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Site-directed Mutagenesis of Evolutionary Conserved Carboxylic Amino Acids in the Chitosanase from *Streptomyces* sp. N174 Reveals Two Residues Essential for Catalysis*

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The comparison of four sequences of prokaryotic chitosanases, belonging to the family 46 of glycosyl hydrolases, revealed a conserved N-terminal module of 50 residues, including five invariant carboxylic residues. To verify if some of these residues are important for catalytic activity in the chitosanase from *Streptomyces* sp. N174, these 5 residues were replaced by site-directed mutagenesis. Substitutions of Glu-22 or Asp-40 with sterically conservative (E22Q, D40N) or functionally conservative (E22D, D40E) residues reduced drastically specific activity and k_{cat}, while K_m was only slightly changed. The other residues examined, Asp-6, Glu-36, and Asp-37, retained significant activity after mutation. Circular dichroism studies of the mutant chitosanases confirmed that the observed effects are not due to changes in secondary structure. These results suggested that Glu-22 and Asp-40 are directly involved in the catalytic center of the chitosanase and the other residues are not essential for catalytic activity.

Chitosan is a polysaccharide consisting of β-1,4-linked D-glucosamine residues, partially substituted with *N*-acetyl groups. This polymer can be considered as a partly deacetylated derivative of chitin. In nature, chitosan is widely distributed as a component of the cell wall in Zygomycetes (1) and of insect cuticles, even if not being abundant. In the last years, the chitosan itself as well as the products derived from its hydrolysis received much attention because of many potential applications in biomedical, agricultural, and environmental sciences (2).

A number of enzymes such as chitinases, lysozymes, and chitosanases can hydrolyze partially *N*-acetylated chitosan by an endo-mechanism. Chitosanases were described in prokaryotes, fungi (3), and more recently in plants (4). The distinction between chitinases and chitosanases became elucidated with the works of several groups that analyzed the structure of the hydrolysis products generated from chitosan by various enzymes of bacterial origin. They have shown that chitinases cleave specifically the *N*-acetyl-β-D-glucosaminidic bonds in partially *N*-acetylated chitosan (5, 6) while chitosanases are specific toward the β-D-glucosaminidic bonds (7, 8). Recently, these two groups of enzymes were delimited more precisely (9). Chitosanases were defined as enzymes able to hydrolyze all kinds of linkages in chitosan except the GlcNAc–GlcNAc bond, while chitinases hydrolyzed only GlcNAc–GlcNAc and GlcNAc–GlcN bonds.

The cloning and sequencing of the first two chitosanase genes from *Bacillus circulans* MH-K1 (10) and from *Streptomyces* sp. N174 (11, 12) allowed the comparison of their deduced amino acid sequences with those of known chitinases of plant, fungal, or bacterial origin (reviewed in Refs. 13 and 14). While all the known chitinases fell into two classes (classes I and II as defined in Ref. 13 or, respectively, families 19 and 18 of glycosyl hydrolases (14)) no homologies were found between the chitosanases and either class I or class II chitinases (10, 12, 14). On the other hand, both chitosanases showed extensive homologies in their N-terminal segments (12). Two more recently available sequences, that of chitosanase from *Nocardioides* sp. N106 (15) and a partial amino acid sequence of chitosanase from *Nocardioides* sp. K-01, obtained by direct sequencing of polypeptide fragments (16), were found to be highly homologous to the *Streptomyces* sp. N174 chitosanase. All these biochemical and molecular data showed that chitosanases are distinct from other chitosan-hydrolyzing enzymes. Thus, they were classified into family 46 of glycosyl hydrolases.[1]

So far, no information concerning essential catalytic residues is available for this enzyme family. However, in the case of glycosyl hydrolases, most, if not all, catalytic amino acids are aspartate or glutamate residues conserved in regions sharing amino acid sequence similarities (14, 17). The N-terminal chitosanase region (the only one having significant homology with the chitosanase of *B. circulans* MH-K1 and those from actinomycetes) includes 5 Asp or Glu residues conserved in all the four known sequences. Here we present some properties of the mutated forms of the *Streptomyces* sp. N174 chitosanase in which these carboxylic residues were modified by site-directed mutagenesis of the *csn* gene.

