-1}$)
Wild type	121.224 ± 8.15	0.475 ± 0.04	150.79	329.96
D139A	0.660 ± 0.004	0.292 ± 0.05	0.974	3.336
E141A	0.019 ± 0.01	0.089 ± 0.02	0.058	0.652
E141D	0.086 ± 0.01	0.097 ± 0.03	0.074	0.763
D164E	3.591 ± 0.28	1.485 ± 0.32	7.026	4.731
D164N	0.922 ± 0.10	0.102 ± 0.01	0.852	8.353

E141A, E141D, D164G, D164E, and D164N) bearing the histidine tag were purified using a Ni-NTA column. But we could not determine the kinetic parameters of the E121A, E121D, and D164G enzymes because these mutants do not retain measurable activities. Substitution of the Glu-141 residue also drastically impaired the catalytic activity, since the k_{cat} values of mutants E141A and E141D were 1/2,600 and 1/2,040, respectively, of the wild-type value. While the activity of the D164G enzyme was undetectable, the D139A, D164E, and D164N substitutions led to lower than wild type but still significant k_{cat} values. The D139A, D164E and D164N mutants also exhibited lower k_{cat}/K_m values than the wild type, but higher ones than the Glu-141 mutants. Considering the relatively moderate effect of these substitutions, we thought that the Asp-139 and Asp-164 residues are not essential for ChoA activity. It is clear, however, that Glu-121 and Glu-141 are critically important residues.

CD Spectra for Wild-Type and Mutant ChoAs—To eliminate the possibility that the reduced activity of chitosanases mutated at Glu-121 and Glu-141 residues was the result of incorrect folding of the mutated proteins, we obtained CD spectra. CD spectra in the UV region are commonly used to probe for alterations in the tertiary structure, especially if aromatic residues are near the mutated residues in the three-dimensional structure. The CD spectra were identical, within experimental error, for the wild type, E121A, E141A, D164G, and D139A (Fig. 3). We also obtained spectra for E121D, E141D, D164E, and D164N mutants, and they were essentially same as that of the wild type (data not shown).

Chitohexose Hydrolysis by Purified Mutant ChoAs—To further confirm the importance of residues Glu-121 and Glu-141, we measured the activities of mutant enzymes using the six oligomer of GlcN. The profiles of products gen-

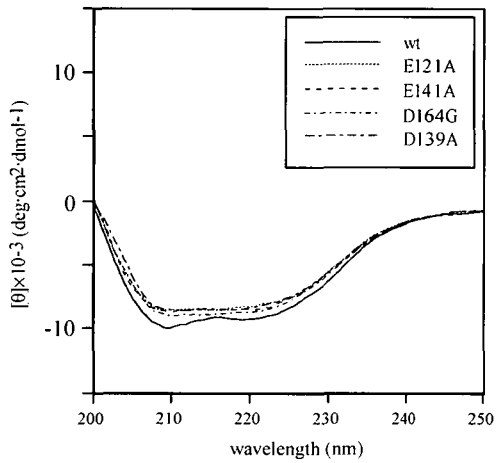

Fig. 3. **CD spectra of the wild-type and mutant forms of ChoA.** Spectra were generated with 4.2 μM protein samples and a 0.1-cm path length. The abscissa is in units of $[\theta] \times 10^{-3}$ (deg·cm^2·dmol^{-1}), using the molar concentration of amino acids present.

TABLE V. **Mutants obtained on random mutagenesis with hydroxylamine.** Asterisks (*) indicate a termination codon. [1]This mutant contained a synonymous mutation at 762.

Mutant	Mutation site	Base number
E141K[1]	C → T	762
	G → A	949
D100N	G → A	826
R150H	G → A	977
G151D	G → A	980
C231Y	G → A	1220
G280D	G → A	1367
W327*	G → A	1508
C340Y	G → A	1547
W341*	G → A	1551
Q364*	C → T	1618

