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# Cloning, Sequence, and Expression of a Chitinase Gene from a Marine Bacterium, *Alteromonas* sp. Strain O-7

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**The gene encoding an extracellular chitinase from marine *Alteromonas* sp. strain O-7 was cloned in *Escherichia coli* JM109 by using pUC18. The chitinase produced was not secreted into the growth medium but accumulated in the periplasmic space. A chitinase-positive clone of *E. coli* produced two chitinases with different molecular weights from a single chitinase gene. These proteins showed almost the same enzymatic properties as the native chitinase of *Alteromonas* sp. strain O-7. The N-terminal sequences of the two enzymes were identical. The nucleotide sequence of the 3,394-bp *Sph*I-*Hin*dIII fragment that included the chitinase gene was determined. A single open reading frame was found to encode a protein consisting of 820 amino acids with a molecular weight of 87,341. A putative ribosome-binding site, promoter, and signal sequence were identified. The deduced amino acid sequence of the cloned chitinase showed sequence homology with chitinases A (33.4%) and B (15.3%) from *Serratia marcescens*. Regardless of origin, the enzymes of the two bacteria isolated from marine and terrestrial environments had high homology, suggesting that these organisms evolved from a common ancestor.**

Chitin is a major component of the exoskeletons of insects and crustaceans, and it is abundantly distributed throughout nature. This polysaccharide is also an important nutrient source of both carbon and nitrogen in the marine environment. Yu et al. (38) pointed out that the oceans would be completely depleted of carbon and nitrogen in a relatively short time if chitin could not be returned to the ecosystem in a biologically usable form. However, marine sediment contains relatively little chitin, despite the production of huge amounts of this insoluble polysaccharide by crustaceans. ZoBell and Rittenberg (39) reported that chitinolytic bacteria, which are abundant and widely distributed in the sea, play an important role in converting insoluble chitin into a biologically usable form. Chitinases (EC 3.2.1.14) are enzymes which degrade chitin, and they have been detected in various microorganisms (3, 25, 28, 30), plants (1, 18), insects (5, 13), and crustaceans (19). *Alteromonas* sp. strain O-7 is a gram-negative, flagellated, motile, and aerobic rod-shaped bacterium of marine origin. This strain excretes chitinase into the growth medium in the presence of chitin (32). We have already reported the purification, properties, and partial amino acid sequence of the enzyme from this strain (33). Recently, bacterial chitinase genes from terrestrial and marine bacteria such as *Serratia marcescens* (6, 10), *Bacillus circulans* (35, 36), *Vibrio harveyi* (28), and *Vibrio vulnificus* (37) have been cloned and sequenced. However, the mechanism of hydrolysis, the relationship between structure and function, and the regulatory system involved in enzyme induction are still unclear. In this report, we describe the cloning of a gene coding for chitinase and the purification of the gene products. The nucleotide sequence of the gene was determined, and the deduced amino acid sequence of the chitinase was compared with those of other microbial and plant chitinases.

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## MATERIALS AND METHODS

**Bacterial strains, plasmids, and culture conditions.** *Alteromonas* sp. strain O-7, which was isolated from a sediment sample collected at Sagami Bay and requires seawater for growth, was used as the source of chromosomal DNA. The bacterium was grown at 27°C in Bacto Marine Broth 2216 (Difco). The pH was adjusted to between 7.6 and 7.8 with NaOH before autoclaving. *Escherichia coli* JM109 was used as the recipient strain for recombinant plasmids. *E. coli* was grown at 37°C on LB medium for the selection of transformants. Purified agar (Nacalai Tesque, Kyoto, Japan) was used at a final concentration of 1.5% (wt/vol) for agar media. Plasmids pUC18 and pUC19 were used as the cloning vectors.