MATERIALS AND METHODS

Bacterial Strains and Plasmids—Escherichia coli JM109 (*end*A1, *thi*, *gyr*A96, *hsd*R17 (r_K^-, m_K^-), *rel*A1, *sup*E44, Δ(*lac-pro*AB), [F′, *tra*D36, *pro*AB, *lac*I^qZΔM15]) obtained from Promega, Madison, WI and *Streptomyces lividans* TK24 (obtained from D. A. Hopwood) were used for cloning experiments. The *E. coli* strain BMH 71–18 (*thi*, *sup*E, Δ(*lac-*

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[1] B. Henrissat, personal communication.

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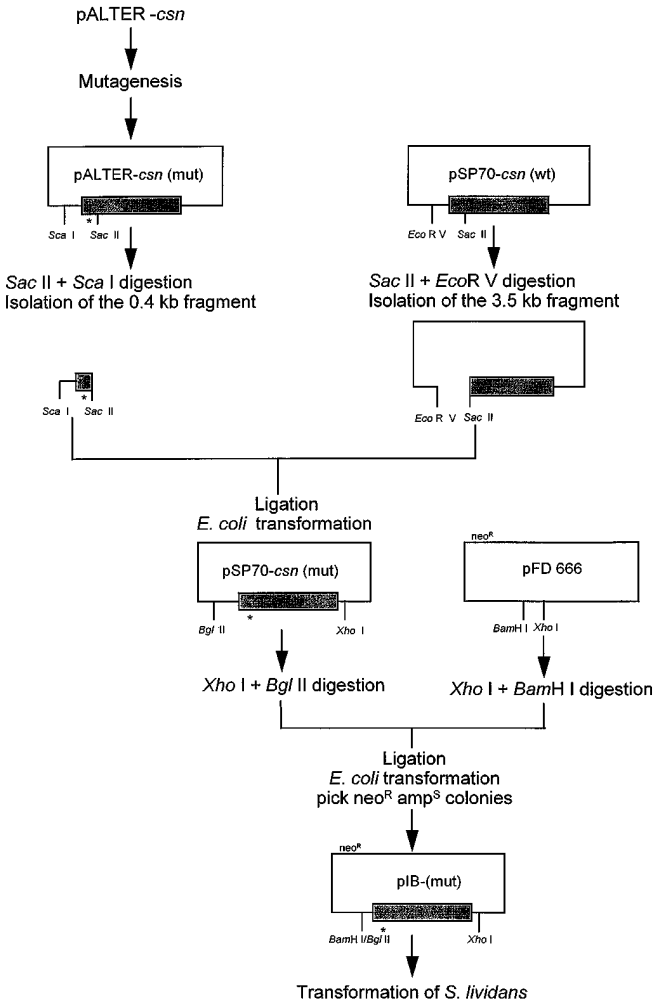

FIG. 1. **Strategy for subcloning of the mutated *csn* gene segments into the shuttle vector pFD666.** The *shaded area* represents the *csn* gene; *asterisks* indicate the mutation site. Only restriction sites used in cloning are shown. *kb*, kilobase(s); *wt*, wild type; *mut*, mutant.

*pro*AB), [*mut*S::Tn10] [F′, *pro*AB, *lac*I^{q}ZΔM15]), deficient in mismatch repair and used as transformation host after *in vitro* mutagenesis, and the vectors pALTER-1, used for site-directed mutagenesis, and pSP70, used for subcloning of mutated DNA segments, were obtained from Promega. The shuttle vector pFD666 (used for expression of wild-type and mutated genes in *S. lividans*) and the plasmid pRL270 (a pFD666 derivative carrying the chitosanase-encoding gene, *csn*, from *Streptomyces* sp. N174) were described previously (18, 19).

DNA Manipulation and Transformation—Extraction of plasmid DNA and preparation and transformation of streptomycete protoplasts were as described (20). Standard methods (21) were used for genetic manipulation of *E. coli*.

Site-directed Mutagenesis—The mutants were constructed using a commercial kit, the Altered Sites system supplied by Promega. The vector used in this method is pALTER-1, in which we subcloned the wild-type *csn* gene, to obtain pALTER-*csn* (Fig. 1). The oligonucleotides (cited in Table I) were synthesized using the Oligo 1000 DNA synthesizer (Beckman Instruments). The mutated *Sca*I-*Sac*II segments were sequenced (22) using the oligonucleotide 5′-CTGGACGAGTTCCAGCA-3′ as primer.

Subcloning of the Mutated Segments—Subcloning strategy is illustrated in Fig. 1. The pALTER-1-derivatives carrying the mutated *csn* genes were digested with *Sca*I and *Sac*II. The resulting 0.4-kilobase segments (encoding the N-terminal amino acids of the chitosanase) were purified from agarose electrophoresis gels and ligated to a pSP70 derivative carrying the wild-type *csn* gene and digested with *Eco*RV and *Sac*II. By such a procedure, only the mutated segments that were entirely sequenced were transferred into subclones.