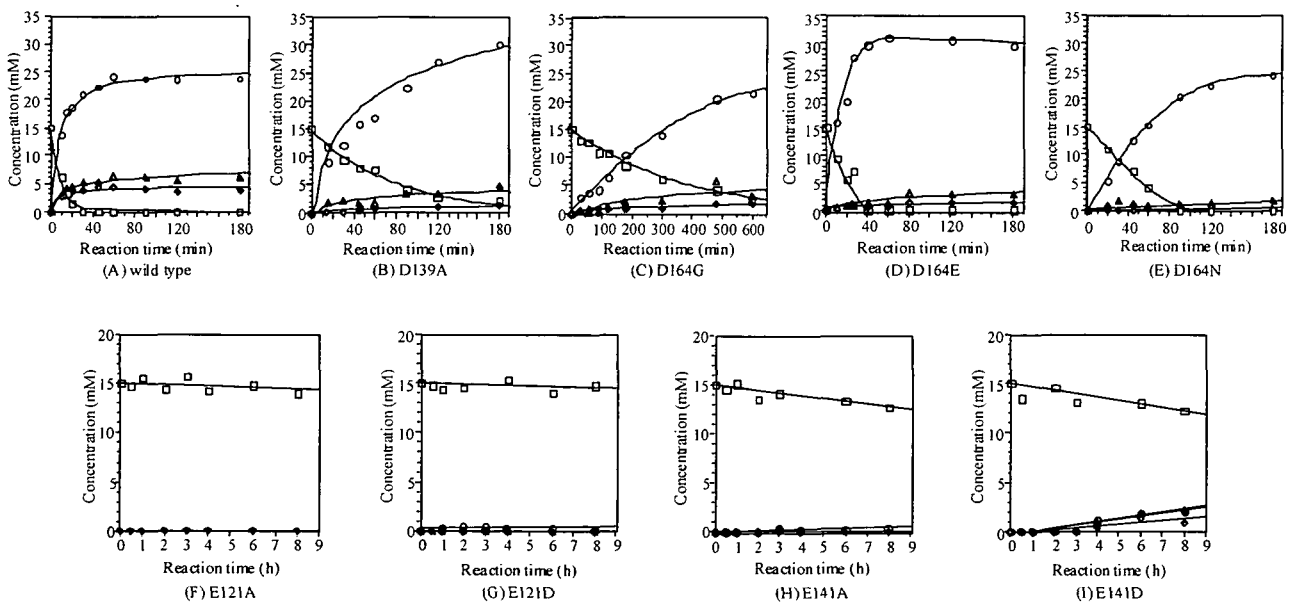

Fig. 4. **Time courses of $(GlcN)_6$ degradation by the wild-type and mutant ChoAs.** The $(GlcN)_n$ concentrations were calculated from the peak areas in HPLC profiles. Open triangles, $(GlcN)_2$; open circles, $(GlcN)_3$; open diamonds, $(GlcN)_4$; open squares, $(GlcN)_6$. Enzymes were reacted with 15 mM $(GlcN)_6$ in 10 mM phosphate buffer, pH 7.0, at 40°C.

erated from $(GlcN)_6$ as a substrate for the Glu-121, Asp-139, Glu-141, and Asp-164 mutants were analyzed (Fig. 4). The wild-type chitosanase produced abundant $(GlcN)_3$, and lesser amounts of $(GlcN)_2$ and $(GlcN)_4$ from $(GlcN)_6$ within 30 min. The product distribution did not change even when the D139A, D164G, D164E, and D164N mutant chitosanases were used, although the overall reaction speed was affected by each of the mutations. To hydrolyze most of the $(GlcN)_6$, the D139A, D164G, D164E, and D164N mutants took 180, 600, 40, and 90 min, respectively. It is now clear that the E121A and E121D mutants do not retain enzymatic activity, and that the E141A and E141D mutants retain extremely low activity.

Random Mutagenesis—To examine the existence of important residues other than Glu-121 and Glu-141 of ChoA in catalysis, random mutagenesis of ChoA with hydroxylamine was performed. *E. coli* DH10B was transformed with randomly mutagenized plasmid pCHOR1821. Mutants not forming clearing halos were then identified and isolated, and the mutation site(s) were determined by DNA sequencing. Ten mutants were found: D100N, E141K, R150H, G151D, C231Y, G280D, W327*, C340Y, W341*, and Q364* (* denotes a termination codon) (Table V). All mutations were due to nucleotide changes of C→T or G→A, which is a known property of hydroxylamine. All mutants produced the ChoA protein, as shown by Western blotting with chitosanase antiserum, but W327*, W341*, and Q364* had low molecular weights relative to the wild type (data not shown). Because the W327*, W341*, and Q364* mutants synthesized imperfect proteins, no chitosanase activity was detected. Random mutagenesis produced the Arg-150 and Glu-141 mutants, which had already been produced by site-directed mutagenesis and had already been analyzed (see Fig. 1 and Table II). The isolation of Arg-150 and Glu-141 mutations by random mutagenesis supports the reliability of the results of site-directed mutagenesis. Two Gly mutations at positions 151 and 280 obtained on random mutagenesis are novel sites of importance and will be briefly discussed later. That the C231Y and C340Y mutants had poor enzymatic abilities suggests that cysteine residues might be important for ChoA activity and consequently we changed all six ChoA cysteine residues to glycines by site-directed mutagenesis. None of the mutants formed halos on the colloidal chitosan medium (Fig. 2A), but all expressed ChoA, as determined by Western blotting (Fig. 2B).

