BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

A novel goose-type lysozyme gene with chitinolytic activity from the moderately thermophilic bacterium *Ralstonia* sp. A-471: cloning, sequencing, and expression

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Abstract In this study, we cloned the gene encoding goose-type (G-type) lysozyme with chitinase (Ra-ChiC) activity from *Ralstonia* sp. A-471 genomic DNA library. This is the first report of another type of chitinase after the previously reported chitinases ChiA (Ra-ChiA) and ChiB (Ra-ChiB) in the chitinase system of the moderately thermophilic bacterium, *Ralstonia* sp. A-471 and also the first such data in *Ralstonia* sp. G-type lysozyme gene. It consisted of 753 bp nucleotides, which encodes 251 amino acids including a putative signal peptide. This ORF was modular enzyme composed of a signal sequence, chitin-binding domain, linker, and catalytic domain. The catalytic domain of Ra-ChiC showed homologies to those of G-type lysozyme (glycoside hydrolases (GH) family 23, 16.8%) and lysozyme-like enzyme from *Clostridium beijerincki* (76.1%). Ra-ChiC had activities against ethylene glycol chitin, carboxyl methyl chitin, and soluble chitin but not against the cell wall of *Micrococcus lysodeikticus*. The enzyme produced α-anomer by hydrolyzing β-1,4-glycosidic linkage of the substrate, indicating that the enzyme catalyzes the hydrolysis through an inverting mechanism. When *N*-acetylglucosamine hexasaccharide [(GlcNAc)6] was hydrolyzed by the enzyme, the second and third glycosidic linkage from the non-reducing end were split producing (GlcNAc)2+(GlcNAc)4 and (GlcNAc)3+ (GlcNAc)3 of almost the same concentration in the early stage of the reaction. The G-type lysozyme hydrolyzed (GlcNAc)6 in an endo-splitting manner, which produced (GlcNAc)3+(GlcNAc)3 predominating over that to (GlcNAc)2+(GlcNAc)4. Thus, Ra-ChiC was found to be a novel enzyme in its structural and functional properties.

Keywords Goose-type lysozyme · *Ralstonia* · Chitinase · Oligosaccharides · Molecular cloning

Introduction

Chitin, a β-1,4 polymer of *N*-acetyl-D-glucosamine (GlcNAc), is the second abundant biopolymer found in nature after cellulose. At least, 10 gigatons (1×10^{13} kg) of chitin are synthesized and degraded each year in the biosphere (Muzzarelli 1999). This natural resource is relatively easily accessible, e.g., as crab and shrimp shell waste.

N-acetyl-chitooligosaccharides and chitooligosaccharides have varied biological functions and many potential applications in a wide range of fields (Tokoro et al. 1988; Hirano and Nagano 1989). To obtain enzyme that can be applied to develop environmentally friendly techniques in chitin-oligosaccharide production, we have isolated a thermophilic strain belonging to the genus *Ralstonia*, which is capable of degrading chitin in a chitin-containing waste (Sutrisno et al. 2004). Chitinase A (Ra-ChiA) was excreted into culture medium and was constantly produced until the colloidal chitin was wholly degraded. The other chitinase, chitinase B (Ra-ChiB), showed a trace amount of protein in the culture medium, and had weaker activity than *Ralstonia* chitinase A. We have isolated and purified Ra-ChiA and

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Ra-ChiB from *Ralstonia* sp. A-471. The enzymatic properties of these two enzymes were similar to each other, e.g., as thermo-stability and high activities against various partially *N*-acetylated chitosans (Ueda et al. 2005). To understand the genetic basis for the production of chitinases in *Ralstonia* sp. A-471, we attempted to clone and sequence the chitinase genes. In the cloning of the chitinase genes from *Ralstonia* sp. A-471, we got several positive clones and one of them indicated a homology to amino acid sequences of G-type lysozyme (Pooart et al. 2005) and *Clostridium* lysozyme-like enzyme (NCBI accession no. YP_001309935). This transformant had activities against ethylene glycol chitin, CM-chitin, and soluble chitin (50% deacetylated chitin) but not against the cell wall of *Micrococcus lysodeikticus*. In this paper, we report the cloning, sequencing and expression of G-type lysozyme gene with chitinolytic activity from *Ralstonia* sp. A-471. The enzymatic properties are also described and discussed in comparison with those of G-type lysozyme and family 19 chitinase.