To express the mutated genes in *S. lividans* TK24, the pSP70 derivatives carrying the mutated *csn* genes were digested with *Bgl*II and *Xho*I; the resulting 1.6-kilobase segments (encoding the mutated chitosanases) were purified from agarose electrophoresis gels and ligated to the pFD666 vector digested with *Bam*HI and *Xho*I. The resulting plasmids (pIB-D6N, pIB-E22Q, pIB-E22D, pIB-E22A, pIB-E36Q, pIB-D37N, pIB-D37E, pIB-D40N, and pIB-D40E) were used to transform *S. lividans* TK24.

TABLE I
Synthetic oligonucleotide primers used for site-directed mutagenesis

Mutation	Oligonucleotide sequence (5′-3′)
D6N	TGGGGATCGT**T**GAGGCCGGCA
E22Q	GAGGAGTTCT**G**GGCGCTGGAG
E22D	GGGAGGAGTT**G**TCGGCGCTGG
E22A	GGAGGAGTTC**G**CGGCGCTGGA
E36Q	CCGATGTCCT**G**GATGTACTTG
D37N	TCACCGATGT**T**CTCGATGTAC
D37E	CGTCACCGAT**C**TCCTCGATGT
D40N	CCGCGGCCGT**T**ACCGATGTCC
D40E	AGCCGCGGCC**C**TCACCGATGT

Enzyme Production and Purification—The ten *S. lividans* TK24 clones carrying, respectively, the pRL270 plasmid or one of the nine pIB plasmids, were grown in 50 ml of tryptic soy broth (Difco) supplemented with 50 μg ml^{-1} kanamycin. Each culture was centrifuged (10 min, 800 $\times$ *g*), and 5 ml of the mycelial pellet were used for inoculation of 500 ml of glucosamine medium consisting of 1% D-glucosamine in minimal salts (11). After 72 h of growth at 30 °C, the culture was filtered, and the filtrate was adjusted to pH 4.5 with acetic acid. Then the conductivity of the filtrate was adjusted to 9 millisiemens with distilled water, and the solution was loaded onto a 65 $\times$ 16-mm column of S-Sepharose Fast Flow (Pharmacia Biotech Inc.) previously equilibrated with 50 ml of 50 mM sodium acetate buffer, pH 4.5 (buffer A) followed by 50 ml of buffer A and 60 mM NaCl. The adjusted culture filtrate was loaded on the column through a hollow fiber on-line filter (MediaKap-5, 0.2 μm, Microgon). The column was then washed successively with 25 ml of buffer A, 60 mM NaCl and 20 ml of buffer A, 0.22 M NaCl. Elution of chitosanase was done with 20 ml of buffer A and 0.27 M NaCl, collecting 2-ml fractions. The five most active fractions were pooled and further purified by size-exclusion chromatography as described (23).

Circular Dichroism Spectroscopy—Spectra were obtained on a Jasco J-600 spectropolarimeter at 20–22 °C. Four spectra were averaged and the data smoothed by the Jasco software. The instrument was calibrated with ammonium (+)-10-camphorsulfonate. Peptide concentrations were calculated from the absorption at 280 nm, using the coefficient of 30,300 M^{-1}, calculated from the amino acid composition of chitosanase. Spectra were measured at peptide concentrations between 0.1 and 0.2 mg ml^{-1}, and data are expressed per mol of residue.

Enzyme Assays and Kinetics—Chitosanase activity was determined by measuring the quantity of reducing sugars generated by hydrolysis of soluble chitosan. The substrate for this assay was chitosan (Sigma, d.a.[2] = 0.21), dissolved in 50 mM sodium acetate buffer, pH 5.5. Highly deacetylated chitosan (Katakura Chikkarin Co., d.a. <0.01) was also used in some experiments.

For the determination of specific activity, reaction mixtures contained 0.8 mg ml^{-1} chitosan. Incubation was for 10 min at 37 °C. The reaction was terminated by transferring 200 μl of the reaction mixture into 400 μl of a 1% solution of *p*-hydroxybenzoic acid hydrazide in 0.5 N NaOH (24). After heating for 5 min at 95 °C, cooling into an ice bath, and pelleting of the unhydrolyzed chitosan by centrifugation (5 min, 800 $\times$ *g*), the reducing sugars were determined by reading the optical density at 405 nm, and comparison with a standard curve was prepared with D-glucosamine. This method was adapted for microtiter plates. One unit of chitosanase was defined as the amount of enzyme releasing 1 μmol min^{-1} D-glucosamine equivalents. Protein concentration was determined from the absorbance at 280 nm.