Effect of a Reducing Agent on Chitopentamer Hydrolysis by ChoA—If the disulfide bond is important for ChoA activ-

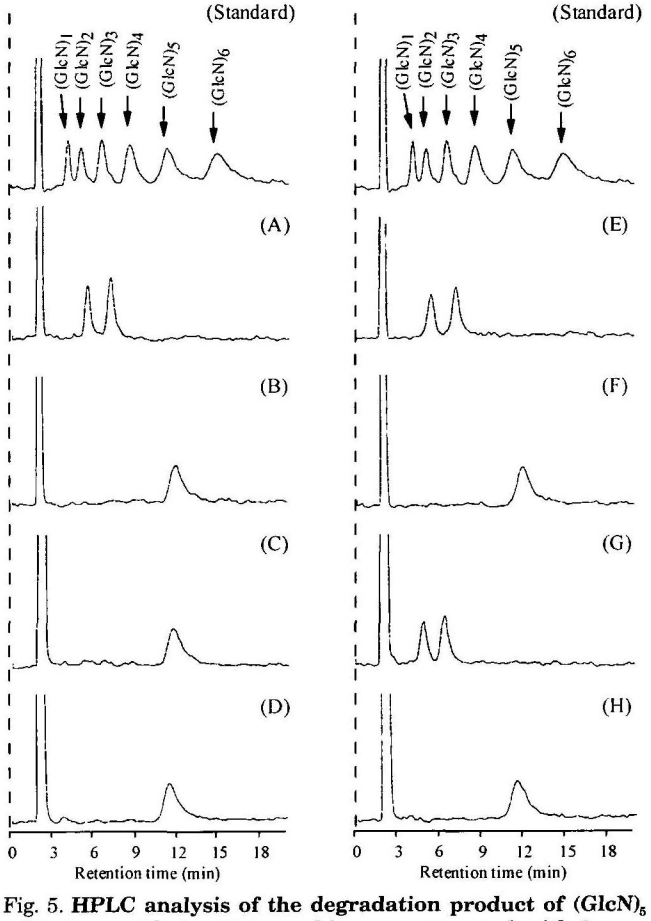

Fig. 5. **HPLC analysis of the degradation product of $(GlcN)_5$ with the purified wild-type chitosanase treated with 2-mercaptoethanol.** The chitosanase activities of wild type 6× His-ChoA treated (C, D) and not treated (A, B) with 2-mercaptoethanol were measured using chitosan oligosaccharide $(GlcN)_5$ as a substrate. As a control, the activities of *Streptomyces* sp. N174 chitosanase treated (G, H) and not treated (E, F) with 2-mercaptoethanol were measured. The enzymes that had been boiled (B, D, F, H) for 10 min were used in the same reaction as a negative controls.

