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Molecular Cloning and Characterization of 58 kDa Chitinase Gene from *Serratia marcescens* KCTC 2172

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Abstract A chitinase gene (pCHi58) encoding a 58 kDa chitinase was isolated from the *Serratia marcescens* KCTC 2172 cosmid library. The chitinase gene consisted of a 1686 bp open reading frame that encoded 562 amino acids. *Escherichia coil* harboring the pChi58 gene secreted a 58 kDa chitinase into the culture supernatant. The 58 kDa chitinase was purified using a chitin affinity column and mono-S column. A nucleotide and *N*-terminal amino acid sequence analysis showed that the 58 kDa chitinase had a leader peptide consisting of 23 amino acids which was cleaved prior to the 24th alanine. The 58 KDa chitinase exhibited a 98% similarity to that of *S. marcescens* QMB 1466 in its nuclotide sequence. The chitinolytic patterns of the 58 kDa chitinase released N,N'-diacetyl chitobiose (NAG2) as the major hydrolysis end-product with a trace amount of *N*-acetylglucosamine. When a 4-methylumbellyferyl-*N*-acetylglucosamin monomer, dimmer, and tetramer were used as substrates, the 58 kDa chitinase did not digest the 4-Mu-NAG monomer (analogue of NAG_2), thereby indicating that the 58 kDa chitinase was likely an endochitinase. The optimum reaction temperature and pH of the enzyme were 50°C and 5.0, respectively.

Keywords: *Serratia marcescens*, chitinase, endochitinase

INTRODUCTION

Chitinases (EC 3.2.1.14) hydrolyze the β-1,4-glucosidic linkages of chitin and are commonly found in a wide variety of organisms, including fungi [1,2] plants [3,4], insects [5], crustacea [6], and bacteria [7,9]. However, the functions of these enzymes are belivered to be different in various hosts. In fungi, chitinases play a physiological role in the apical growth and morphogenesis of fungal hypae [1,2]. The production of chitinases in higher plants is considered part of their defense system against fungal infection [10]. Bacterial chitinases appear to have a nutritional or scavenging function in the decomposition of insoluble chitin and the utilization as chitin as a carbon and energy source.

Serratia marcescens is a gram-negative soil bacterium and well known chitin decomposer characterized by five types of chitinolytic activities [11].

Four chitinase genes *chi*54, *chi*52, *chi*35, and *chi*22 have been cloned from *Serratia marcescens* KCTC 2172 and sequenced [15,16].

Other chitinase gene. have also been cloned from several bacteria, *e.g. Altermonase*[12], *Bacillus circulans* [9], and *Vibrio vulnificus* [13]. The current report describes the cloning and characterization of an unreported gene of *Serratia marcescens* KCTC 2172 that encodes a 58 kDa chitinase.

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MATERIALS AND METHODS

Bacterial Strains, Culture Conditions, Genomic Library Construction, and Nucleotide Sequence Analysis

The bacterial strains, culture conditions, genomic library construction, and nucleotide sequence analysis have all been described previously [15].

Isolation of 58 kDa Chitinase Gene

Using the N-terminal amino acid sequences of the purified 58 kDa chitinase of *S. marcescens* KCTC 2172 and the conserved consensus sequence (FDGVDIDWE) of a 54 kDa and 52 kDa chitinase [15,16], bidirectional PCR primers were synthesized. The sequences of the primers were 5'-CTCCCAGTCGATATCCACGCCGT-CGAA-3' and 5'-GCNGCNCCNGGNAAGCCNCCN-3'. The chromosomal DNA used as the template in the PCR reaction was prepared from *S. marcescens* KCTC 2172 according to the method described by Sambrook *et al.* [14]. PCR amplification was performed for 30 cycles consisting of 94°C for 30 sec, 50°C for 30 sec, and 70°C for 1 min followed by a final incubation at 72°C for 10 min. The PCR product was subcloned into a pBluescript SK (-) vector, sequenced, and used as a probe for the isolation of the 58 kDa chitinase gene. The recombinant cosmids were allowed to hybridize to the PCR product probe after it was randomly labeled with [α-^{32}P]ATP. All colony hybridization experiments were performed ac-

cording to the method of Sambrook *et al.* [14]. The chitinolytic *Sau*3A fragment of the cosmid insert that hybridized with the 58 kDa chitinase probe was subcloned into pBluescript SK (-), The clone that produced the 58 kDa chitinase was designated *pChi*58 and the complete nucleotide sequence was analyzed.