Materials and methods

Bacterial strains and media

Ralstonia sp. A-471 was used as a DNA donor. *Escherichia coli* DH5α (*supE*44, Δ*lac*U169 (φ80*lac*ZΔM15), *hsdR*17, *recA*1, *endA*1, *gyrA*96, *thi*-1, *relA*1) and *E. coli* JM109 (*recA*1, *endA*1, *gyrA*96, *thi*, *hsdR*17, *supE*44, *relA*1. λ⁻, Δ(*lac-proAB*), (F′, *proAB*, *lacI*^{q}ZΔM15, *traD*36)) were used as host strains. The plasmid pUC19 was used for gene library construction, subcloning, and sequencing studies. *Ralstonia* sp. A-471 was cultured in the medium as described previously (Sutrisno et al. 2004). *E. coli* strains were cultured and maintained appropriately in LB and 2× YT medium supplemented with ampicillin (100 μg/ml) if necessary. Chromogenic substrate X-gal (40 μg/ml) and IPTG (10 μg/ml) were added to the medium to detect recombinant plasmids.

Genomic library construction

Ralstonia sp. A-471 genomic DNA was partially digested with *Sau*3AI and 2.0–9.0 kb fragments were collected by agarose gel electrophoresis. The partially digested DNA fragments and pUC 19, which had been digested by *Bam*HI and dephosphorylated thereafter, were mixed and ligated with T4 DNA ligase. *E. coli* DH 5α cells were transformed with the ligated DNA. In order to select recombinants, transformants were spread on LB agar plate containing 0.1% (w/v) carboxy methyl (CM) chitin, 0.01% trypan blue and 100 μg/ml ampicillin. The plates were incubated at 37 °C

overnight. Colonies of enzyme-positive transformants developed clear halos on the blue background of the medium, because CM-chitin was hydrolyzed by the enzyme liberated from the cells due to natural autolysis. The plasmid coding Ra-ChiC was named pRa-C2.
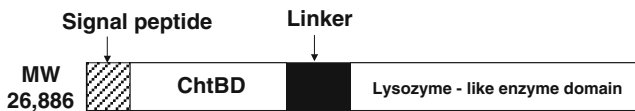
Construction of expression vector

A forward (5′-CGC CAT ATG GCA TGG GCT CCT AAT ACT TCC T-3′, the *Nde*I site is underlined) and reverse primers (5′-TGC TCT AGA ATA TCT GCC GCC ATA GCT CTT A-3′, the *Xba*I site is underlined) for PCR were synthesized on the regions corresponding to amino acid residues 37–43 and 246–252 of Ra-ChiC, respectively. PCR was done in a reaction mixture (50 μl in Prime STAR buffer) containing a pRa-C2 plasmid DNA, 0.2 μmol of each primer, 200 μM each of deoxynucleotide triphosphates (dNTPs), and 2.5 U of Prime STAR DNA polymerase (Takara Bio Inc., Kyoto, Japan). Thermocycling was done by 25 cycles of 98 °C for 10 s, 55 °C for 10 s, and 72 °C for 1 min. A DNA fragment of 0.65 kb obtained by PCR was cloned into pCR II-Blunt-Topo vector (Invitrogen Co., CA, USA). The nucleotides of the amplified fragments were confirmed by sequencing after ligation. The pCR II-Blunt-Topo vector containing the DNA fragment was treated with *Nde*I and *Xba* I, and the fragment was recovered by agarose gel electrophoresis. The DNA fragments and pCold I vector, which had been digested by *Nde*I and *Xba*I, were mixed and ligated with T4 DNA ligase. This expression plasmid coding a matured Ra-ChiC was named pCRa-C2.

Expression and purification of recombinant chitinolytic enzyme

Expression plasmid pCRa-C2 was transformed into *E. coli* JM109 and transformant was cultured in LB medium (ca. 4 l) containing ampicillin at 37 °C to OD600 of 0.4 and induced with IPTG (1 mM) at 15 °C overnight. *E. coli* cells cultured were harvested by centrifugation (9,000 ×*g*, 20 min, 4 °C). The cells were resuspended in 20 mM Tris–HCl buffer (pH 7.0), sonicated, and centrifuged (9,000 ×*g*, 20 min, 4 °C). The supernatant was applied to HisTrap HP column (GE healthcare UK Ltd., Little Chalfont, Buckinghamshire, England) equilibrated with 50 mM phosphate buffer (pH 8.0) containing 5 mM imidazol and 300 mM NaCl. The enzyme was eluted with the gradient of 5–300 mM imidazol in the buffer. Active fractions were applied onto a Superdex 75 column equilibrated with 20 mM Tris–HCl buffer (pH 7.0) containing 0.2 M NaCl and eluted with the same buffer. Active fractions were pooled and used as the purified enzyme solution.