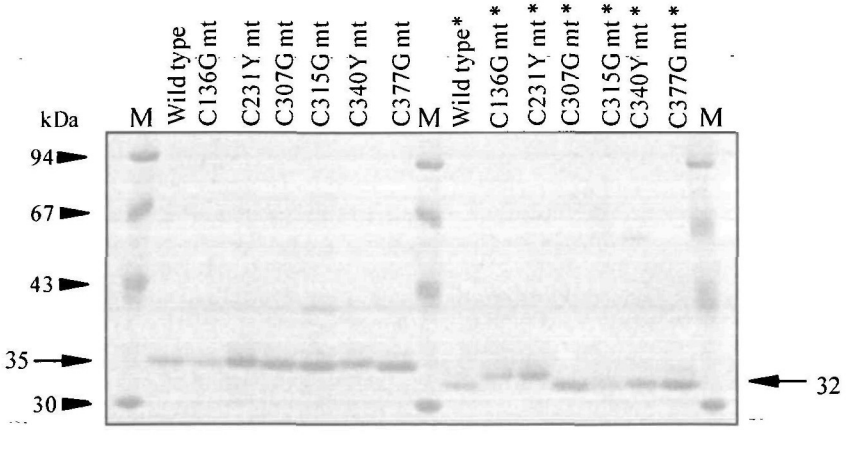

Fig. 6. **Band patterns of the native and reduced 6× His-ChoA mutant proteins on SDS-PAGE.** Samples were run towards the anode in a 10% SDS–polyacrylamide gel at 20 mA. Asterisks (*) indicate native samples prepared in 8× sample-migration buffer. The other samples were denatured ones prepared in 8× sample-migration buffer containing DTT.

ity, the ChoA protein must be affected by a reducing agent such as 2-mercaptoethanol. The products of the enzymatic hydrolysis of a substrate, $(GlcN)_5$, with and without treatment with 2-mercaptoethanol were analyzed by HPLC (Fig. 5). As a result, the ChoA protein treated with 2-mercaptoethanol did not hydrolyze $(GlcN)_5$ (Fig. 5C), meanwhile ChoA and *Streptomyces* sp. N174-chitosanase without treatment with 2-mercaptoethanol, and *Streptomyces* sp. N174-chitosanase treated with 2-mercaptoethanol hydrolyzed $(GlcN)_5$ to produce $(GlcN)_2$ and $(GlcN)_3$ (Fig. 5, A, E, and G). The enzyme activities of the boiled ChoA protein and *Streptomyces* sp. N174-chitosanase were abolished and so they did not hydrolyze $(GlcN)_5$ (Fig. 5, B, D, F, and H). These results indicated that the ChoA protein, but not *Streptomyces* sp. N174-chitosanase, was affected by a reducing agent, supporting the importance of the disulfide bond formation of ChoA.

Analytical SDS-PAGE of Cysteine Mutants of ChoA— That all cysteine mutants of ChoA were important for catalysis suggests that a disulfide bond between the cysteines may be necessary to maintain the ChoA structure required for enzyme activity. To determine which of the cysteine mutations eliminated the disulfide bonding in ChoA, the wild type and mutant ChoA proteins were treated with or without a reducing agent (DTT) and then subjected to SDS-PAGE. The wild type and mutant ChoA proteins prepared with DTT all gave a single band corresponding to an approximate molecular weight of 35,000 (Fig. 6). In the absence of DTT, the wild type, C307G, C315G, C340Y, and C377G mutants exhibited approximate molecular weights of 32,000, while in contrast the C136G and C231Y mutants continued to run to the 35,000 position (asterisks in Fig. 6). The protein migration of the C136G or C231Y mutants was equivalent whether DTT was present or not, indicating that the normal disulfide bond formation in ChoA is disrupted in these mutants, and that the Cys-136 and Cys-231 residues form a disulfide bond in the wild type enzyme.

DISCUSSION

The catalytic sites of *Streptomyces* sp. N174 chitosanase have been found to be Glu-62 and Asp-80 by site-directed mutagenesis (*31*), while Glu-79 and Asp-97 were found, from the three-dimensional structure, to be the catalytic sites of *B. circulans* MH-K1 chitosanase (*29*). When the *M. chitosanotabidus* 3001 ChoA amino acid sequence was compared with those of other chitosanases, three ChoA amino acids, Glu-121, Asp-148, and Arg-150, were found to be conserved, except in the one from *Fusarium* (Fig. 1). Upon site-directed mutagenesis and enzymatic function testing by means of various assays, however, the mutation of only one of these residues, Glu-121, consistently had a severely detrimental effect on catalysis. Replacement of Glu-121 with alanine completely eliminated the chitosanase activity, indicating that the Glu-121 carboxyl group is essential. That the carboxyl group of Glu-121 could also not be substituted by that of aspartic acid indicated that the relative disposition of the Glu-121 carboxyl group as to a substrate is critical for catalytic activity (Tables III and IV). Site-directed mutagenesis was also performed on six other Glu and Asp residues around the putative catalytic site determined on sequence comparison, and enzyme function analysis showed that Glu-141 is probably also a catalytic residue. The mutation of Glu-141 to aspartic acid severely impaired the catalytic activity and drastically reduced the kinetic parameters. This effect was obvious when the six oligomer of chitosan was used as a substrate (Fig. 4). However, while the carboxyl group of Glu-141 appeared to be essential it is not as critical as in the case of Glu-121 (Fig. 4). Nevertheless, it appears that Glu-121 and Glu-141 are directly involved in the catalytic mechanism of chitosanase.

