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Expression and Characterization of Endochitinase C from *Serratia marcescens* BJL200 and Its Purification by a One-Step General Chitinase Purification Method

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In this study we cloned, expressed, purified, and charaterized chitinase C1 from *Serratia marcescens* strain BJL200. As expected, the BJL200-ChiC1 amino acid sequence of this strain was highly similar to sequences of ChiC1 identified in two other strains of *S. marcescens*. BJL200-ChiC1 was overproduced in *E. coli* by the T7 expression system, and purified by a one-step hydrophobic interaction chromatography (HIC) with phenyl-sepharose. BJL200-ChiA and BJL200-ChiB had an approximately 30-fold higher k_{cat} and 15 fold-lower K_m than BJL200-ChiC1 for the oligomeric substrate 4-methylumbelliferyl-*β*-*D*-*N*-*N*′-*N*″-triacetylchitotrioside, while BJL200-ChiC1 was 10–15 times faster than BJL200-ChiB and BJL200-ChiA in degrading the polymeric substrate CM-chitin-RBV. BJL200-ChiC1 degradation of *β*-chitin resulted in a range of different chito-oligosaccharides $(GlcNAc)_2$ (main product), GlcNAc, $(GlcNAc)_3$, $(GlcNAc)_4$, and $(GlcNAc)_5$, indicating endo activity. The purification method used for BJL200-ChiC1 in this study is generally applicable to family 18 chitinases and their mutants, including inactive mutants, some of which tend to bind almost irreversibly to chitin columns. The high specificity of the interaction with the (non-chitinous) column material is mediated by aromatic residues that occur in the substrate-binding clefts and surfaces of the enzymes.

Key words: *Serratia marcescens*; chitinase C; purification; hydrophobic interaction chromatography; endochitinase

Chitin, a 1,4-*β*-linked polymer of *N*-acetyl-*β*-D-glucosamine (GlcNAc), is the second most abundant biopolymer in nature, and is a major structure component of the exoskeleton of insects and crustaceans and the cell walls of some fungi. Hydrolysis of the glycosidic bonds in chitin is catalyzed by chitinases, which are found in all types of organisms, from viruses to humans. On the basis of sequence similarities, chitinases are classified into two different families, families 18 and 19, that differ in structure and mechanism of catalysis.[1] The catalytic domains of family 18 chitinases have an $(\alpha/\beta)_8$ (TIM-barrel) fold.[2–4] In addition to the catalytic domain, many family 18 chitinases contain one or more domains that are involved in binding to the substrate (chitin-binding domains).[5–7]

The soil bacterium *Serratia marcescens* produces up to four different chitinases (A, B, C1, and C2), a chitin binding protein (CBP21), and a chitobiase when grown on chitin.[8–12] The crystal structures of ChiA and ChiB have been determined.[3,4,13] Based on structural considerations and the outcomes of a variety of experiments, it has been suggested that ChiA and ChiB are exoenzymes that degrade chitin chains from opposite directions.[4,10,14–17] Even so, both enzymes have shown endo activity on the soluble substrate chitosan.[18] Both enzymes contain a deep substrate-binding groove, which is extended by an aromatic surface of a putative chitin-binding domain. The suggestion that these enzymes degrade chitin chains from opposite ends was originally based on the fact that the chitin-binding domains are located on opposite sides of the catalytic center,[4] and it has recently been confirmed by elegant experiments with end-labeled substrates by Hult *et al.*[16]

The *chiC* gene has been described for two different *S. marcescens* strains.[10,19] Judged by the deduced amino acid sequence, ChiC1 contains two chitin-binding domains, which are located on the C-terminal side of the molecule. ChiC2 is a proteolytic derivative of ChiC1 that can emerge under certain experimental conditions, and that corresponds to the catalytic domain only.[19,20] The crystal structures of ChiC1 or ChiC2 are not known.

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Abbreviations: 4MU-GlcNAc$_3$, 4-methylumbelliferyl-*β*-D-N-N′-N″-triacetylchitotrioside; ChiA, chitinase A; ChiB, chitinase B; ChiC, chitinase C; GlcNAc, *N*-acetyl-*β*-D-glucosamine; HIC, hydrophobic interaction chromatography