**Isolation and cloning of DNA.** Chromosomal DNA was prepared from *Alteromonas* sp. strain O-7 by the method of Marmur (21). The DNA was partially digested with *Hin*dIII and electrophoresed on a 0.6% agarose gel. Fragments between 4 and 6 kb in size were then collected by electroelution. These *Hin*dIII fragments were ligated into the dephosphorylated *Hin*dIII site of pUC18. The ligation mixture was inserted into competent *E. coli* JM109, and the transformants were screened on LB agar plates containing ampicillin (100 μg/ml), isopropyl-β-D-thiogalactopyranoside (IPTG, 120 μg/ml) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal, 50 μg/ml). The resulting white colonies, indicating clones carrying pUC18 with genomic inserts, were transferred to LB agar plates containing 0.25% colloidal chitin. Colonies forming clear halos indicated putative clones containing hybrid plasmids with genomic inserts coding for chitinase activity. Transformants containing only pUC18 did not form clear halos on colloidal chitin plates.

**Analysis of cloned chitinase gene.** Restriction enzyme cleavage maps were constructed by using single and multiple digests of hybrid plasmids. Restriction enzymes were purchased from Toyobo (Tokyo, Japan) and were used according to the manufacturer's specifications. Plasmid DNA from

a transformant was prepared by an alkaline lysis procedure (26).

**Localization of chitinase in *E. coli*.** A chitinase-positive clone of *E. coli*, designated pCHI997, was grown in 20 ml of LB medium containing ampicillin (100 μg/ml). Fractionation of extracellular, periplasmic, and cellular enzymes was based on the procedure of Koshland and Botstein (14). β-Lactamase (27) and malate dehydrogenase (12) were assayed as markers for periplasmic and cellular proteins, respectively.

**Purification of chitinases.** *E. coli* carrying pCHI997 was cultured to the early stationary phase at 37°C with vigorous shaking. The cells (4.4 g) were collected by centrifugation, and the periplasmic fraction containing chitinases (162 ml) was prepared. The enzyme was partially purified by successive chromatography with DEAE-Toyopearl 650M and Sephadex G-100 according to the previous report (32). Ammonium sulfate (final concentration, 1.0 M) was added to the enzyme solution, and the mixture was applied to a phenyl-Toyopearl 650M column (1.1 by 25 cm; Tosoh, Tokyo, Japan) equilibrated with 50 mM Tris-HCl buffer (pH 7.5) containing 1.0 M ammonium sulfate. The column was washed with the same buffer, and the enzyme was then eluted with a linear gradient of 1.0 to 0 M ammonium sulfate at a flow rate of 18 ml/h. Two active fractions were eluted at a concentration of 0.4 and 0.25 M ammonium sulfate. These purified enzymes were named Chi-78 and Chi-85, respectively.

**Enzyme assay.** Chitinase was assayed by the procedure of Schales (9) with the previously described modification (32). Enzymatic hydrolysis of chitooligosaccharides from dimer to hexamer (Seikagaku Corporation, Tokyo, Japan) was performed by the method previously described (33). β-Lactamase activity was measured by the method of Sawai and Takahashi (27). Malate dehydrogenase activity was measured by the method of Kitto (12).

**SDS-PAGE.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a 7.5% gel was done by the method of Laemmli (17). Low-molecular-weight kit E (Pharmacia) was used as a molecular weight marker.

**Amino-terminal amino acid sequence.** The amino acid sequences of the cloned chitinases (Chi-78 and Chi-85) were determined on an Applied Biosystems model 477A gas-phase sequencer, and the phenylthiohydantoin derivatives of amino acids were identified by an automatic on-line analysis system.

**Nucleotide sequence determination.** Restriction fragments from pCHI997 were subcloned by using pUC18 or pUC19. Plasmids of subclones were prepared for sequencing by using a plasmid purification kit (QIAGEN, Hilden, Germany). Dideoxy DNA sequencing was performed with an AmpliTaq sequencing kit (Takara Shuzo Co., Kyoto, Japan) as specified by the manufacturer. Reactions were performed in a DNA thermal cycler (Perkin-Elmer Cetus). DNA fragments were analyzed on a DNA sequencer (Hitachi SQ-3000).

**Protein estimation.** Protein concentration was measured by the method of Bradford (2). Bovine serum albumin was used as the standard.