Enzyme activity

Chitinase activity was measured as described previously using ethylene glycol chitin as substrate (Imoto and Yagishita 1971). The enzyme activities were measured by standard assay method with ethylene glycol chitin as substrate in 0.1 M sodium phosphate buffer (pH 6.0) at 37 °C for 15 min. One unit of activity was defined as the amount of enzyme that liberated 1 μmol of reducing sugar per minute.

Effects of pH and temperature on activity and stability

The optimum pH of the chitinolytic enzyme was measured after incubation with ethylene glycol chitin in the buffer at various pH (2–13) at 37 °C for 15 min. The optimum temperature of the enzyme was measured after incubation with ethylene glycol chitin at various temperatures (10–80 °C) for 15 min. The pH and thermal stabilities of the enzyme were measured from residual activities after incubation in the buffer at various pH at 37 °C for 30 min, and in 0.1 M sodium phosphate buffer, pH 6.0, at various temperatures for 30 min, respectively. The residual activities of the enzyme were measured by standard assay. The buffers used were: 0.1 M acetic acid–HCl buffer (pH 2.0–4.0), 0.1 M sodium acetate buffer (pH 4.0–6.0), 0.1 M sodium phosphate buffer (pH 6.0–8.0), 0.1 M Tris–HCl buffer (pH 7.0–9.0), and 0.1 M glycine–NaOH buffer (pH 9.0–13.0).

Electrophoresis of protein and N-terminal amino acid sequence analysis

SDS-polyacrylamide gel electrophoresis was done by the method of Laemmli (1970) using the molecular weight

Fig. 1 Nucleotide sequence of a novel G-type lysozyme gene and deduced amino acid sequence of the gene product. The −10 and −35 regions of a possible promoter sequence are *underlined*. The SD sequence is *double-underlined*. Horizontal *arrows* indicate terminator-like, inverted repeat sequences. The putative signal cleavage site is indicated by a *vertical arrow*. Chitin-binding domain is indicated by *italic letters*. Catalytic domain is indicated by *shaded letters*. The nucleotide sequence data reported in this paper have been deposited in DDBJ, EMBL, and GenBank databases under accession number AB445458

Signal peptide **Linker**

MW
26,886 | ChtBD | | Lysozyme - like enzyme domain

Fig. 2 Domain structure of Ra-ChiC from *Ralstonia* sp. A-471. *ChtBD* chitin-binding domain

marker "Daiichi" II (Daiichi Pure Chemicals Co., Tokyo, Japan) as a standard. Protein bands were detected by staining with Coomassie brilliant blue R-250. N-terminal amino acid sequence analysis was done by Edman degradation with a protein sequencer (Applied Biosystems, Model Procise® Model 491HT, Foster City, CA, USA).

HPLC analysis of enzymatic products

To identify the cleavage pattern and anomeric form of the hydrolysis products of the purified enzyme, 5 mM *N*-acetylglucosamine oligosaccharide substrates (GlcNAc)n were dissolved in 50 mM sodium acetate buffer (pH 6.0), and several microliters of the enzyme solution was added to 200 µl of the each substrate solution. After the enzyme reaction was carried out at 37 °C for an appropriate period, a portion of the reaction mixture was withdrawn and mixed with the same volume of chilled acetonitrile (−20 °C) in order to terminate the enzymatic reaction. The resultant solution was applied onto a column of TSK-GEL AMIDE 80 (4.6×250 mm, Tosoh Co., Tokyo, Japan). The elution was conducted with 70% acetonitrile at a flow rate of 0.7 ml/min, and the substrates and the products were monitored by absorbance at 210 nm (Koga et al. 1998).

RNA analysis

The *Ralstonia* cells were cultured in the media with or without 0.3% colloidal chitin (Sutrisno et al. 2004). The

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Ral-E   37  AWAPNTSYAVNDTVTYNGSTYTCIQAHTSLVGWEPPVVPALWSL-  80
B-chiA1 655 AWQANTAYYVGQLVTYNGKTYKCLQSHTFLTGWEPSNVAALWEL- 698
K-Chi   655 AWQANTAYTVGQLVTYSGKTYKCLQSHTSLPGWEPSNVPALW-   696
Clo-E   101 AWDSSKAYVSGDIVSYQNSNY---KAKWWTQG-DKPGSADVWEL- 140
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Fig. 4 Alignment of chitin-binding domains from Ra-ChiC (*Ral*-E), *Bacillus circulans* chitinase A1 (*Bac*-ChiA1) (Watanabe et al. 1990), *Kurthia zopfii* chitinase (*Kur*-Chi) (NCBI accession no. D63702), and *Clostridium beijerincki* lysozyme-like enzyme (*Clo*-E). All sequences are numbered from Met-1 of the peptide. Three tryptophans (*) play a very important role to bind chitin