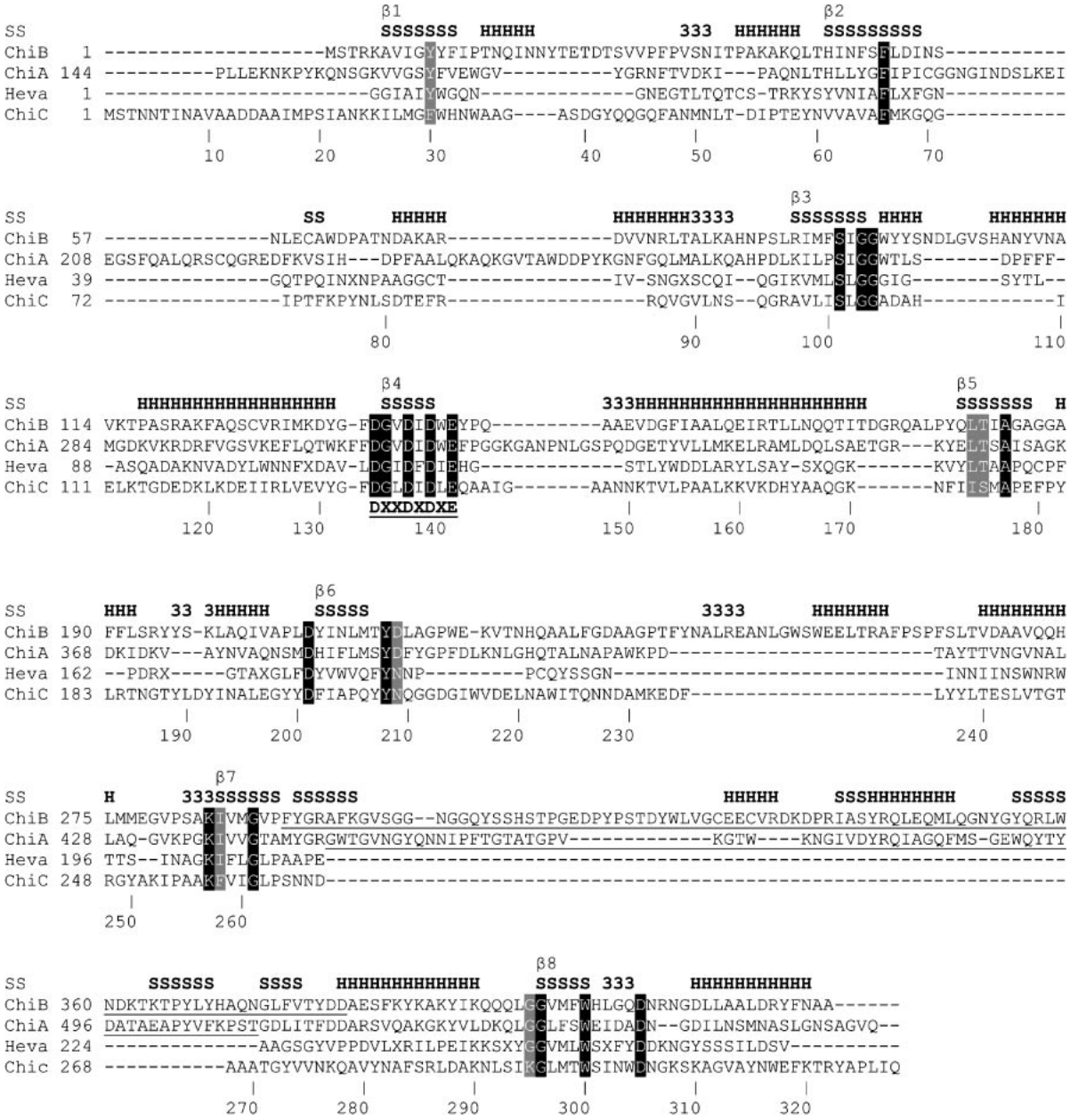

Fig. 1. Sequence Alignment of the Catalytic Domains of ChiA (chitinase A from *S. marcescens* BJL200), ChiB (chitinase B from *S. marcescens* BJL200), ChiC1 (chitinase C1 from *S. marcescens* BJL200), and Heva (Hevamine from *Hevea brasiliensis*).

Sequence numbering and secondary structure (SS) is based on the sequence and structure of ChiB.[4] Conserved resides are highlighted in black. Residues conserved in ChiA, ChiB, and hevamine, but not in ChiC1, are highlighted in gray. The stretches of residues constituting the α/β-domain present in ChiA and ChiB, but lacking in ChiC1 and hevamine, are underlined. An x in the sequences indicates a small insertion in hevamine.

Sequence alignments indicate that BJL200-ChiC differs widely from BJL200-ChiA and BJL200-ChiB in that it lacks the so-called α/β-domain, which makes up one of the walls of the deep substrate-binding clefts in the two exoenzymes (Fig. 1). Judged by its sequence, the catalytic domain of BJL200-ChiC resembles that of hevamine, a plant endochitinase with a shallow active site cleft (Fig. 1).[21]

Synergistic effects on the rate of degradation of colloidal chitin have been observed in combining BJL200-ChiA and BJL200-ChiB.[15,22] Clear synergism on the hydrolysis of powdered chitin has also been observed for several combinations of *Serratia* chitinases, including BJL200-ChiC1.[10,22] These observations indicate that ChiA, B, and C1 have different roles in the degradation of chitin, and suggests different modes of action in the three enzymes. Indeed, recent studies with chitosan as a substrate combined with work by Hult *et al.*[16] strongly indicate that ChiA and ChiB act as processive exo-enzymes on chitin, whereas ChiC1 is a non-processive endo-enzyme.