**Nucleotide sequence accession number.** The nucleotide sequence data reported here have been submitted to the DDBL, EMBL, and GenBank data bases under accession number D13762.

## RESULTS

**Cloning of chitinase gene from *Alteromonas* sp. strain O-7.** Approximately 1,500 transformants were obtained, and all

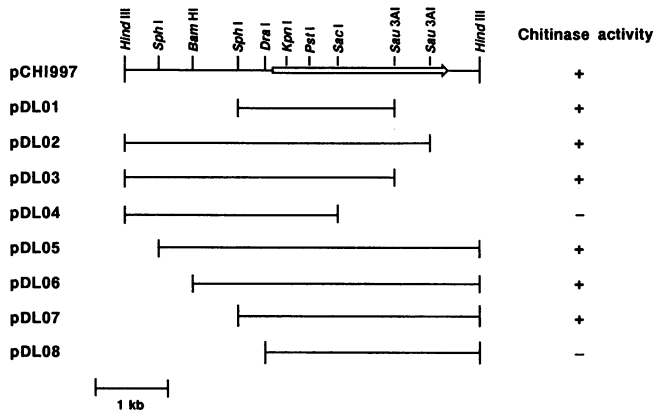


FIG. 1. Restriction map and deletion analysis of pCHI997. The transformants carrying the plasmids with appropriate deletions were transferred to an LB agar plate containing 0.25% colloidal chitin and ampicillin. Production of chitinase was judged by the formation of clear halos of colloidal chitin around the colonies. +, visible halo; −, no halo. The arrow indicates the coding sequence for the chitinase protein.

were transferred to colloidal chitin plates. Four colonies formed clear halos, indicating the expression of chitinase activity. Plasmids isolated from the four clones contained common inserts (5.0-kb *Hin*dIII fragment) with identical restriction maps. Further analysis was performed on pCHI997, which was one of the four clones. This plasmid contained a 5.0-kb *Hin*dIII fragment from *Alteromonas* sp. strain O-7.

**Restriction mapping and subcloning.** A restriction map and deletion analysis of pCHI997 are shown in Fig. 1. To determine the location of the chitinase gene in the 5.0-kb inserted DNA, we prepared various subclones. The subclones were inserted into competent *E. coli* JM109, and expression of chitinase in *E. coli* was determined by the formation of clear halos around the colonies. Deletions of 1.4 kb from left to right (pDL07) and of 1.0 kb from right to left (pDL03) did not affect the expression of chitinase activity on the chitin plate. On the other hand, deletions of 1.8 kb from left to right (pDL08) and of 2.2 kb from right to left (pDL04) caused the loss of chitinase activity. Thus, the 2.3-kb *Sph*I-*Sau*3AI fragment was the region necessary for chitinase

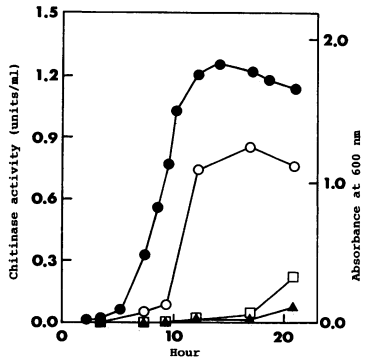


FIG. 2. Location of chitinase activity in *E. coli* carrying pCHI997. *E. coli* JM109 carrying pCHI997 was grown in LB medium containing 100 μg of ampicillin per ml. Cultivation was done at 37°C on a rotary shaker. Symbols: ●, bacterial growth. Chitinase activity in the extracellular fraction (□), the periplasmic fraction (○), and the cytoplasmic fraction (▲) was determined by a modification of the Schales procedure (9).