Purification of Chitinases

An *Escherichia coli* transformant harboring *pChi*58 was cultured up to the early stationary phase at 37°C with vigorous shaking in 1l of an LB broth containing 0.1% glycol chitin. The culture supernatant was collected after centrifugation and supplemented with ammonium sulfate to 80% saturation. The precipitated proteins were dissolved in 50 mL of a 20 mM sodium bicarbonate buffer (pH 8.4) and dialyzed for 8 h against the same buffer. After washing with 400 mL of the same buffer, 100 mL of the regenerated chitin solution (2.5%) was allowed to react with 50 mL of the enzyme solution for 12 h at 4°C. The mixture was then packed into a column (26 ×400 mm) and washed with 400 mL of a 20 mM sodium bicarbonate buffer (pH 8.4). The Chitinase was eluted by the stepwise addition of 200 mL of a 20 mM acetic acid buffer (pH 3.3) and 2.5 M acetic acid buffer (pH 2.0). The eluted fractions were immediately adjusted to pH 5.0 with saturated Tris and dialzed against a 10 mM sodium acetate buffer (pH 5.0) for 18 h. The 58 kDa chitinase from the *S. marcescens* KCTC 2172 culture supernatant was used for N-terminal amino acid sequencing and purified according to the above method.

Analysis of Chitinase Activity

The chitinase activity of both the native and recombinant chitinase was assessed in three ways according to the method described by Vinetz *et al.* [18]. First, the enzyme preparations were analyzed for their ability to degrade polymeric chitin, as described previously [17]. Second, microfluorometry (TD-700, Turner Designs, Sunnyvale, CA, USA, excitation 360 nm and emission 460 nm) was used to measure the hydrolysis of 4-MU-GlcNAc, 4-MU-GlcNAc2, and 4-MU-GlcNAc3 (Sigma). The enzymatic activity was than detected as fluorescence intensity. Third, TLC was used to analyze the products of the chitinolytic enzyme with a native chitin polymer and chitin oligosaccharides (GlcNA2-3, Calbiochem). 6 μL of a 5 mM substrate was mixed with 4 μL of a 20 mM sodium acetate buffer pH 6.0 to which was added 10 μg/10 μL of the enzyme. The reaction mixtures were incubated at 37°C overnight and then analyzed by TLC. 3 μL of the reaction mixture was applied to Silica Gel-60 TLC plates, 20 × 20 cm (EM Science, Gibbstown, NJ), and chromatographed in isopropyl alcohol:ethanol:water (5:2:1). The plates were developed by spraying with 10% sulfuric acid in ethanol followed by heating at 120°C for 10-20 min to detect any dark spots.

SDS-polyacrylamide Gel Eelectrophoresis, Detection of Chitinolytic Activity and Enzyme Characterization

SDS-polyacrylamide gel electrophoresis, the detection of chitinolytic activity, and enzymatic characterization were performed as described previously [15]

N-terminal Amino Acid Sequencing

The *N*-terminal amino acid sequences of the purified 58 kDa chitinase secreted by the *E. coli* transformants and *S. marcescens* KCTC 2172 were determined using an automatic peptide sequencer (ABI 431).