We have cloned, purified, and characterized ChiA and

ChiB from *S. marcescens* BJL200.[15,23,24] To obtain a complete picture of the chitinolytic machinery of this strain and to obtain more detailed insight into the enzymatic properties of BJL200-ChiC1, we have cloned and expressed its *chiC*. Previously, BJL200-ChiA and BJL200-ChiB from this *S. marcescens* strain were purified to homogeneity by an easy one-step procedure involving hydrophobic interaction chromatography.[15,23] As the second part of this study, we wanted to determine whether this potentially general purification method would also be applicable to a chitinase quite different from ChiA and ChiB, ChiC1 (see below). The availability of such a potentially general purification method is important, since some chitinases, and especially their mutants (G. Vaaje-Kolstad and V. G. H. Eijsink, unpublished observations) are not easily purified by common purification methods that are based on interactions with the substrate (*e.g.*, chitin beads). After purification of BJL200-ChiC1 by this method, the enzyme was characterized with respect to its activity towards oligomeric (4MU-$GlcNAc_3$) and polymeric substrates (CM-chitin-RBV; β-chitin). In order to provide a structural perspective on the ChiC catalytic domain for discussion of the enzymatic mode of action, the BJL200-ChiC1 sequence was aligned against a structural alignment of BJL200-ChiA, BJL200-ChiB, and hevamine.

Methods

Bacterial strains and culture conditions. Serratia marcecens BJL200 was grown in Luria-Bertani (LB) medium at 30 °C for chromosomal DNA extraction. *Escherichia coli* TOP 10 cells containing *chiC* in plasmid TOPO TA were grown in LB medium supplemented with 100 µg/ml ampicillin for plasmid preparation. *E. coli* BL21(DE3) star cells (Invitrogen Carlsbad, CA) transformed with the *chiC* gene cloned in pRSETB were grown in NZCYM medium supplemented with 100 µg/ml of ampicillin for chitinase production. In the latter two cases, the cultures were grown at 37 °C.

DNA extraction and cloning of the chiC *gene from* S. marcescens *in* E. coli. Chromosomal DNA from *S. marcescens* was isolated according to the method described by Saito and Miura,[25] with some small modifications.[26] The *chiC* gene was amplified from *S. marcescens* strain BJL200 chromosomal DNA by PCR using two primers constructed based on nucleotide sequences upstream of a putative promoter and downstream of putative inverted repeats of *chiC* from *S. marcescens* strain KCTC2172.[19] The sense primer upstream of *chiC* was 5′-CCACGCTTGAGCGACATTGAAACCATGC-3′, and the antisense primer downstream of *chiC* was 5′-GGGCAGCATTGCCGATTTATCAGCG-3′. The amplification consisted of 25 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C using Tag polymerase (Promega, Madison, WI). The resulting PCR product was cloned into TOPO TA cloning vector (Invitrogen) and sequenced. For control of the sequence, see DNA sequencing and sequence analysis below. To verify the amplified *chiC* sequence and to ensure that no insertions or deletions occurred during the PCR reaction, the DNA sequence of the *chiC* gene with upstream and downstream sequences was verified in three different clones, yielding identical and correct sequences. For overexpression of ChiC in *E. coli* using T7 promoter vector pRSETB (Invitrogen), two primers, CCGGGAATTCCATATGAGCACACAAATAACAC (NdeIChiC1) and M13 forward primer, were used in a PCR reaction using the TOPO clone as a template. This PCR reaction produces the *chiC* gene with an in-frame ATG and a *Nde*I site at the start of the gene. The PCR product was digested with *Nde*I and *Xho*I and ligated into pRSETB digested with the same enzymes. The resulting plasmid (pRSETB-chiC1) was transformed into *E. coli* BL21(DE3) star.

DNA sequencing and sequence analysis. The sequence of *chiC* was determined using the ABI PRISM TM Dye Terminator Cycle Sequencing Ready Reaction Kit and and an ABI PRISM 37878 DNA Sequencer (Perkin Elmer Applied Biosystems, Foster City, CA). Computer analysis of the nucleic acid sequence was done using the program BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). ORF analyses were done with the translation tool of the ExPasy Proteomix tools. BLAST searches were carried out with software available from the National Center for Biotechnology Information (NCBI) of USA. The sequence of the BJL200-ChiC catalytic domain (BJL200-ChiC2, residues 1-327) was aligned with the profile of the structure-based multiple sequence alignment of BJL200-ChiA, BJL200-ChiB, and hevamine (see van Aalten *et al.*[4]), using Clustal-V.[27] Due to limited sequence similarity between the initial part of the sequence and the profile, the alignment of the first 90 residues was guided by multiple alignment of BJL200-ChiC2 and its 20 most similar sequences (identified by a BLAST search).

One-step purification of BJL200-ChiC1. In the production of BJL200-ChiC1 in *E. coli*, BL21(DE3) expression of *chiC1* was induced by 0.4 mM isopropyl-b-D-thiogalactoside (IPTG) when the OD_{600} was between 0.5 and 0.7. After growth for 150 min at 37 °C, the cultures were harvested. Harvested cells were fractionated by the osmotic shock method,[28] and BJL200-ChiC1 was purified from the resulting periplasmic extracts in a one-step purification step using a Phenyl-Superose HR 5/5 column in an FPLC system (GE Healthcare, Oslo, Norway) as previously reported by Brurberg *et al.*[15,23]

Enzymology. Determination of specific activities of BJL200-ChiC1 fractions during purification was done

using 4-methyllumbelliferyl-β-D-N,N′-diacetylchitobioside [4MU-$(GlcNAc)_2$] (Sigma, St. Louis, MO) as described previously.[29,30] A 69-µM substrate solution was used in the assays. The enzyme activity was expressed as nmol 4MU released per s and mg protein.