In most glycosyl hydrolases, the catalytic site appears to involve the carboxyl group of an amino acid, as the catalytic site(s) of many lysozymes, chitinases and chitosanases have been shown to be aspartic acid and/or glutamic acid. Some chitinases have only one glutamic acid at a catalytic site (*31, 35, 36*). β-*N*-Acetylhexosaminidase from *Streptomyces plicatus* uses aspartic acid and glutamic acid as catalytic sites (*37*). Two glutamic acids have also been reported to be the catalytic sites in human β-glucuronidase belonging to family 2, *Pseudomonas fluorescens* mannanase A belonging to family 26, and *P. fluorescens* ssp. *cellulosa* galactanase A belonging to family 53 (*38–40*). Hydrophobic cluster analysis of the galactanase A from *P. fluorescens* ssp. *cellulosa* suggested that Glu-161 and Glu-270 were, respectively, involved in the acid-base and nucleophilic catalysis by this enzyme (*39*). Here we show that *M. chitosanotabidus* 3001 ChoA uses Glu-121 and Glu-141 as putative catalytic residues. This may be the first example among the chitosanases and chitinases of two glutamic acids being used as the catalytic residues.

In addition, we found that replacement of Asp-164 with glycine so severely impaired the catalytic activity that the kinetic parameters could not be determined. Replacement with asparagine also decreased the catalysis, while replacement with glutamic acid resulted in somewhat higher chitosanase activity than that of the D164N mutant (but it was still lower than the wild type ChoA level) (Fig. 2B). The D164E and D164N mutations also severely decreased the k_{cat} value, although not as much as the Glu-141 mutations. If some modification is introduced at a subsite apart from the catalytic site, the mode of binding of an oligosaccharide to the chitosanase should be affected, resulting in a different product distribution in the time course (*31*). The time course experiment involving chitohexose suggested that Asp-139 and Asp-164 participate in the enzymatic reaction near the catalytic site. This is compatible with the proposed function of catalytic residues Glu-121 and Glu-141. Asp-139 and Asp-164 should participate in the saccharide binding near the catalytic site or in some assistance of the catalytic dyad, Glu-121 and Glu-141.

Replacement of Arg-150 by alanine or histidine decreased the chitosanase activity, but replacement of Arg-150 by lysine did not affect the chitosanase activity (Fig. 2A). Other studies have shown that the p*K*a value of the aspartic acid in the catalytic site is controlled by an arginine that is conserved in the fifteen known β-hexosaminidase glycosidases (*41, 42*). Thus, it is possible that the Ala or His mutations of Arg-150 may have altered the stereochemical structure of ChoA.

The W327*, W341*, and Q364* (* indicates a termination codon) mutants generated on random mutagenesis of *choA* were found to lack enzymatic activity due to imperfect protein synthesis, as our Western blot analysis indicated. Random mutagenesis also revealed that the G151D and G280D mutants lacked catalytic activity. While Gly-151 is

conserved in chitosanases, Gly-280 is not. That glycine residues affected glycosidase enzymatic activity has been found for T4-lysozyme (*43–45*). Mutations at Glu-139, Asp-148, and Asp-152 had low or inconsistent effects on enzymatic activity, and thus it was concluded that these sites did not play important roles in ChoA activity.

There was only one cysteine residue in *Streptomyces* sp. N174 chitosanase, indicating that disulfide bonding did not occur in this protein. In contrast, the three-dimensional structure of *B. circulans* MH-K1 chitosanase revealed that Cys-50 and Cys-124 formed a disulfide bond (*29*). In our study, mutation of all six *M. chitosanotabidus* 3001 ChoA cysteines abrogated the enzymatic activity (Table II). At least disulfide bonding of Cys-136 and Cys-231 could be detected. In addition, the ChoA protein treated with a reducing agent was not able to degrade the chitopentamer (Fig. 5). These results support that the disulfide bond is important for ChoA activity. We speculate that the disulfide bond of ChoA is important for protein folding.