cultured cells were collected by centrifugation (27,000 ×g, 10 min), and then total RNAs were prepared using an ISOGEN (Nippon gene Co. Ltd., Tokyo, Japan) according to the manufacturers' instruments and treated with DNase. For RT-PCR analysis, cDNAs were synthesized from RNAs treated with DNase. These cDNAs were used as templates in a PCR reaction with two specific primers Ra-ChiC-forward primer (5′-GCATGGGCTCCTAATAC-3′) and Ra-ChiC-reverse primer (5′-GCTGTGTTATAGGCATG-3′). As a PCR control, specific primers of the 16s rRNA gene were used in this experiment (forward primer 5′-AGAGTTT GATCCTGGCTCAG-3′, reverse primer 5′-ACGGTTACC TTGTTACGACTT-3′) (Sutrisno et al. 2004). We also used two specific primers of Ra-ChiA from *Ralstonia* sp. A-471 (Ral-Chi-F, 5′-ATCGTTGCCTACTATCCA-3′; Ral-Chi-R, 5′-ATGATCGTCTTCAGGTG-3′) (DDBJ accession no. AB443938).

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Ral-E  133  LLSAVIKKESYFGDGL---SGSPSAGDGLMQVEPNTRNAYLSQFSAKYGH
Clo-E  192  LICAVIKKECNYGAGL---GGSPSSGDGLMQVEPNTRNAYSSEFSSTYGH
G-lyso  65  VIAGIISRESHAGKVLKNGWGDRGNGFGLMQVDKRSHKPQGTWNGEVHIT
                            *

Ral-E  179  AYNHSSEQDQVYMGSLILNEKIVRFGSIYSGLLHYNGGDYWYPGATDSYG
Clo-E  239  AYNHSNLQDQVYMGSMIINDNIKQFGNIYNGLLHYNGGPNWYPGATDSYG
G-lyso 115  QGTTILINFIKTIQKKFPSWTKDQQLKGGISAYNAGAGNVRSYARMDIGT

Ral-E  236  RPILADQYANTVYAQYK-------
Clo-E  289  RPILADQYANAVYATYKSYGGKN-
G-lyso 162  IGTTHDDYANDVVARAQYYKQHGY
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Fig. 3 Alignment of Ra-ChiC from *Ralstonia* sp. (*Ral*-E) and *Clostridium beijerincki* (*Clo*-E) (NCBI accession No. ZP_00908239), and G-type lysozyme (*G*-lyso) (Simpson and Morgan 1983). Conserved amino acid sequences are indicated by *asterisks*. All sequences are numbered from Met-1 of the peptide