Kinetic parameters for ChiA, B and, C1 were determined using 4MU-$(GlcNAc)_3$ by initial rate measurements at various substrate concentrations. The reactions were carried out in 125 µl of 50 mM citrate-phosphate buffer, pH 6.3, with substrate concentrations ranging from 2–8 µM (BJL200-ChiA), 1–10 µM (BJL200-ChiB), and 40–120 µM (BJL200-ChiC1). The enzyme concentrations in assays were 0.15 nM, 0.2 nM, and 10 nM for BJL200-ChiA, BJL200-ChiB, and BJL200-ChiC1 respectively. The reactions were stopped by adding 125 µl 1 M Glycin/NaOH, pH 10.5. The amount of 4-MU released was measured with a Cary Eclipse Fluorescence Spectrophotometer (Varian Scientic Instruments, Palo Alto, CA) with excitation at 365 nm and emission at 460 nm, using a 4MU standard curve. Product formation was monitored at four time points for each reaction, and was found to be linear for at least 12 min for all substrate concentrations. While BJL200-ChiA and BJL200-ChiC1 quantitatively liberate the 4MU group from 4MU-$(GlcNAc)_3$, BJL200-ChiB converts this substrate exclusively to 4MU-GlcNAc and $(GlcNAc)_2$. Hence, the activity of BJL200-ChiB was monitored in a mixed assay based on measuring the disappearance of substrate. To achieve this, substrate remaining at the end of the reactions was quantitatively converted to free 4MU by adding an overdose of 60 nM BJL200-ChiA for 15–30 s, before stopping the reactions.

For pH dependency measurements, concentrations of 30, 60, and 120 µM of 4MU-$(GlcNAc)_3$ (substrate inhibition was not observed until concentrations greater than 220 µM) and 53 nM (pH 3.9 to 6.39) and 106 nM (pH 7 to 9) of BJL200-ChiC1 were used. Aliquots were taken out and analyzed at 13, 30, and 45 seconds (pH levels, 3.5 and 3.9), 1, 2, and 3 min (pH levels, 4.4, 4.8, and 5.3), and 2.5, 5.0, and 7.5 min (pH levels 6.3, 7.0, 8.0, and 9.0). Sample volumes were 50 µl, and the reactions were stopped by addition to solution of 1.95 ml of 0.2 M Na_2CO_3.

K_m- and k_{cat}-values were determined using hyperbolic regression with the program Hyper (J.S. Easterby, University of Liverpool, obtainable from homepage http://www.liv.ac.uk./~jse/software.html).

Chitinase activity was also measured using the polymeric substrate, carboxymethyl chitin Remazol Brilliant Violet (CM-chitin-RBV, Loewe Biochemica, Munich, Germany). A 5-ml reaction mixture containing 37.5 mM NaAcetate, pH 5.5, and 0.6 mg/ml CM-chitin-RBV was preincubated at 37 °C before the chitinase was added to the solution. The reaction was monitored every third min up to 15 min by transfer of a sample of 800 µl to a new tube containing 200 µl 0.2 M HCl to stop the reaction. After mixing, the samples were cooled on ice for 10 min and centrifuged at maximum speed for 5 min in an Eppendorf centrifuge. Absorbance in the supernatants was measured at 550 nm and corrected for adsorption in a control sample without chitinase. The enzyme concentration was 2 nM for BJL200-ChiA and BJL200-ChiB and 0.5 nM for BJL200-ChiC1. With these enzyme concentrations and the actual substrate concentrations, we found a linear release increase in adsorption with time for at least 12 min. The specific activities were expressed as $\Delta A550\,min^{-1}$ per pmole protein.

Hydrolysis of squid pen β-chitin (3 µm in size; Seikagaku, Tokyo) was carried out at 37 °C in 50 mM sodium acetate buffer, pH 6.1.

Protein concentrations were determined by the method of Bradford[31] using a protein assay from Bio-Rad. Bovine Serum albumin (BSA) was used as the standard.

Chromatography of chito-oligosaccharides. Sizes and anomers of chito-oligosaccharides were determined by HPLC using a Tosoh TSK Amide 80 column (0.46 × 25 cm) with an Amide 80 guard-column. A 10 µl-sample was injected onto the column, and chitin fragments were eluted isocratically at 0.7 ml/min with 70% acetonitrile at room temperature. UV absorbance of the chito-oligosaccharides was measured at 210 nm, and the peaks were identified using chito-oligosaccharide standards purchased from Sigma (St. Louis, MO).

Results

Nucleotide sequence and deduced amino acid sequence

The *chiC* gene with flanking sequences was isolated from the genomic DNA of *S. marcescens* BJL200 by PCR amplification using two primers homolog to a upstream and downstream region of *chiC* from *S. marcescens* KCTC2172.[19] The resulting PCR product was cloned into TOPO TA cloning vector. Three individual clones from three individual PCR reactions were sequenced to ensure that the obtained sequence, of 1,862 nucleotides, was correct. The same nucleotide sequence was obtained in the three individual clones.