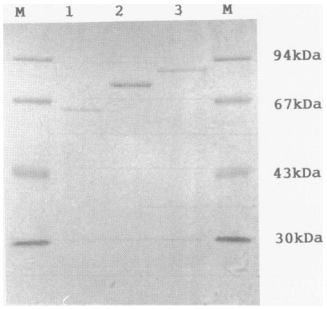


FIG. 3. SDS-PAGE of the purified chitinases. Lane M, molecular size standards: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa). Lanes: 1, chitinase (Chi-A) from *Alteromonas* sp. strain O-7; 2, Chi-78; 3, Chi-85.

activity. However, pDL01 did not contain the full-length insert of the chitinase-encoding gene. The *Sph*I-*Hin*dIII fragment of pCHI997 was subcloned by using pUC18 (pDL07) and pUC19 (pDL071). The transformants carrying pDL07 or pDL071 showed detectable clear halos on an agar plate containing colloidal chitin, indicating that transcription of the gene occurred through the use of its own promoter.

**Localization of chitinase activity in *E. coli*.** The location of the cloned chitinase in *E. coli* harvested at intervals of 2 to 5 h was determined by separation of bacterial proteins into extracellular, periplasmic, and cytoplasmic fractions. The chitinase activity was located mainly in the periplasmic fraction for up to 21 h of cultivation, as shown in Fig. 2. Low chitinase activity was detected in both the culture supernatant and the cytoplasmic fraction. To ensure correct fractionation, we measured β-lactamase and malate dehydrogenase activities as marker enzymes in the periplasm and cytoplasm, respectively. When β-lactamase activity was determined, 97% was found in the periplasmic fraction, and 82.3% of the malate dehydrogenase was present in the cytoplasmic fraction after 18 h of cultivation.

**Purification of chitinase produced by the clone.** *E. coli* carrying pCHI997 produced two types of chitinases (Chi-78 and Chi-85) with different molecular masses, 78 and 85 kDa, respectively, as shown in Fig. 3. *E. coli* carrying pDL07 also produced Chi-78 and Chi-85, indicating that both cloned chitinases are encoded by the same gene (data not shown). The two proteins from one gene comigrated like a single protein on the DEAE-Toyopearl 650M and Sephadex G-100

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CATCCGATGTTT GGAAAGCAATTT AAAGAAAACCCA AATTAATTGGGT ACAACATGTAAC TACAATAAGGTT GAAGATATGAAA CTTAATAAAATA ACCAGCTATATA  432
                                                                                  M  K  L  N  K  I  T  S  Y  I
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CCGAGCTTGAGA AGGGCTTAATTG ATGTCCTGCCAT CCGCAAGCTT
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FIG. 4. Nucleotide sequence of a 3.4-kb DNA fragment from pCHI997. The putative ribosome-binding site (TAAGG) is underlined. The −10 and −35 regions of a possible promoter sequence are boxed. The deduced amino acid sequence of Chi-85 is given below the nucleotide sequence. The amino-terminal amino acid sequence of Chi-85 protein was determined by using an Applied Biosystems model 477A gas-phase sequencer and is underlined. The signal peptide cleavage site is shown with an arrow (↓). The putative inverted repeat sequence is indicated by facing arrows with solid lines.