RESULTS AND DISCUSSION

Cloning of 58 kDa Chitinase Gene

S. marcessens KCTC 2172 secretes chitinases with five distinct molecularweights of 58, 54, 52, 35, and 22 kDa into a culture broth. Previously [15,16], the current authors isolated four genes encoding the 54, 52, 35 and 22 kDa chitinase from the PLAFR3 cosmid genomic DNA library and characterized the gene organizations and enzymatic properties of the enzymes. In order to isolate the 58 kDa chitinase gene from a cosmid library of *S. marcescens* KCTC 2172, a PCR probe was synthesized with the help of two primers which corresponded to the N-terminal amino acid sequence of the 58 kDa chitinase and a conserved sequence, common to most bacterial chitinases, including 54 and 52 kDa, FDGVDI-DWE. After PCR amplification, a PCR product of about 750 bp was obtained and used as a probe for screening the cosmid clones. Eight clones, which hybridized to the PCR probe, were isolated, and four clones of them secreted a 58 kDa chitinase into the culture supernatant. The insert sizes at the four clones were about 20-30 Kb. From the smaller clone, a 4.0 kb *Sau*3A DNA fragment, which produced a 58 kDa chitinase in *E. coli* transformants, was subcloned into pBluscript SK (-). After restriction enzyme mapping, A DNA fragment about 2.6 kb, which was digested with *Sal*1 and *Eco*R1, also produced 58 kDa chitinase and was designated *pChi*58. The complete nucleotide sequence of *pChi*58 was determined to be 1,686 bp with a single open reading frame encoding 562 amino acids (Fig. 1).

A putative ribosomal binding site(AAAGGA) and promoter motifs at-10(TTTA) and 35(AATG) were also identified. The *rho*-dependent termination sites CCGGGCGATATCCTTTCGCCCCCGG) were located 17 bp downstream of the translation stop codon(TAA). A comparison of the deduced amino acid sequence of the 58 kDa chitinase gene with the N-terminal amino acid sequence of the secreted protein, underlined in Fig. 1, revealed that the chitinase contained a N-terminal signal peptide consisting of 23 amino acids.

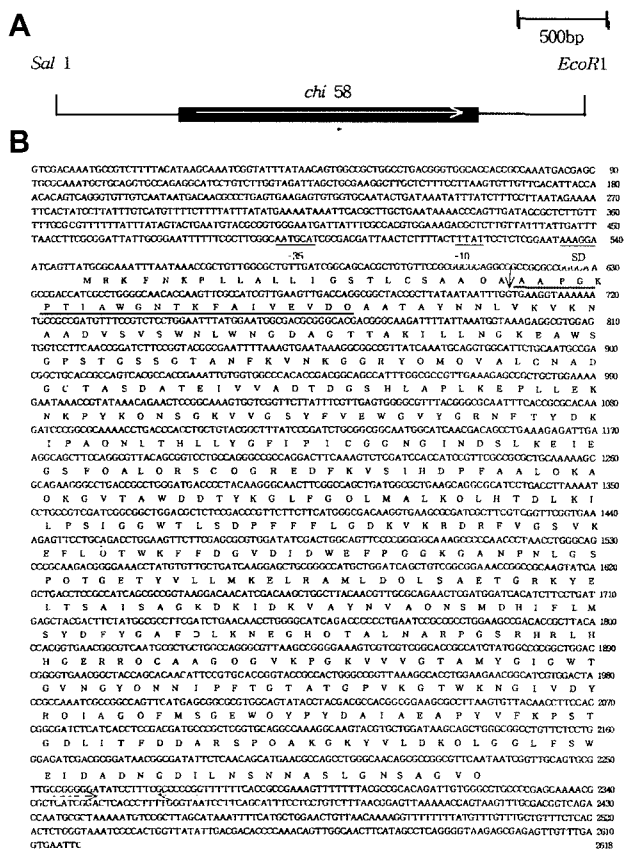

Fig. 1. Physical map (A) and nucleotide sequence of *pChi*58. The putative ribosome binding site (SD) and promoter sequences (-10 and -35) are underlined (thin lines). The N-terminal amino acid sequence of the 58 kDa chitinase is underlined (thick line). The singal peptide cleavage site is indicated by a vertical arrow. The inverted repeat sequences (palindrom) are indicated by horizontal arrows facing each other.