One open reading frame (ORF) of 1,440 nucleotides encoding a protein of 480 amino acids was identified in the sequenced DNA region. The degrees of identity to the two previously described enzymes were 95% and 96% at the DNA level and 93% and 98% at the protein level for the enzymes from strain KCTC2172[19] and strain 2170,[20] respectively. This clearly indicates that the gene cloned here is the counterpart of the two previously described *chiC* genes. A possible Shine Dalgarno sequence (GAGG) five bases upstream of the ATG start codon and a putative terminator nine bases downstream of the UAA stop codon were identified (data not shown). These sequences are also found in the upstream and downstream regions of the two other *chiC* genes published for *S. marcescens*.[19,20]

The sequences of BJL200-ChiC1 and the corresponding gene *chiC* with upstream and downstream regions

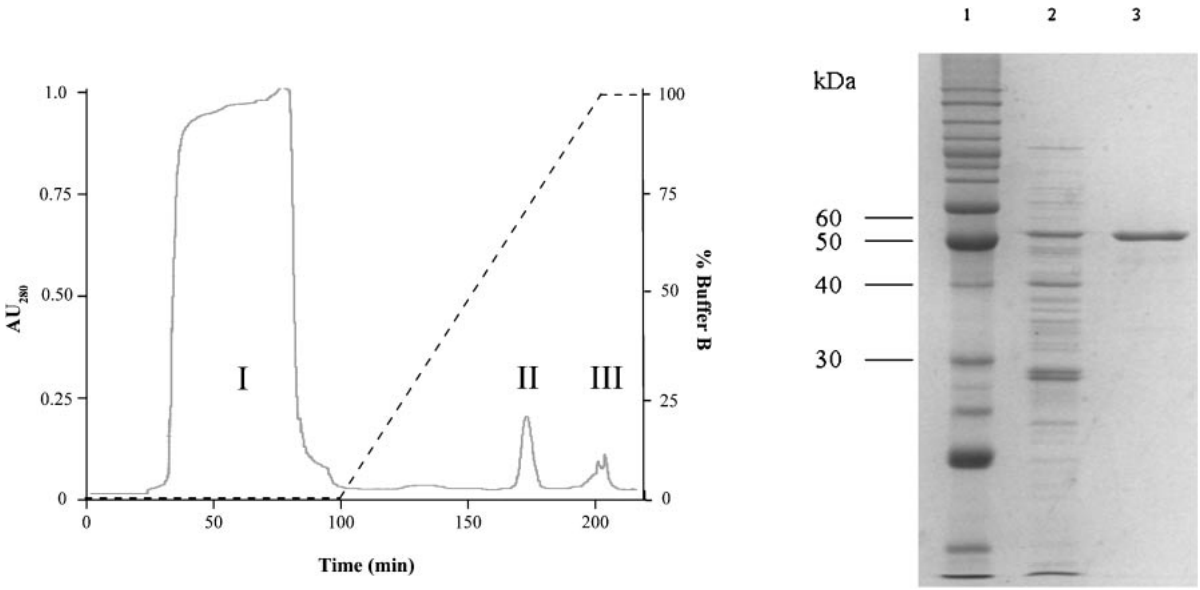

Fig. 2. Purification of ChiC1.

Left panel, hydrophobic interaction chromatography. The picture shows the A_{280} trace and the gradient (- - - - - - -) from 100% buffer A (20 mM Tris, 1 mM EDTA, and 0.5 M $(NH_4)_2SO_4$, pH 8.0) to 100% buffer B (20 mM Tris and 4% isopropanol, pH 8.0). The first large peak in the chromatogram (marked I) represents waste proteins, whereas the second peak contains ChiC1 (marked II). The ChiC1 peak is symmetrical and shows good separation characteristics. The third peak (marked III) does not contain ChiC1 as controlled by SDS–page analysis. Right panel, SDS–page gel analysis of ChiC1 purification. Lane 1, protein standard; lane 2, the periplasmatic extract; lane 3, purified protein from peak II.

Table 1. One-Step Purification of *S. marcescens* Chitinase BJL200-ChiC1 by Hydrophobic Interaction Chromatography

Fraction	Volume (ml)	Total protein (mg)	Total activity (nmol s^{-1})	Recovery (%)	Specific activity (nmol s^{-1} mg^{-1})
Periplasmatic extract	2.0	2.92	4.4	100	1.5
Peak fractions	1.7	0.31	3.8	86	12.3

from *S. marcescens* strain BJL200 have been deposited in the EMBL Nucleotide Sequence Database under accession no. AJ630582.