Kinetic constants were determined on chitosan (Sigma). 0.5-ml reactions were set up containing from 0.02 to 0.8 mg ml^{-1} chitosan. Wild-type chitosanase was used at 1.2 μM concentration. Mutant proteins were used at concentrations that gave the same overall hydrolysis level as the wild-type control. Liberation of reducing sugars was measured as above. K_m and k_{cat} values were obtained from direct linear plots (25) using the program COSY (provided on Internet by M. Eberhard, Biozentrum, Basel).

[2] The abbreviations used are: d.a., degree of *N*-acetylation; $(GlcN)_n$, β-1,4-linked oligosaccharide of GlcN with a polymerization degree of *n*; HPLC, high performance liquid chromatography.

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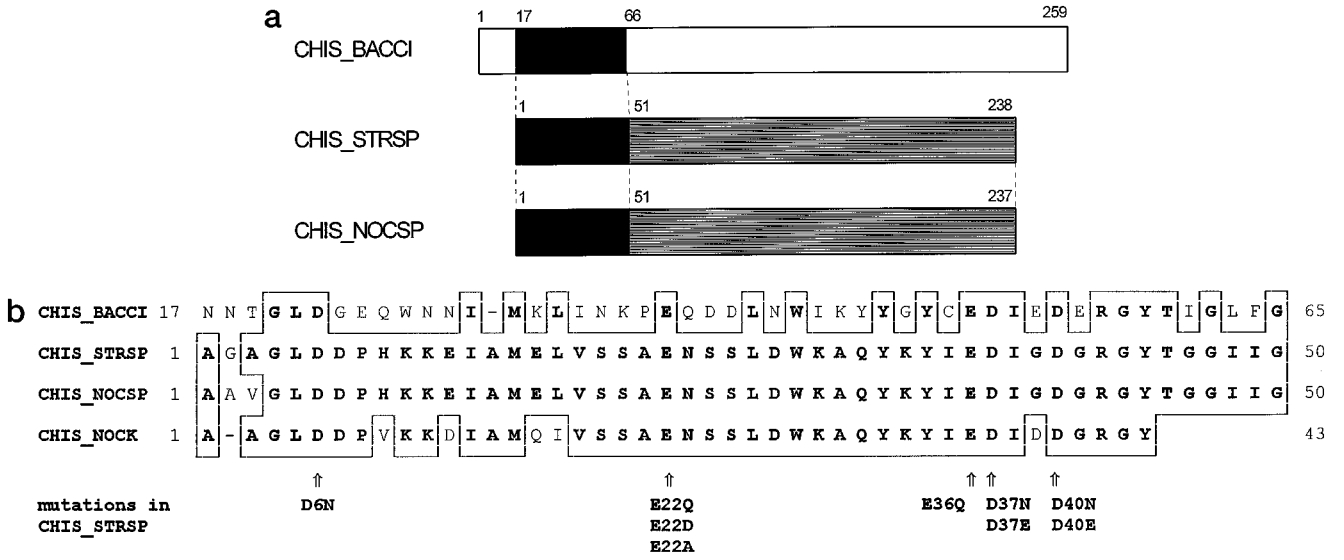

FIG. 2. *a*, schematic representation of homologies among chitosanase sequences. *Black bar*, conserved N-terminal module; *shaded bar*, actinomycete-type C-terminal module; *white bar*, *Bacillus*-type modules. *b*, amino acid sequence alignment of N-terminal modules in chitosanases. *Arrows* indicate target amino acid residues for site-directed mutagenesis of chitosanase from *Streptomyces* sp. N174. Chitosanases are from the following microorganisms: CHIS_BACCI, *B. circulans* MH-K1 (GenBank D10624); CHIS_STRSP, *Streptomyces* sp. N174 (LO7779); CHIS_NOCSP, *Nocardioides* sp. N106 (L40408); CHIS_NOCK, *Nocardioides* sp. K-01 (Ref. 16).