Fig. 5 SDS-PAGE pattern of the purified Ra-ChiC. Marker: phosphorylase B (97.4 kDa), bovine serum albumin (66.3 kDa), aldose reductase (42.4 kDa), carbonic anhydrase (30.0 kDa). I: purified Ra-ChiC
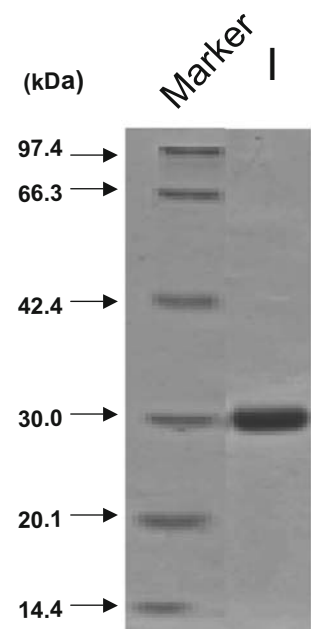
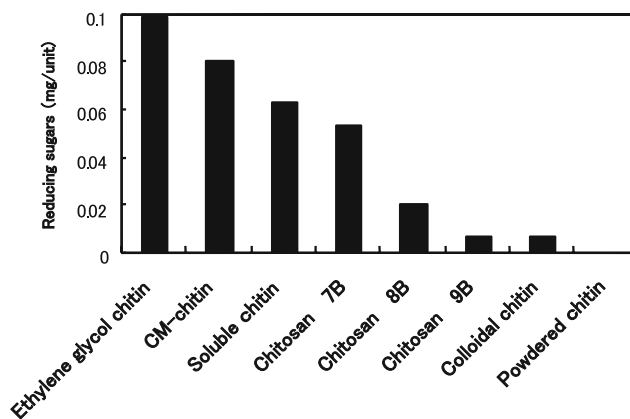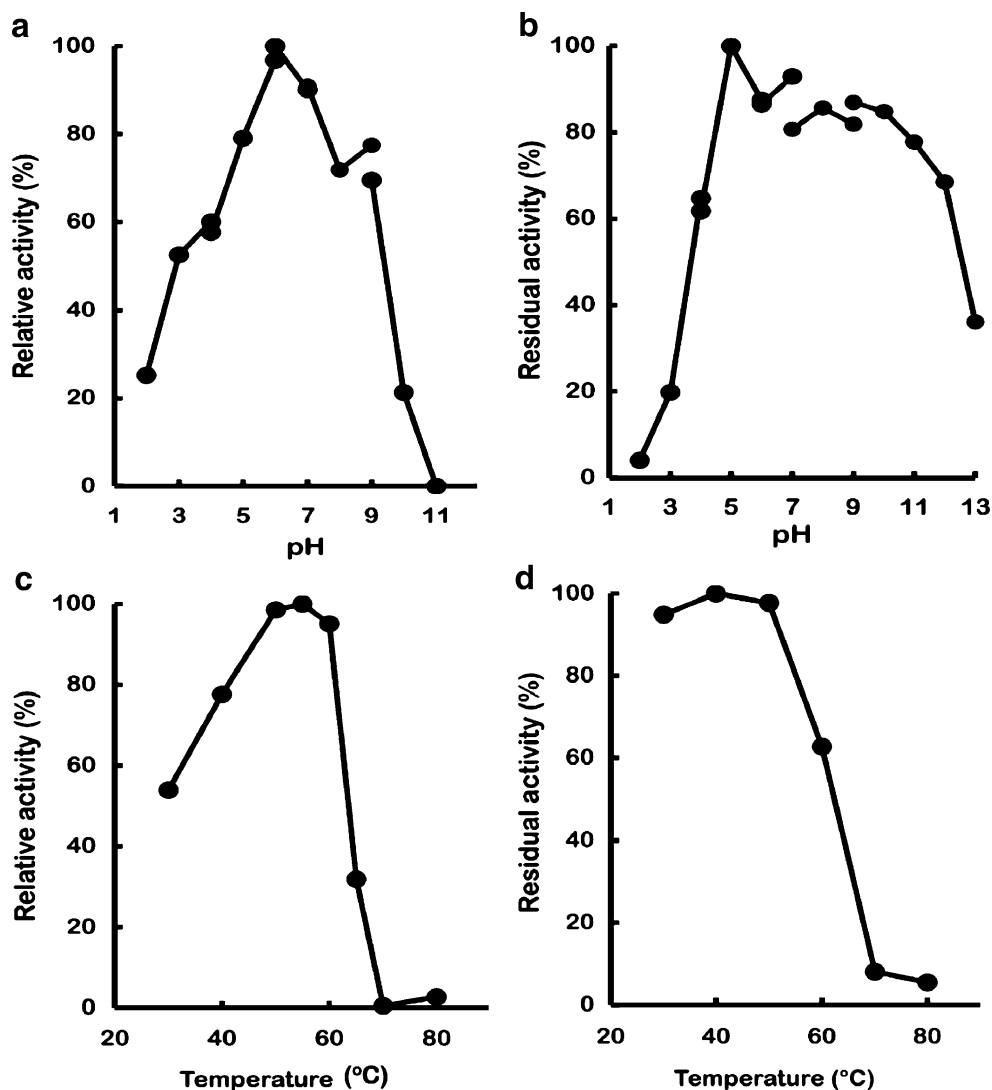**Table 1** Purification of Ra-ChiC from *Ralstonia* sp. A-471

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Yield (%)	Fold
Cell-free extract	362	1,270	3.51	100	1
HisTrap	111	460	4.11	36.2	1.17
Superdex 75	55.8	278	4.98	21.9	1.42

Results

Cloning of a novel G-type lysozyme gene

Among 10,000 transformants, several colonies showed chitinolytic enzyme activity on the LB agar plate containing CM-chitin. The gene encoding Ra-ChiC was found in one of the positive clones. The plasmid coding Ra-ChiC was named pRa-C2. The pRa-C2 contained the 2.8-kb DNA fragment. Southern blot analysis of the *Ralstonia* sp. A-471