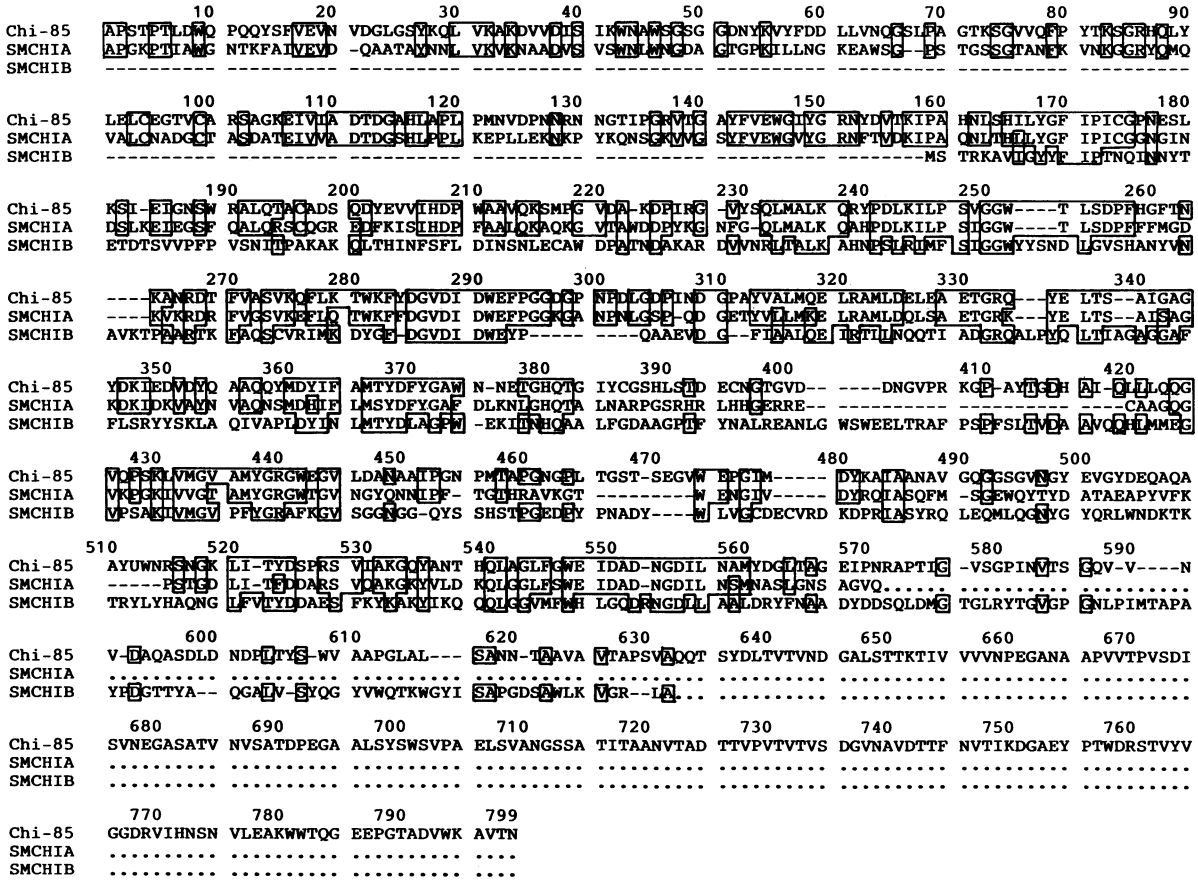

FIG. 5. Comparison of the amino acid sequence of *Alteromonas* chitinase with those of *S. marcescens* chitinases. The sequence of Chi-85 is compared with those of *S. marcescens* chitinase A (SMCHIA) and *S. marcescens* chitinase B (SMCHIB). The numbering above the sequences refers to Chi-85. Dashed lines represent spacers inserted into sequences for alignment. Amino acids that are identical between Chi-85 and other sequences are boxed.

chromatograms. However, these two proteins were separated by phenyl-Toyopearl 650M column chromatography. In this procedure, Chi-78 and Chi-85 were purified about 14-fold. The recovery of activity was about 6.0 and 23.2% of the periplasmic fraction, respectively. The proteins showed almost the same enzymatic properties as the native chitinase (Chi-A) from *Alteromonas* sp. strain O-7, that is, specific activities of 9.8 U/mg of protein (Chi-78), 9.9 U/mg of protein (Chi-85), and 10.0 U/mg of protein (Chi-A); pH and temperature optima of pH 8 to 9 and 50°C; and chitobiose as the main reaction product. The N-terminal sequences of the two enzymes were found to be identical (APSTPTLDWQ PQQYSFVEVNVDGLGSY-). This sequence is in good agreement with the sequence deduced from the nucleotide sequence and also with the amino-terminal sequence of Chi-A.