Time Course of the Enzymatic Reaction of $(GlcN)_6$—The substrate, $(GlcN)_6$, was dissolved in 50 mM sodium acetate buffer, pH 5.5, to give 16.6 mM solution. The enzyme (0.14–0.19 nmol) was added to 0.5 ml of the substrate solution, and the reaction mixture was incubated at 40 °C. After an appropriate reaction time, a portion of the reaction mixture was withdrawn and mixed with an equal volume of 0.1 N NaOH in order to terminate the enzymatic reaction. The reaction products thus obtained were analyzed by HPLC on a gel filtration column of TSK-GEL G2000PW (0.5 × 600 mm, Tosoh) using a Shimadzu RID-2A differential refractometer. The elution was done with 0.5 M NaCl at room temperature and a flow rate of 0.3 ml min^{-1}. $(GlcN)_n$ product concentrations were calculated from peak areas in the HPLC profiles using the standard curves obtained from pure saccharide solutions.

RESULTS AND DISCUSSION

Choice of Residues for Mutagenesis—Mutational analysis coupled with kinetic and structural studies has proven to be a valuable approach for identification of amino acid residues involved in the catalytic function of glycosyl hydrolases (26). In order to determine the carboxylic residues that were possible candidates for such function in chitosanase, we profited from the rather unusual homology between the first two chitosanases for which the primary sequences were determined. The chitosanases from *B. circulans* MH-K1 (27) and from *Streptomyces* sp. N174 (23) are similar in several ways. Both are alkaline proteins of similar M_r (27,000–29,000); both hydrolyze preferentially chitosans with a low acetylation degree by an endo-mechanism, generating end products of similar length; and both are inactive against chitin and carboxymethylcellulose. However, inspection of their primary sequences reveals that significant homology can be found only for a 50-residue-long box situated at the N terminus of the mature proteins (Fig. 2*a*). Only five carboxylic amino acids were found to be conserved in these two sequences, making them obvious targets for mutagenesis. These residues were found to be conserved in two more recently published chitosanase sequences from two actinomycete strains, members of the genus *Nocardioides*, isolated independently in Japan and Canada (Fig. 2*b*).

Production of Mutated Chitosanase Forms—The *csn* gene was modified by site-directed mutagenesis using *E. coli* as cloning host. However, neither the wild-type nor the mutated chitosanase forms could be obtained at satisfactory levels from periplasmic extracts of *E. coli*. For this reason, all the mutated genes were transferred into the pFD666 vector and expressed in *S. lividans*.

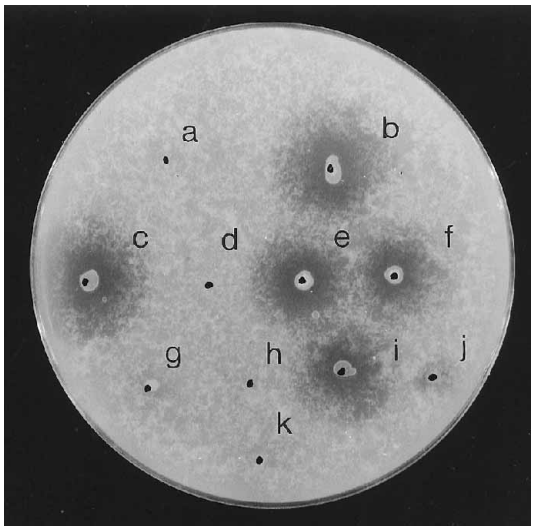

FIG. 3. **Activity of *S. lividans* TK24 clones on chitosanase detection medium (15).** Colonies were grown for 48 h at 30 °C. *a*, *S.lividans* TK24; *b–k*, *S.lividans* TK24 transformants carrying pFD666 derivatives with the following versions of the *csn* gene: *b*, wild type; *c*, mutant D6N; *d*, E22Q; *e*, E36Q; *f*, D37N; *g*, D40N; *h*, E22D; *i*, D37E; *j*, D40E; *k*, E22A.

The chitosanase activities of mutants were first estimated from clearing halos around colonies of *S. lividans* transformants growing on mineral medium with chitosan as the sole carbon source (Fig. 3). Clearing was absent around colonies carrying E22Q, E22D, E22A, or D40N mutations and was extremely reduced around the colony carrying the D40E mutation. The intensities of clearing of the other mutants were close to the wild-type control.