Purification and Characterization of 58 kDa Chitinase and Other Chitinase Isoforms

To compare the properties of the 58 kDa chitinase with other chitinase isoforms, four chitinase isoforms, 54, 52, 35, and 22 kDa chitinase, were purified by previous methods [15]. The 58 kDa chitinase was isolated from the culture supernatant of an *E. coli* transformant containing *pChi*58 using chitin affinity column chromatography (Fig. 2). The purified five chitinase isoforms were compared on acrylamide gel stained with coomassie and activity as shown in Fig. 3. The optimal pH and temperature for the 58 kDa chitinase were pH 5.0 and 50°C, respectively as shown in Table 1. The catalytic specificity of the 58 kDa chitinase was assayed using swollen chitin, chitobiose, and chitotriose as substrates. The major product of the enzymatic reaction was (GlcNAc)2 (Fig. 4, A, B, C). When using(GlcNAc)2 as a substrate, as shown in Fig. 4C, the 58 kDa chitinase degraded it to a monomor form with a very low activity because no chitinolytic product was observed during the enzymatic reaction until after the first 24 h. Meanwhile, when 4-methylumbellyferyl *N*-acetic glucosaminne was used as a substrate analogue of a chitin dimmer, the 58 kDa chitinase did not split it into 4-methylumbellyferon and *N*-acetyl glucosamine, as shown in Fig. 5A. Kless *et al.* [19] previously reported on the chitobiase from *Serratia marcescens* spp. The current authors also partially purified a 95 kDa chitinase which exhibited chitobiase activity as shown in Fig. 5C. The 52 and 35 kDa chitinases were not detected by the fluorometric assay system. As such, 4-Mu-chitin oligomers would appear to be much more sensitive for checking chitinolytic activities than chitin oligomers.

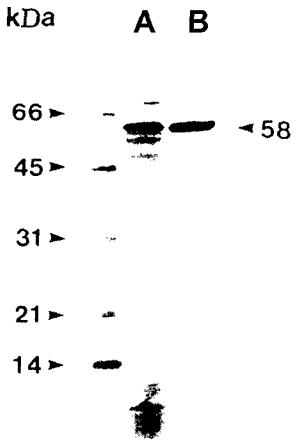

Fig. 2. SDS polyacrylamiamide gel electrophoresis of 58 kDa chitinase purified from culture superatant of *E. coli* carrying *pChi*158. A: culture supernxatant of *E.coli* harboring *pChi*58. B: The purified 58 kDa chitinase.

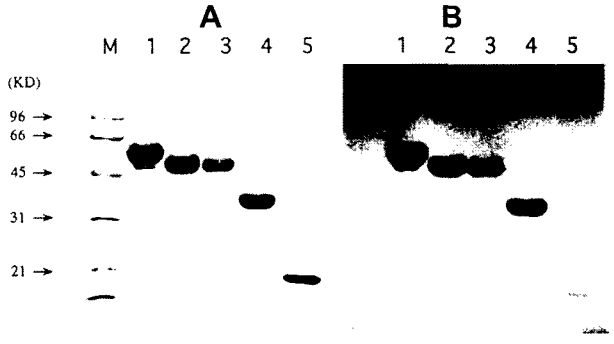

Fig. 3. SDS-PAGE of five chitinase isoforms purified from culture supzernatant of *E. coli* harboring *pChi*58, *pChi*54, *pChi*52, *pChi*35, and *pChi*22. A. coomassie blue staining, B. active staining by calcofluoro white M2R in the gel containing 0.01%(w/v) glycol chitin. M. molecular weight markers, 1. purified 58 kDa chitinase, 2. 54 kDa chitinase, 3. 52 kDa, 4. 35 kDa, 5. 22 kDa.

Table 1. Comparison of properties of five chitinase isoforms from *S. marcescens* KCTC 2172

Isozymes	Properties						
	Mol mass (kD)	Km[a] (uM)	Kcat[a] s^{-1}	pH (Optimum)	Temp. (Optimum)	Products of chitin[b] degradation	Proposed mode of action
Chi A	58	38	25.7	5.0	50°C	NAG, $(NAG)_2$ $(NAG)_5$	Endochitinase
Chi B	54	66	44	5.5	55°C	$(NAG)_2$	Chitobiosidase
Chi C	52	59	39.9	5.5	45°C	NAG	Exochitinase
Chi D	35	42	28.8	5.5	45°C	NAG	Exochitinase
Chi E	22	710	4.46×10^{-2}	5.0	55°C	$(NAG)_2$	Chitobiosidase

[a] when using 4-methylumbellyferyl *N, N'*-diacetylchitobiose as a substrate, [b] when using chitin or chitin-oligomers as substrates
ND; were determined by TLC and fluorometric assay in this work.