Fig. 7 Substrate specificity of Ra-ChiC. Each substrate solution (0.3% *w/v*) was reacted with the enzyme for 15 min in 0.1 M phosphate buffer (pH 6.0) at 37 °C. Chitosan 7B (approximately 30% *N*-acetylated chitosan), chitosan 8B (approximately 20% *N*-acetylated chitosan), and chitosan 9B (approximately 10% *N*-acetylated chitosan) were purchased from Funakoshi Co. (Tokyo, Japan). Soluble chitin (50% deacetylated chitin) was a gift from Yaizu Suisankagaku Industry Co., Ltd. (Shizuoka, Japan)

Fig. 6 Effects of pH and temperature on the enzyme activity and stability of Ra-ChiC. The enzyme activities were measured by the standard assay method with ethylene glycol chitin as substrate at various pH and temperatures. The buffer systems used were 0.1 M acetic acid–HCl (pH 2.0–4.0), 0.1 M sodium acetate buffer (pH 4.0–6.0), 0.1 M sodium phosphate buffer (pH 6.0–8.0), 0.1 M Tris–HCl buffer (pH 7.0–9.0), 0.1 M glycine–NaOH buffer (pH 9.0–13.0) (**a**), and 0.1 M sodium phosphate buffer (pH 6.0) (**c**). For assessment of stability, the enzyme solutions were held at various pH for 30 min at 37 °C (**b**) and at various temperatures for 30 min in 0.1 M sodium phosphate buffer (pH 6.0) (**d**). The residual activities were measured under the standard assay

genome, in which the cloned DNA fragment was used as a hybridization probe, indicated that the DNA fragment was derived from the *Ralstonia* sp. A-471 genome (data not shown).

DNA sequence and deduced amino acid sequence

The 2.8-kb DNA fragment in the pRa-C2 was sequenced. An ORF consisting of 756 bases starts at the ATG codon and ends with the stop codon TAT, encoding 251 amino acids (Fig. 1). The molecular mass of the unprocessed protein was calculated to be 26,886. The initiation codon is preceded by a GGAGGT (Shine-Dalgarno) sequence typical of ribosome binding sites. Possible promoter sequences, TGTATC for −35 region and TATATT for the −10 region with 22 bp spacing between them, were observed. An inverted repeat sequence was found downstream from the termination codon of ORF. The protein derived from ORF of pRa-C2 appears to be a modular enzyme composed of a signal sequence, chitin-binding domain, linker, and lysozyme-like enzyme domain (Fig. 2). The deduced amino acid sequence of Ra-ChiC from *Ralstonia* sp. A-471 was compared with those of lysozymes and other enzymes. The catalytic domain of Ra-ChiC showed significant homologies to those obtained from Rhea egg white (16.8%) and *Clostridium beijerincki* (76.1%) (Fig. 3). Chitin-binding domain of Ra-ChiC had a significant similarity to that of *Bacillus circulans* ChiA1 (Watanabe et al. 1990) (Fig. 4).

Expression of a novel G-type lysozyme gene and purification of the recombinant protein

After cultivation of *E. coli* JM 109 transformed with the expression vector (pCRa-C2), the crude enzyme solution was obtained as a cell-free extract. The chitinase activity of the extract from *E. coli* JM 109 harboring pCRa-C2 was determined 0.363 U/ml culture. The recombinant enzyme was purified from *E. coli* JM 109 harboring pCRa-C2 as described above. The purified protein appeared as a single band as determined by SDS-PAGE (Fig. 5). The yield and purity of the recombinant chitinolytic enzyme at each purification step was summarized in Table 1. The recombinant enzyme was purified about 1.42-fold with a recovery of 21.9%. The molecular mass of the recombinant enzyme was estimated to be 29 kDa from SDS-PAGE.

Optimum pH and temperature

The ethylene glycol chitin was used as the substrate in the assay of the effects of pH and temperature. The enzyme

was active at pH 6.0 and 9.0 and stable over a pH range 5.0–10.0 (Fig. 6a and b). The purified enzyme showed maximum activity at 55 °C and was stable up to 50 °C when incubated for 30 min (Fig. 6c and d).

Substrate specificity

Various chitin-derivative polymers such as powdered chitin, colloidal chitin, ethylene glycol chitin, CM-chitin, soluble chitin, and partially *N*-acetylated chitosans were used to determine the substrate specificity of purified enzyme. The enzyme showed a wide range of substrate specificity and high activities toward soluble chitins such as ethylene glycol chitin (0.10 mg reducing sugars/unit), CM-chitin (0.0812 mg reducing sugars/unit), and soluble chitin (0.0630 mg reducing sugars/unit) (Fig. 7). Among the substrates, the activity against insoluble substrate powdered chitin was the lowest. Its specific activities toward the partially acetylated chitosans (chitosan 7B, 8B, and 9B) were higher than that of colloidal chitin. The enzyme also did not show any activity toward *para*-nitrophenyl β-D-*N*-acetylglucosaminide.