In previous works, a medium with chitosan as the main carbon source was used for chitosanase production by recombinant *S. lividans* strains in shaken cultures. However, we have found that this medium is inefficient for the production of mutated forms of chitosanase that have lost their enzymatic activity (data not shown). Thus, a new medium composition

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TABLE II
Relative specific activities of purified chitosanases

Enzyme	Relative specific activity against		
	Chitosan (d.a. = 0.21)[a]	Chitosan (d.a. < 0.01)[a]	$(GlcN)_6$[b]
	%	%	%
Wild type	100	100	100
D6N	83.5	85.4	ND[c]
E22Q	0.040	ND	ND[c]
E22D	0.23	ND	ND[c]
E22A	0.025	ND	ND[c]
E36Q	88.7	91.0	ND[c]
D37N	51.7	41.2	48.7
D37E	22.8	13.5	16.4
D40N	0.21	0.23	0.23
D40E	0.78	0.87	0.91

[a] Activity estimated by measuring the release of reducing sugars from substrate.
[b] Activity estimated from degradation rate of $(GlcN)_6$ substrate during time course analysis (see also Fig. 5). For wild type, the degradation rate was 48.3 s^{-1}.
[c] ND, not determined.

TABLE III
Kinetic parameters of purified wild-type and mutant Streptomyces sp. N174 chitosanases

Enzyme	K_m[a]	k_{cat}	k_{cat}/K_m
	$\mu g\ ml^{-1}$	min^{-1}	$min^{-1}\ (\mu g\ ml^{-1})^{-1}$
Wild type	29.3 ± 2[b]	727.5	24.8
D6N	28.9 ± 3	605.4	20.9
E22Q	57.3 ± 10	0.33	0.0057
E22D	23.4 ± 3	1.76	0.075
E22A	17.4 ± 8	0.20	0.011
E36Q	27.2 ± 1	676.5	24.9
D37N	50.5 ± 3	423.1	8.40
D37E	46.5 ± 7	183.8	3.95
D40N	27.5 ± 2	1.5	0.054
D40E	21.1 ± 4	5.9	0.27

[a] Substrate is chitosan (Sigma).
[b] Standard deviation.

with D-glucosamine as sole carbon source was used. In this medium, mutated chitosanases were produced almost as efficiently as the wild-type enzyme (data not shown). Chitosanase, protein, and protease levels as well as conductivity were monitored every 12 h in culture supernatants. Usually, the culture supernatant was recovered by filtration once the total protein level reached 150–175 $\mu g\ ml^{-1}$, while conductivity was not higher than 10–12 millisiemens.

Enzyme Purification and Kinetic Analysis—Wild-type and mutant chitosanases were purified to homogeneity (as judged by SDS-polyacrylamide gel electrophoresis and Coomassie Blue staining). Western blot using an anti-chitosanase rabbit serum showed that all the purified enzymes reacted in similar way with antibodies (not shown).

Relative specific activities, determined with standard chitosan (d.a. = 0. 21), highly deacetylated chitosan (d.a. <0.01), and $(GlcN)_6$ as substrates are shown in Table II. The relative activities determined by using chitosan as substrate appear to be consistent with those determined by $(GlcN)_6$. These determinations confirmed what was seen on the plate assay; mutants of residues 6 or 36 had nearly normal chitosanase activity, while mutants of residues 22 or 40 were severely affected in their activity. The activities of mutants of residue 37 were reduced two to six times as compared with the wild type.

Kinetic parameters (Table III) were determined under conditions that corresponded to the optimum previously found for the wild-type enzyme (23). Substitutions of the Glu-22 residue drastically impaired the catalytic activity. The k_{cat} values of mutants E22Q and E22A were, respectively, 1/2200 and 1/3600 of the wild-type chitosanase. Thus Glu-22 seems to be essential for catalytic activity and could not be efficiently substituted by an Asp residue, as the k_{cat} of the E22D mutant had only 0.4% of the value of the wild type.

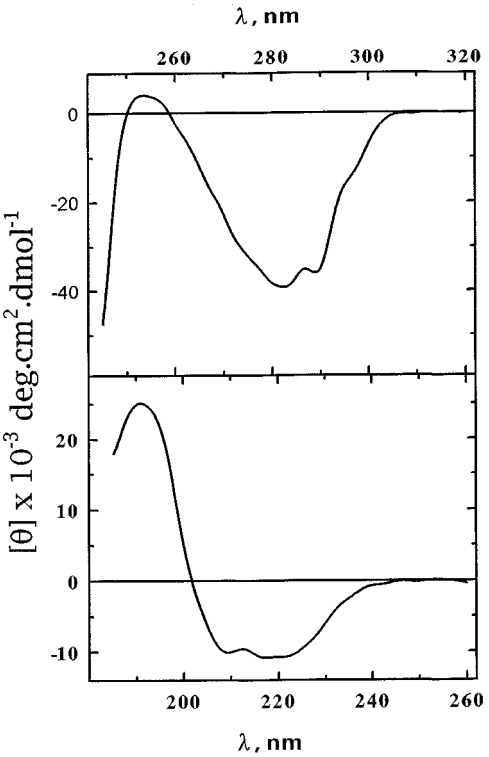

FIG. 4. **Near UV (*upper*) and far UV (*lower*) CD spectra of wild-type chitosanase.**