Comparison of the catalytic domain of BJL200-ChiC1 with other family 18 chitinases

A structural alignment of the catalytic domain of BJL200-ChiC1 with the corresponding domains in BJL200-ChiA and BJL200-ChiB from *S. marcescens* and with hevamine is shown in Fig. 1. Generally, there were very few fully conserved residues. The few regions with full sequence conservation included the catalytically crucial SXGG sequence at the end of the third β-strand and the DXXDXDXE motif with the catalytic glutamic acid that spans the fourth β-strand. The main difference among the four catalytic domains was the absence of the so-called α/β-domain in hevamine and BJL200-ChiC1.[3] In BJL200-ChiA and BJL200-ChiB, this domain is situated between the seventh β-strand and α-helix, and it consists of residues 295–373 in BJL200-ChiB and residues 447–509 in BJL200-ChiA. This domain, comprising five β-sheets and two helixes, makes up one of the sides of the deep substrate binding cleft in BJL200-ChiA and BJL200-ChiB.

Overproduction and purification of BJL200-ChiC1

BJL200-ChiC1 was overproduced in *E. coli* by the T7 expression system.[32] After induction with IPTG, the cells were fractionated by osmotic shocking. It has been found that the osmotic shocking method used here somehow leaves most of recombinantly produced BJL200-ChiA (containg an N-terminal signal peptide for sec-dependent secretion) or BJL200-ChiB (not containing a recognizable secretion signal[24]) in the periplasmic fraction, providing a chitinase-enriched starting material for purification. Although BJL200-ChiC1 does not contain any recognizable signal for secretion, approximately 88% of the chitinase activity was detected in the periplasmic extract. BJL200-ChiC1 was purified from this extract using hydrophobic interaction chromatography (HIC) (Fig. 2). In a typical experiment, the recovery of enzyme activity is between 80% and 90% (Table 1). This is in good agreement with the yields of BJL200-ChiA and BJL200-ChiB by the same purification method.[15,23] SDS–PAGE showed that the chitinase peak contained a highly pure protein of approximately 50 kb (Fig. 2).

Since the results with BJL200-ChiC1 indicated that the periplasmic extraction procedure combined

Table 2. Kinetic Parameters and Hydrolytic Activity of *Serratia marcescens* Chitinases Using 4MU-$(GlcNAc)_3$[22] and CM-Chitin-RBV as Substrates

	4MU-$(GlcNAc)_3$		CM-chitin-RBV	
Enzyme	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\,\mu M^{-1}$)	Sp. Activity ($\Delta OD550\,min^{-1}$, $pmole^{-1}$)
BJL200-ChiA	4.2 ± 0.4	67.4 ± 3	16	0.0035 ± 0.0005
BJL200-ChiB	6.8 ± 1.0	56.8 ± 4.2	8.4	0.0047 ± 0.0010
BJL200-ChiC1	79.8 ± 7.5	2.0 ± 0.1	0.025	0.066 ± 0.001

with hydrophobic interaction chromatography does indeed provide a general method for purification of family 18 chitinases, we applied the same one-step procedure to a variety of chitinase variants, including inactive mutants such as BJL200-ChiC1-E141Q, BJL200-ChiB-E144Q, and BJL200-ChiA-E315Q, which we could not easily purify using chitin-affinity chromatography. All these mutants were successfully purified, with yields and purities comparable to those of the wild-type enzymes. BJL200-ChiB variants with mutations in the chitin-binding domain or in aromatic residues in the substrate-binding groove were also purified by this method, but they eluted from the column earlier than other BJL200-ChiB variants (see below for further discussion).

Enzymatic characterization of BJL200-ChiC1 and comparison with BJL200-ChiA and BJL200-ChiB

The hydrolytic activities of BJL200-ChiA, BJL200-ChiB, and BJL200-ChiC1 against various chitinous substrates were determined. Extensive reports on comparative studies with a variety of substrates may be found in the work of Horn *et al.*[22,33] For the purpose of this study, we compared activity of the three enzymes towards an oligomeric substrate, 4MU-$(GlcNAc)_3$, with the activity towards a polymeric substrate, CM-chitin-RBV. In addition, we studied product formation by BJL200-ChiC1 from β-chitin.

Table 2 summarizes the activities of BJL200-ChiA, BJL200-ChiB, and BJL200-ChiC1 against 4MU-$(GlcNAc)_3$ and CM-chitin-RBV. Both BJL200-ChiA and BJL200-ChiB had higher activity against 4MU-$(GlcNAc)_3$ than did BJL200-ChiC1. When kinetic parameters k_{cat} and K_m were determined using this substrate, BJL200-ChiA and BJL200-ChiB had approximately 30 fold higher k_{cat} and 15 fold lower K_m than BJL200-ChiC1. On the other hand, the specific activity of BJL200-ChiC1 towards the polymeric substrate CM-chitin-RBV was approximately 10 and 15 times higher respectively than that of BJL200-ChiB and BJL200-ChiA.