Effects of metal ions and organic compounds

As shown in Table 2, the enzyme was slightly increased by the addition of 1 mM $CoCl_2$. The addition of 1 mM

Table 2 Effects of various metal ions and organic compounds on enzyme activity

Metal ion/compound	Concentration	Relative activity (%)
None	1 mM	100
Li^+	1 mM	101
Na^+	1 mM	103
K^+	1 mM	101
Mg^{2+}	1 mM	102
Co^{2+}	1 mM	118
Ba^{2+}	1 mM	97
Al^{3+}	1 mM	103
Cu^{2+}	1 mM	90
Ag^+	1 mM	96
Fe^{2+}	1 mM	79
Mn^{2+}	1 mM	96
Zn^{2+}	1 mM	98
Hg^{2+}	1 mM	56
EDTA	1 mM	95
Acetonitrile	50% (*v/v*)	88
Methanol	50% (*v/v*)	101
Ethanol	50% (*v/v*)	107

The enzyme activity was determined with the standard assay using ethylene glycol chitin following the pre-incubation of the enzyme in 50 mM sodium acetate buffer, pH 6.0 containing each compound at 37 °C for 30 min

HgCl$_2$ considerably inhibited the enzyme activity. Interestingly, the enzyme showed high residual activity when incubated with organic compounds such as acetonitrile, methanol, and ethanol.

HPLC analysis of hydrolysis products from (GlcNAc)2–6

To identify the cleavage pattern and anomeric form of hydrolysis products catalyzed by the purified enzyme,