**Nucleotide sequence of chitinase gene.** The nucleotide sequence of both strands of the *Sph*I-*Hin*dIII fragment of pCHI997 was determined by using an automated sequencing system (Fig. 4). In this sequence, a single open reading frame starting at base 403 and ending at base 2865 was found, as shown by the arrow in Fig. 1. The putative Shine-Dalgarno sequence, TAAGG, was found upstream of the start codon (ATG) of the open reading frame. The open reading frame of 2,460 bp would encode a protein of 820 amino acids with a molecular weight of 87,341. The deduced N-terminal 21-amino-acid sequence showed the typical features of signal peptides, which are composed of a positively charged region, a hydrophobic region, and a signal sequence cleavage site. From the characterization of the cleavage sites, it is presumable that the putative site of cleavage might be between alanine residues 21 and 22, which is compatible with the −3,−1 rule of von Heijne (34). The N-terminal sequences of Chi-78 and Chi-85 coincided precisely with the sequence starting from alanine residue 22 of the deduced amino acid sequence encoded by the chitinase gene. Thus, we clarified that cleavage of the signal peptide occurred between alanine residues 21 and 22. The putative −10 and −35 regions, which showed relatively weak homology to the consensus sequence in *E. coli*, were detected in the A+T-rich region upstream of the Shine-Dalgarno sequence. The inverted repeat, which was composed of an 8-bp stem and a loop of five bases, was located downstream of the chitinase terminal codon (TAA).

**Comparison of amino acid sequence of Chi-85 with other enzymes.** The protein sequence of Chi-85 was compared with available protein sequences from data bases (GenBank and EMBL) as well as those from the literature. The protein sequence of Chi-85 particularly showed homology with chitinases A (33.4%) and B (15.3%) from *S. marcescens* (Fig. 5). Although common high sequence homology was not found in the N- and C-terminal regions from the sequence

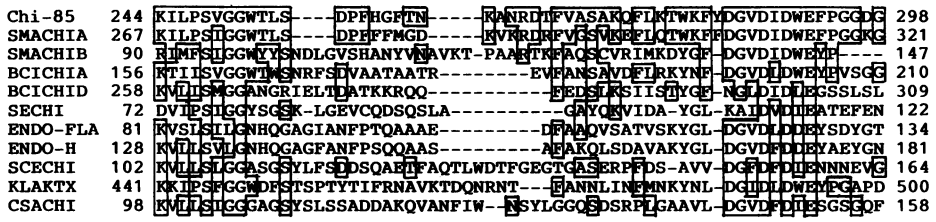


FIG. 6. Sequence comparisons of *Alteromonas* chitinase with other chitinases and the related enzymes. The middle part of Chi-85 is aligned with other chitinases or related enzymes. Amino acids that are identical between Chi-85 and other sequences are boxed. SMACHIA, *S. marcescens* chitinase A; SMACHIB, *S. marcescens* chitinase B; BCICHIA, *B. circulans* chitinase A1; BCICHID, *B. circulans* chitinase D; SECHI, *S. erythraeus* chitinase; ENDO-FLA, *Flavobacterium* sp. endo-β-*N*-acetylglucosaminidase; ENDO-H, *S. plicatus* endo-β-*N*-acetylglucosaminidase; SCECHI, *S. cerevisiae* chitinase; KLAKTX, *K. lactis* killer toxin; CSACHI, *C. sativus* chitinase.

comparison of the three chitinases, two middle parts of the proteins (residues 244 to 258 and residues 285 to 298 of Chi-85) revealed extensive homology. The conservation of these regions in other chitinases and the related enzymes was examined by using a protein data bank. As a result of the computer search, the two regions were found to be well conserved not only in chitinases from *S. marcescens* but also in chitinases from *B. circulans* (35, 36), *Saccharomyces cerevisiae* (15), *Streptomyces erythraeus* (11), *Kluyveromyces lactis* (29), and *Cucumis sativus* (22) and glycosidases from *Streptomyces plicatus* (24) and *Flavobacterium* sp. (31), as shown in Fig. 6.