As Fig. 3 shows, initial product mixtures formed during degradation of β-chitin with BJL200-ChiC1 contained not only $(GlcNAc)_2$ and GlcNAc, but also the longer oligosaccahrides $(GlcNAc)_3$, $(GlcNAc)_4$, and $(GlcNAc)_5$. Degradation of β-chitin by BJL200-ChiA and BJL200-ChiB initially yields $GlcNAc_2$ and a small amounts of GlcNAc and $(GlcNAc)_3$.[33] The fact that longer oligomers are initially produced by BJL200-ChiC1 is in accordance with the notion that this enzyme is an endo-acting one. However, as the reaction proceeds, the longer oligosaccharides are degraded and the final product is a mixture of only GlcNAc and $(GlcNAc)_2$, as for BJL200-ChiA and BJL200-ChiB.[33]

The pH dependency of 4MU-$(GlcNAc)_3$ on BJL200-ChiC1 was investigated (Table 3). The results show that BJL200-ChiC1 has a pH optimum at 3.9 ($16.1 \pm 3.0\,s^{-1}$) and that enzyme turnover decreases with increasing pH. Substrate efficiency is also highest at pH 3.9 ($0.114\,s^{-1}\,\mu M^{-1}$), with subsequent decreases with increasing pH. The enzyme was not stable for the time window of the experiment at pH levels lower than 3.5, and hence the kinetic parameters were not determined at pH levels lower than this.

Discussion

In this study, we cloned, expressed, and purified ChiC1 from *S. marcescens* strain BJL200. The BJL200-ChiC1 amino acid sequence resembled those of the two previously described ChiCs from *S. marcescens*. Generally, family 18 chitinases are highly conserved between *S. marcescens* strains. For the BJL200 and 2170 strains, the sequence identities at the amino acid level are 99.3%, 98.3%, and 98.3% for ChiA, ChiB, and ChiC1 respectively. ChiC1 from *Serratia marcescens* strain 2170 has been characterized by Suzuki *et al.*[20] This enzyme consists of a catalytic domain and two putative C-terminal chitin-binding domains. The catalytic domain lacks the α/β-domain, which makes up one of the walls of the substrate binding clefts in ChiA and ChiB. The absence of this domain and the consequent shallowness of the predicted substrate-binding groove are in accordance with the idea that ChiC1 is an endochitinase, like hevamine.[34]

The experimental results presented above also confirm the idea that BJL200-ChiC1 acts as an endo-enzyme: BJL200-ChiC1 is much less active than the the processive exo-chitinases towards oligomeric substrates, whereas it is much more active towards complex polymeric substrates, *i.e.*, substrates that are likely to be the primary target of the endo-acting member of the chitinolytic enzyme machinery. Our results are in agreement with those obtained by Suzuki *et al.*,[10] who reported that 2170-ChiC1 hydrolyzes $(GlcNAc)_4$ more slowly than do 2170-ChiA and 2170-ChiB.

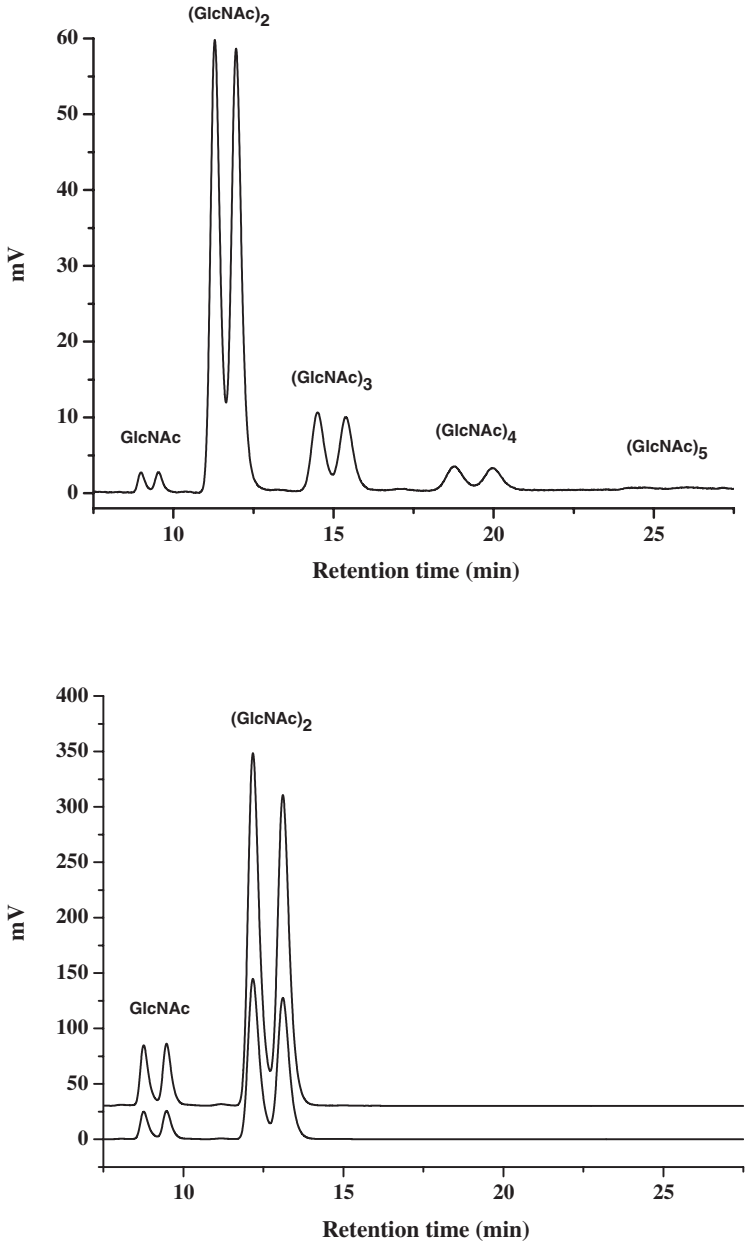

Fig. 3. HPLC Analysis of Degradation Products of β-Chitin by ChiC after 10 min (top) and 1 (bottom, lower panel) and 48 h (bottom, upper panel). The α-anomer eluted before the β-anomer; thus, two peaks were observed for each chito-oligosaccharide. (There was no temperature control of the column during experiments, and retention time and shape of the peaks can vary with column temperature).