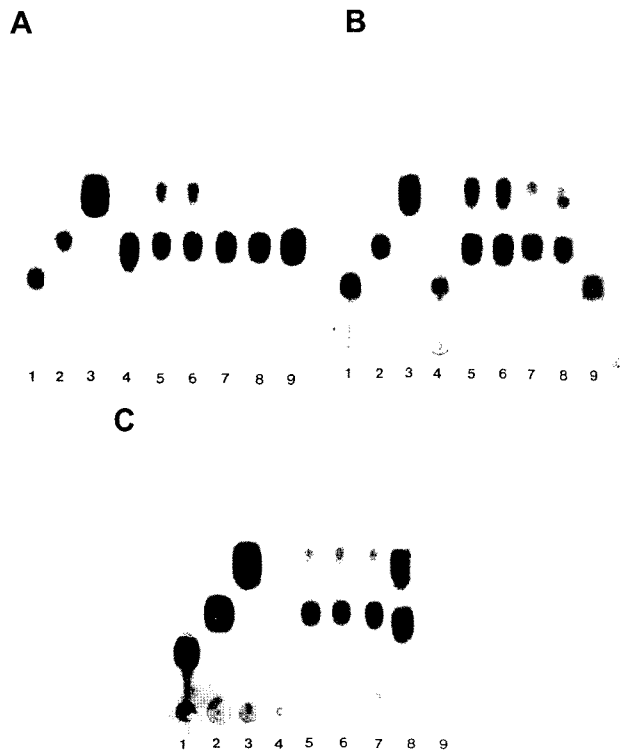

Fig. 4. TLC amalysis of chitinolytic end-products by the chitinase isoforms. A: NAG dimer, B: NAG Trimer, C: chitin polymer. 1: *N*-acetylglucosamine(NAG) Trimer, 2: NAG dimmer. 3: NAG monomer, 4: chitinolytic products from 22 kDa chitinase, 5: chitinolytic products from 35 kDa chitinase, 6: chitinolytic products from 52 kDa chitin, 7: chitinolytic products from 54 kDa chitin, 8: chitinolytic products from 58 kDa chitinase, 9: substrate only.

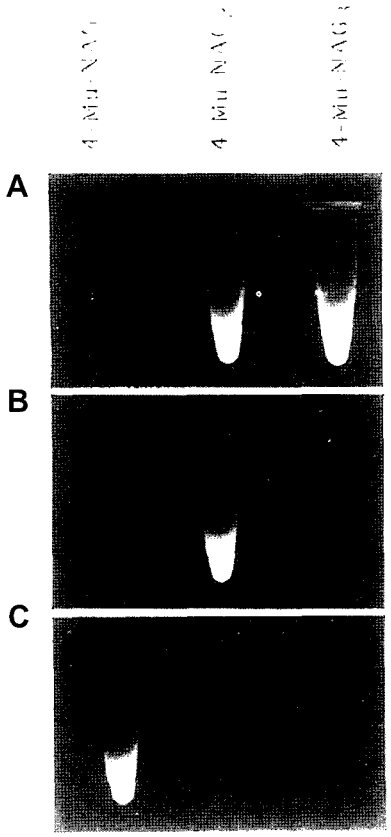

Fig. 5. Fluorometric analysis of chitinolytic end-products by purified chitinase isozymes. A: 58 kDa chitinase, B: 54 kDa and 22 kDa chitinase, C: 95 kDa chitinase.

When taken together, even though there was a slight obscurity in the results it was concluded that the 58 kDa chitinase was an endo-type chitinase.

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