In conclusion, we have found that the catalytically important residues of *M. chitosanotabidus* 3001 ChoA are Glu-121 and Glu-141, and the involvement of some other residues including all cysteines in the enzyme activity. Sequence comparison of ChoA with other proteins indicated poor homology, suggesting that ChoA is a unique protein. Supporting this idea, it was proposed that ChoA from *M. chitosanotabidus* 3001 should be classified in glycosylhydrolase family 80 of the Henrissat classification (*46*). Recently, we used Western and Southern blotting to assess the presence of this type of chitosanase in various bacteria. We found that ChoA-like genes are widely distributed in the β- and γ-subclasses of *Proteobacterium* and *Flavobacterium* (unpublished observation). These ChoA-like chitosanases have not been characterized yet, but the wide distribution of this enzyme in nature suggests to us that active biodegradation of chitosan by bacteria is common.

We wish to thank Prof. T. Fukamizo of Kinki University, Japan, for providing the purified *Streptomyces* sp. N174-chitosanase, and Dr. T. Yamamoto of Shimane University for the advice of measuring CD spectra.

REFERENCES

1. Fukamizo, T., Ohkawa, T., Ikeda, Y., and Goto, S. (1994) Specificity of chitosanase from *Bacillus pumilus*. *Biochim. Biophys. Acta* **1205**, 183–188
2. Masson, J.Y., Denis, F., and Brzezinski, R. (1994) Primary sequence of the chitosanase from *Streptomyces* sp. strain N174 and comparison with other endoglycosidases. *Gene* **140**, 103–107
3. Boucher, I., Dupuy, A., Vidal, P., Neugebauer, W.A., and Brzezinski, R. (1992) Purification and characterization of a chitosanase from *Streptomyces* N174. *Appl. Microbiol. Biotechnol.* **38**, 188–193
4. Okajima, S., Kinouchi, T., Mikami, Y., and Ando, A. (1995) Purification and some properties of a chitosanase of *Nocardioides* sp. *J. Gen. Appl. Microbiol.* **41**, 351–357
5. Cheng, C.Y. and Li, Y.K. (2000) An *Aspergillus* chitosanase with potential for large-scale preparation of chitosan oligosaccharides. *Biotechnol. Appl. Biochem.* **32**, 197–203
6. Fenton, D.M. and Eveleigh, D.E. (1981) Purification and mode of action of a chitosanase from *Penicillium islandium*. *J. Gen. Microbiol.* **126**, 151–165
7. Kim, S.Y., Shon, D.H., and Lee, K.H. (1998) Purification and characteristics of two types of chitosanases from *Aspergillus fumigatus* KH-94. *J. Microbiol. Biotechnol.* **8**, 568–574
8. Shimosaka, M., Nogawa, M., Ohno, Y., and Okazaki, M. (1993) Chitosanase from the plant pathogenic fungus, *Fusarium solani* f. sp. *phaseoli*—purification and some properties. *Biosci. Biotechnol. Biochem.* **57**, 231–235
9. Zhang, X.Y., Dai, A.L., Zhang, X.K., Kuroiwa, K., Kodaira, R., Shimosaka, M., and Okazaki, M. (2000) Purification and characterization of chitosanase and exo-β-D-glucosaminidase from a koji mold, *Aspergillus oryzae* IAM 2660. *Biosci. Biotechnol. Biochem.* **64**, 1896–1902
10. Grenier, J., Benhamou, N., and Asselin, A. (1991) Colloidal gold-complexed chitosanase: a new probe for ultrastructural localization of chitosan in fungi. *J. Gen. Microbiol.* **137**, 2007–2015