Table 3. pH Dependency of 4MU-$(GlcNAc)_3$ Hydrolysis by BJL200-ChiC1

pH	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (s^{-1} μM^{-1})
3.5	14.3 ± 2.3	158 ± 35	0.091
3.9	16.1 ± 3.0	140 ± 38	0.114
4.4	9.3 ± 1.2	98.1 ± 20.8	0.095
4.8	6.9 ± 0.7	95.5 ± 15.5	0.072
5.3	5.0 ± 0.6	94.0 ± 19.8	0.053
6.3	2.0 ± 0.1	80.0 ± 7.5	0.025
7.0	0.74 ± 0.07	83.7 ± 12.9	0.009
8.0	0.31 ± 0.04	113.1 ± 18.3	0.003
9.0	0.06 ± 0.01	90.7 ± 21.6	0.001

Whereas ChiA and ChiB from *Serratia marcescens* (both BJL200 and 2170) have broad neutral pH optima for activity,[10,15] this study indicates that BJL200-ChiC1 has a clear acidic pH optimum, at about pH 4.0. Moreover, BJL200-ChiC1 contains an Asn at position 209, whereas BJL200-ChiA and BJL200-ChiB contain an Asp at the corresponding positions, 391 and 215 respectively (Fig. 1). Using site-directed mutagenesis, Synstad *et al.* have shown for BJL200-ChiB that mutation of Asp215 to asparagine leads to a drastic acidic shift in the pH optimum of this enzyme.[29] Asp215 in BJL200-ChiB stabilizes the observed boat conformation of the sugar in the -1 subsite during hydrolysis by accepting a hydrogen bond from the O6

hydroxy group of the sugar.[29] In addition, Asp215 increases the pK_a values of the Asp142-Glu144 diad, which is a part of the conserved DXXDXDXE motif.[29] For BJL200-ChiB-D215N, the hydrogen bond to the sugar moiety is retained while the increase in the pK_a values for the Asp142-Glu144 diad is abolished, resulting in a pH profile similar to that of BJL200-ChiC1.

Cloned ChiC1 protein from *S. marcescens* BJL200 was recovered from *E. coli* BL21(DE3) by extracting periplasmic proteins using cold osmotic shock treatment of the cells. The same procedure has worked very well for BJL200-ChiA and BJL200-ChiB, as well as for numerous BJL200-ChiB variants produced in our laboratories.[15,23] Remarkably, of these proteins, only BJL200-ChiA contains a typical N-terminal signal sequence for *sec*-dependent secretion. Although the reasons for this are unclear, it is clear that the osmotic shock method produces very good starting preparations for purification of recombinant family 18 chitinases produced in *E. coli*.

The use of perplasmic extracts as starting material permitted the purification of BJL200-ChiC1 by a single fast hydrophobic interaction chromatography step, which has also been used successfully in the purification of BJL200-ChiA and BJL200-ChiB.[15,23] As for the purification of BJL200-ChiA and BJL200-ChiB, BJL200-ChiC1 was bound to the column material in 20 mM Tris-buffer with 0.5 M ammoniumsulphate, and was eluted in a sharp symmertrical peak after the concentration of buffer B, consisting of 20 mM Tris and 4% isopropanol, reached 100% in the chromatographic system (Fig. 2). Thus, remarkably, this one-step chromatographic procedure appears to be a general method of purifying family 18 chitinases from *S. marcescens*. It is tempting to speculate that the interaction between the chitinases and phenyl-sepharose somehow involves stacking interactions between the aromatic phenyl ring and the many aromatic residues that line the substrate-binding grooves of the enzymes and that cover the putative binding surfaces of the chitin-binding domains.[3,4,13,20] In accordance with this idea, it was observed that BJL200-ChiB variants in which residues Trp97 or Trp220 in the substrate-binding groove had been replaced by alanine, as well as a variant truncated at residue 446 (*i.e.*, lacking the C-terminal chitin-binding domain[4]) eluted earlier from the column, at approximately 80% buffer B (data not shown).

The one step hydrophobic interaction chormatography procedure also allowed for the purification of inactive mutants such as BJL200-ChiC1-E141Q, BJL200-ChiB-E144Q, and BJL200-ChiA-E315Q. When chitin affinity column chromatography, a standard chromatographic method for the isolation of chitinases,[10,12,19,20,35] is used, these inactive mutants tend to become irreversibly bound to the chitin beads of the column, and can be eluted only as denatured proteins with a 0.3 M NaOH solution (G: Vaaje-Kolstad and V. G. H: Eijsink, unpublished observations).

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