Substitutions of Asp-40 also had important effects on activity, even if less drastic than for Glu-22. The catalytic constants of mutants D40N and D40E were only 0.2 and 0.8% of the value for wild-type chitosanase, while K_m was essentially unchanged. This aspartic acid could not be substituted efficiently by a glutamic acid. Thus, Asp-40 seems also to be essential for catalytic activity.

In contrast with the above residues, the substitution of Asp-37 with glutamic acid led to an enzyme less active than the Asn-substituted mutant. Both these mutants also had a slightly higher K_m value than the wild type. However, considering the overall moderate effect of these substitutions, the Asp-37 residue cannot be considered as essential for chitosanase activity.

CD Spectra—The reduced activity of chitosanases mutated in Glu-22 and Asp-40 residues could have been the result of incorrect folding of the mutated proteins. CD spectra in the far UV region are commonly used to probe for alterations in tertiary structure, especially if aromatic residues are near the mutated residues in the three-dimensional structure. The near and far UV spectra for the wild-type chitosanase are shown in Fig. 4. The spectra were identical, within experimental error, for all the mutants reported here.

Chitohexaose Hydrolysis—The profiles of products generated from $(GlcN)_6$ were analyzed for mutants in position 37 or 40 (Fig. 5). From $(GlcN)_6$, the wild-type chitosanase produced $(GlcN)_3$ abundantly and $(GlcN)_2$ and $(GlcN)_4$ in lesser amounts. The product distribution was not changed even when each of the mutant chitosanases was used instead. Only the overall reaction rate was affected by each of the mutations. If some modification is introduced at a subsite apart from the catalytic site, the binding mode of oligosaccharide to the chitosanase should be affected resulting in a different product distribution

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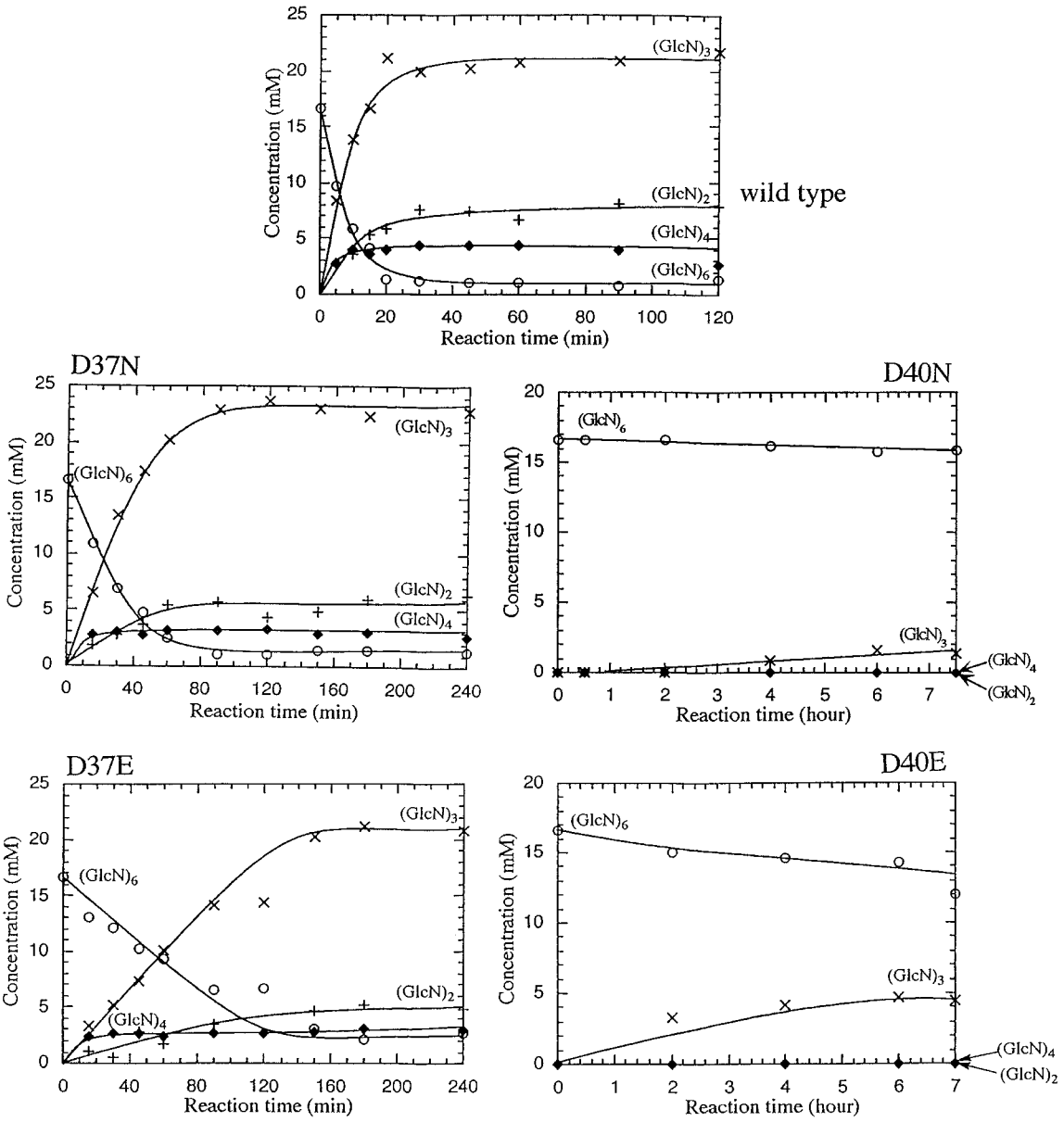