11. Osswald, W.F., Shapiro, J.P., Doostdar, H., McDonald, R.E., Niedz, R.P., Nairn, C.J., Hearn, C.J., and Mayer, R.T. (1994) Identification and characterization of acidic hydrolases with chitinase and chitosanase activities from sweet orange callus tissue. *Plant Cell Physiol.* **35**, 811–820
12. Hedges, A. and Wolfe, R.S. (1974) Extracellular enzyme from *Myxobacter* AL-1 that exhibits both β-1,4-glucanase and chitosanase activities. *J. Bacteriol.* **120**, 844–853
13. Kurakake, M., Yo-u, S., Nakagawa, K., Sugihara, M., and Komaki, T. (2000) Properties of chitosanase from *Bacillus cereus* S1. *Curr. Microbiol.* **40**, 6–9
14. Okajima, S., Ando, A., Shinoyama, H., and Fujii, T. (1994) Purification and characterization of an extracellular chitosanase produced by *Amycolatopsis* sp. CsO-2. *J. Ferment. Bioeng.* **77**, 617–620
15. Pelletier, A. and Sygusch, J. (1990) Purification and characterization of three chitosanase activities from *Bacillus megaterium* P1. *Appl. Environ. Microbiol.* **56**, 844–848
16. Rivas, L.A., Parro, V., Moreno-Paz, M., and Mellado, R.P. (2000) The *Bacillus subtilis* 168 *csn* gene encodes a chitosanase with similar properties to a *Streptomyces* enzyme. *Microbiology* **146**, 2929–2936
17. Yabuki, M., Uchiyama, A., Suzuki, K., Ando, A., and Fujii, T. (1988) Purification and properties of chitosanase from *Bacillus circulans* MH-K1. *J. Gen. Appl. Microbiol.* **34**, 255–270
18. Yamasaki, Y., Hayashi, I., Ohta, Y., Nakagawa, T., Kawamukai, M., and Matsuda, H. (1993) Purification and mode of action of chitosanolytic enzyme from *Enterobacter* sp. G-1. *Biosci. Biotech. Biochem.* **57**, 444–449
19. Grenier, J. and Asselin, A. (1990) Some pathogenesis-related proteins are chitosanases with lytic activity against fungal spores. *Mol. Plant-Microbe Interact.* **3**, 401–407
20. Ando, A., Noguchi, K., Yanagi, M., Shinoyama, H., Kagawa, Y., Hirata, H., Yabuki, M., and Fujii, T. (1992) Primary structure of chitosanase produced by *Bacillus circulans* MH-K1. *J. Gen. Appl. Microbiol.* **38**, 135–144
21. Masson, J.Y., Boucher, I., Neugebauer, W.A., Ramotar, D., and Brzezinski, R. (1995) A new chitosanase gene from a *Nocardioides* sp. is a third member of glycosyl hydrolase family 46. *Microbiology* **141**, 2629–2635
22. Shimosaka, M., Fukumori, Y., Zhang, X.Y., He, N.J., Kodaira, R., and Okazaki, M. (2000) Molecular cloning and characterization of a chitosanase from the chitosanolytic bacterium *Burkholderia gladioli* strain CHB101. *Appl. Microbiol. Biotechnol.* **54**, 354–360
23. Akiyama, K., Fujita, T., Kuroshima, K., Sakane, T., Yokota, A., and Takata, R. (1999) Purification and gene cloning of a chitosanase from *Bacillus ehimesis* EAG1. *J. Biosci. Bioeng.* **87**, 383–385
24. Shimosaka, M., Kumehara, M., Zhang, X.Y., Nogawa, M., and Okazaki, M. (1996) Cloning and characterization of a chitosanase gene from the plant pathogenic fungus *Fusarium solani*. *J. Ferment. Bioeng.* **82**, 426–431
25. Sun, L., Adams, B., Gurnon, J.R., Ye, Y., and Etten, J.L.V. (1999) Characterization of two chitinase genes and one chitosanase gene encoded by chlorella virus PBCV-1. *Virology* **263**, 376–387
26. Park, J.K., Shimono, K., Ochiai, N., Shigeru, K., Kurita, M., Ohta, Y., Tanaka, K., Matsuda, H., and Kawamukai, M. (1999)