## DISCUSSION

A 5.0-kb *Hin*dIII DNA fragment coding for chitinase activity from *Alteromonas* sp. strain O-7 was cloned in *E. coli* by using the vector pUC18. The *Hin*dIII DNA fragment hybridized to chromosomal DNA digests from *Alteromonas* sp. strain O-7 but not those from *E. coli* JM109. Only the 5.0-kb band was visible in the genomic *Hin*dIII digest (data not shown). These results indicate that the cloned fragment was derived from *Alteromonas* sp. strain O-7. In most cases, when genes encoding foreign extracellular proteins are cloned in *E. coli*, the precursor is synthesized, processed, and exported across the inner membrane but not the outer membrane. The cloned chitinase was not secreted into the growth medium but accumulated in the periplasmic space, indicating that the signal peptide is functional in *E. coli*. The native chitinase (Chi-A) of *Alteromonas* sp. strain O-7 was recovered as a single 65-kDa polypeptide from the growth medium (Fig. 3), but was overestimated as 70 kDa in our previous paper (33). *Alteromonas* sp. strain O-7 is an unusual gram-negative bacterium which excretes several enzymes such as chitinase, protease, amylase, DNase, and lipase into the medium (32). It has been reported that the cholera toxin from *Vibrio cholerae* (7), several proteins from *Aeromonas hydrophila* (8) or *Pseudomonas aeruginosa* (4), and the pullulanase from *Klebsiella pneumoniae* (23) transiently entered the periplasmic space, and specific helper proteins play critical roles in their translocation across the outer membrane. Thus, specific helper proteins produced by *Alteromonas* sp. strain O-7 might participate in the secretion of chitinase.

A chitinase-positive clone of *E. coli*, designated pCHI997, produced two chitinases with different molecular weights. Chi-85 was considered to correspond to the intact gene product. On the other hand, Chi-78 appeared to be the product of partial proteolysis of Chi-85. The N-terminal sequences of the two proteins and Chi-A were found to be precisely identical. These results indicate that three forms of enzyme differing in size were the result of partial proteolysis occurring in the C-terminal region.

The chitinase gene encoded a prechitinase protein of 820 amino acids that contained a 21-residue signal sequence. The size of the Chi-85 protein was calculated to be 799 amino acid residues or 85,190 daltons. The calculated molecular weight of Chi-85 was in good agreement with that estimated by SDS-PAGE. The three different forms of chitinases (Chi-78, Chi-85, and Chi-A) which retained the ability to hydrolyze chitin seemed to be the same gene product. The truncated protein encoded by a 2.3-kb *Sph*I-*Sau*3AI fragment of pCHI997 showed a relatively weak halo on the chitin plate. These results suggest that the C-terminal portion of Chi-85 is not essential for chitinase activity.

Comparison of the deduced amino acid sequence of Chi-85 with those of chitinases A and B from *S. marcescens* (6, 10) showed considerable homology. In particular, striking homology was observed in the middle parts of the three enzymes. Considering that these regions were well conserved from prokaryote to eukaryote and that the region (residues 285 to 292 of Chi-85) contained three aspartic acid residues and one glutamic acid residue involved in the active site of lysozyme (20), these portions of chitinases may constitute the catalytic sites of the enzymes. Furthermore, the clusters of identical amino acids indicate that the genes probably evolved from a common ancestor.

It was reported that both the *Saccharomyces* chitinase (15) and the *Bacillus* chitinase (35) are composed of four domains. The chitin-binding domain which exists in both proteins was not detected in the sequence of Chi-85, as there was no binding of chitinase to regenerated chitin by the method of Watanabe et al. (36) (data not shown). Thus, it is suggested that the *Alteromonas* chitinase is divided into three domains, that is, signal sequence, catalytic domain, and noncatalytic C-terminal domain. The function of the C-terminal domain is still obscure; however, the region from amino acid residues 630 to 770 in the C-terminal domain is thought to form a hydrophobic core on the basis of the hydropathic profile calculated by the method of Kyte and Doolittle (16) with a span of 20 amino acid residues. Therefore, the interaction of this hydrophobic region with the membrane might play an important role in exporting the *Alteromonas* chitinase across the outer membrane.

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