FIG. 5. **Time courses of $(GlcN)_6$ degradation by wild-type chitosanase and mutants in positions 37 and 40.** The $(GlcN)_n$ concentrations were calculated from peak areas in HPLC profiles. +, $(GlcN)_2$; [mult], $(GlcN)_3$; ♦, $(GlcN)_4$; ○, $(GlcN)_6$. Reaction conditions were 16.6 mM $(GlcN)_6$ in 50 mM sodium acetate buffer, pH 5.5, at 40 °C. Enzyme concentration was as follows: wild type, 0.372 μM; D37N, 0.230 μM; D37E, 0.281 μM; D40N, 0.301 μM; D40E, 0.322 μM.

in the time course (28). Thus, the results suggest that Asp-37 and Asp-40 participate in the enzymatic reaction at or near the catalytic site. This is compatible with the proposed function of catalytic residue for Asp-40. Asp-37 should participate in the saccharide binding near the catalytic site or in some assistance of the catalytic dyad, Glu-22 and Asp-40.

Conclusions—The analysis of chitosanase from *Streptomyces* sp. N174 by site-directed mutagenesis revealed that two carboxylic residues, Glu-22 and Asp-40, localized within the conserved N-terminal region are essential for catalytic activity. The other residues studied here, Asp-6, Glu-36, and Asp-37, behaved as if they were not essential and, most probably, will be found to be not strictly conserved as more chitosanase sequences will be determined.

The catalytic role of residues Glu-22 and Asp-40 is further sustained by some recently obtained data. While this work was in progress, the three-dimensional structure of the *Streptomyces* sp. N174 chitosanase was solved,[3] following an earlier report on its crystallization (30). In the current structural model, Glu-22 and Asp-40 are proposed as catalytic residues corroborating the conclusions drawn from the present work. On the other hand, the side chain of Asp-37 points away from the active site and makes close interaction with His-90. The lowered activity seen in the Asp-37 mutants is probably due to distortion of the active site cleft resulting from an altered interaction between these two residues.

Furthermore, it was found that this chitosanase hydrolyzes $(GlcN)_6$ with inversion of anomeric configuration (31). The average separation between the catalytic residues, expressed as the average of the four distances measured between each pair of carboxylate oxygen atoms, depends upon the mechanism of glycosidase action (29). The average separation in retaining

[3] E. Marcotte and J. D. Robertus, personal communication.

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β-glycosidases is 5.3 ± 0.2 Å, while in inverting β-glycosidases it is much larger, 9.5 Å. In the *Streptomyces* sp. N174 chitosanase crystal, this spacing is 13.8 Å, which is compatible with the above established inverting mechanism. The longer spacing in chitosanase could be due to the interaction between Glu-22 and Arg-205, which tends to displace Glu-22 away from the active site. Most probably, the spacing will be shortened in crystals of enzyme-substrate complexes. The mutants described in this work are good candidates for further structural studies of chitosanase.

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