Purification, characterization, and gene analysis of a chitosanase (ChoA) from *Matsuebacter chitosanotabidus* 3001. *J. Bacteriol.* **181**, 6642–6649
27. Yoon, H.G., Kin, H.Y., Lim, Y.H., Kim, H.K., Shin, D.H., Hong, B.S., and Cho, H.Y. (2000) Thermostable chitosanase from *Bacillus* sp. strain CK4: Cloning and expression of the gene and characterization of the enzyme. *Appl. Environ. Microbiol.* **66**, 3272–3734
28. Marcotte, E.M., Monzingo, A.F., Ernsy, S.R., Brzezinski, R., and Robertus, J.D. (1996) X-ray structure of an anti-fungal chitosanase from *Streptomyces* N174. *Nat. Struct. Biol.* **3**, 155–162
29. Saito, J., Kita, A., Nagata, Y., Ando, A., and Miki, K. (1999) Crystal structure of chitosanase from *Bacillus circulans* MH-K1 at 1.6-Å resolution and its substrate recognition mechanism. *J. Biol. Chem.* **274**, 30818–30825
30. Monzingo, A.F., Marcotte, E.M., Hart, P.J., and Robertus, J.D. (1996) Chitinases, chitosanases, and lysozymes can be divided into procaryotic and eucaryotic families sharing a conserved core. *Nat. Struct. Biol.* **3**, 133–140
31. Boucher, I., Fukamizo, T., Honda, Y., Willick, G.E., Neugebauer, W.A., and Brzezinski, R. (1995) Site-directed mutagenesis of evolutionary conserved carboxylic amino acids in the chitosanase from *Streptomyces* sp. N174 reveals two residues essential for catalysis. *J. Biol. Chem.* **270**, 31077–31082
32. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning. A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
33. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685
34. Imoto, T. and Yagishita, K. (1971) A simple activity measurement of lysozyme. *Agric. Biol. Chem.* **35**, 1154–1156
35. Hashimoto, Y., Yamada, K., Motoshima, H., Omura, T., Yamada, H., Yasukochi, T., Miki, T., Ueda, T., and Imoto, T. (1996) A mutation study of catalytic residue Asp 52 in hen egg lysozyme. *J. Biochem.* **119**, 145–150
36. Watanabe, T., Kobori, K., Miyashita, K., Fujii, T., Sakai, H., Uchida, M., and Tanaka, H. (1993) Identification of glutamic acid 204 and aspartic acid 200 in chitinase A1 of *Bacillus circulans* WL-12 as essential residues for chitinase activity. *J. Biol. Chem.* **268**, 18567–18572
37. Mark, B.L., Wasney, G.A., Salo, T.J.S., Khan, A.R., Cao, Z., Robbins, P.W., James, M.N.G., and Triggs-Raine, B.L. (1998) Structural and functional characterization of *Streptomyces plicatus* β-*N*-acetylhexosaminidase by comparative molecular modeling and site-directed mutagenesis. *J. Biol. Chem.* **273**, 19618–19624
38. Bolam, D.N., Hughes, N., Virden, R., Lakey, J.H., Hazlewood, G.P., Henrissat, B., Braithwaite, K.L., and Gilbert, H.J. (1996) Mannanase A from *Pseudomonas fluorescens* ssp. *cellulosa* is a retaining glycosyl hydrolase in which E212 and E320 are the putative catalytic residues. *Biochemistry* **35**, 16195–16204
39. Braithwaite, K.L., Barna, T., Spurway, T.D., Charnock, S.J., Black, G.W., Hughes, N., Lakey, J.H., Virden, R., Hazlewood, G.P., Henrissat, B., and Gilbert, H.J. (1997) Evidence that galactanase A from *Pseudomonas fluorescens* subspecies *cellulosa* is a retaining family 53 glycosyl hydrolase in which E161 and E270 are the catalytic residues. *Biochemistry* **36**, 15489–15500
40. Islam, M.R., Tomatsu, S., Shah, G.N., Grubb, J.H., Jain, S., and Sly, W.S. (1999) Active site residues of human β-glucuronidase. Evidence for Glu540 as the nucleophile and Glu451 as the acid-base residues. *J. Biol. Chem.* **274**, 23451–23455
41. Hou, Y., Tse, R., and Mahuran, D.J. (1996) Direct determination of the substrate specificity of the α-active site in heterodimeric β-hexosaminidase A. *Biochemistry* **35**, 3963–3969
42. Tse, R., Vavougios, G., Hou, Y., and Mahuran, D.J. (1996) Identification of an active acidic residue in the catalytic site of β-hexosaminidase. *Biochemistry* **35**, 7599–7607
43. Gray, T.M. and Matthews, B.W. (1987) Structural analysis of the temperature-sensitive mutant of bacteriophage T4 lysozyme, glycine 156→aspartic acid. *J. Biol. Chem.* **262**, 16858–16864
44. Matthews, B.W., Nicholson, H., and Becktel, W.J. (1987) Enhanced protein thermostability from site-directed mutations that decrease the entropy of unfolding. *Proc. Natl. Acad. Sci. USA* **84**, 6663–6667
45. Nicholson, H., Tronrud, D.E., Becltel, W.J., and Matthews, B.W. (1992) Analysis of the effectiveness of proline substitutions and glycine replacements in increasing the stability of phage T4 lysozyme. *Biopolymers* **32**, 1434–1441
46. Tremblay, H., Blanchard, J., and Brzezinski, R. (2000) A common molecular signature unifies the chitosanases belonging to families 46 and 80 of glycoside hydrolases. *Can. J. Microbiol.* **46**, 952–955