Fig. 8 HPLC analysis of hydrolysis product of (GlcNAc)6, (GlcNAc)5, (GlcNAc)4, and (GlcNAc)3 by Ra-ChiC. To identify the cleavage pattern and anomeric form of the hydrolysis products of the purified enzyme, 5 mM of each substrate [(GlcNAc)2, (GlcNAc)3, (GlcNAc)4, (GlcNAc)5, and (GlcNAc)6] were dissolved in 50 mM sodium acetate buffer, pH 6.0, and several microliters of the enzyme solution was added to 200 μl of the each substrate solution. The enzyme reaction was carried out at 37 °C for 30 min, respectively. HPLC analysis of the hydrolysis products was carried out using a Tosoh TSK-Gel amide-80 column (4.6×250 mm) with 70% acetonitrile as an eluent at a flow rate of 0.7 ml/min and detected with a UV detector
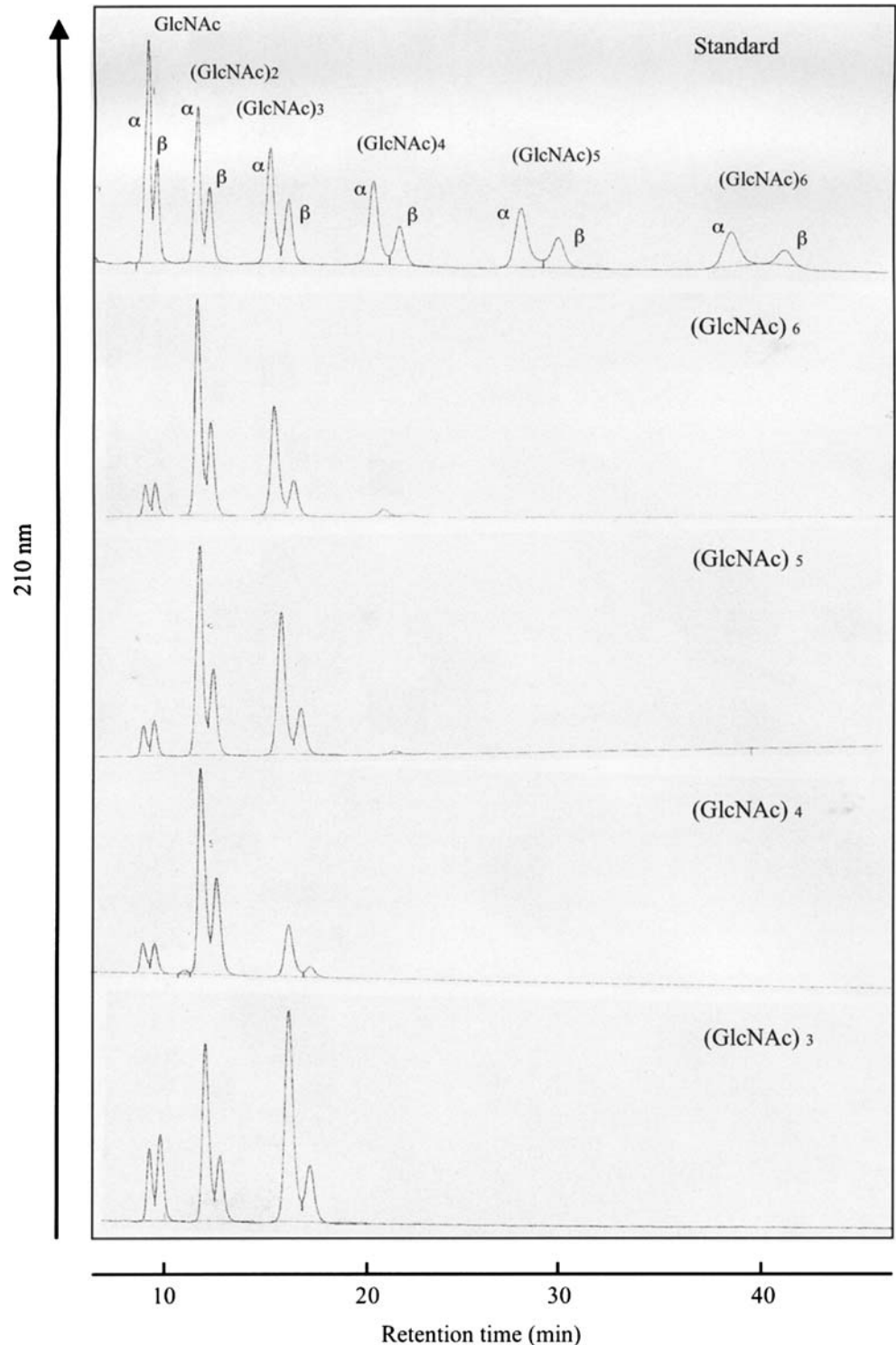
(GlcNAc)2 to (GlcNAc)6 were used as the substrates. HPLC analysis was carried out using TSK-Amide 80 column that can be used to separate α- and β-anomers of the hydrolysis products as well as to estimate the cleavage pattern of the enzyme (Koga et al. 1998). In the case of this enzyme, the major hydrolysis products from (GlcNAc)6 and (GlcNAc)5 were (GlcNAc)2 and (GlcNAc)3, and that from (GlcNAc)4 was (GlcNAc)2 under this condition (Fig. 8). The major hydrolysis products from (GlcNAc)3 were (GlcNAc)2 and GlcNAc. (GlcNAc)2 was not degraded by the chitinolytic enzyme. In Fig. 9, almost the same concentration of (GlcNAc)4, (GlcNAc)3, and (GlcNAc)2 were obtained as hydrolysis product of (GlcNAc)6 in the early stage of the reaction. Since the newly created reducing end of the products was largely α (e.g., (GlcNAc)2 in Fig. 9), it appears that this enzyme is an inverting enzyme. The amount and α/β-anomer ratio of the products indicated that this enzyme mainly hydrolyzed the second and/or third linkage from the non-reducing end-side of the substrate.
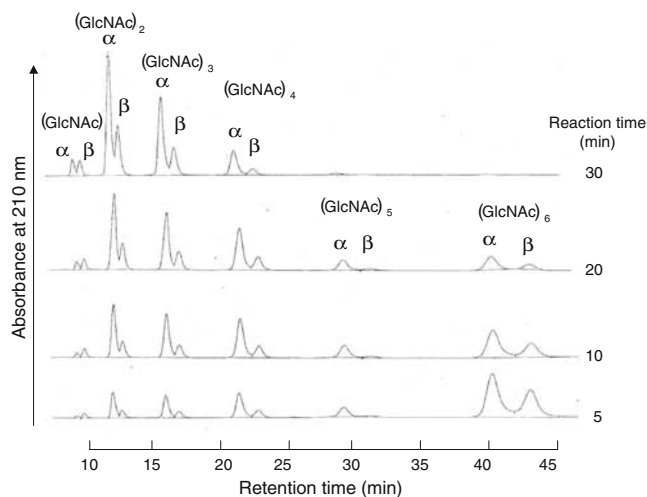
RNA analysis

We have not yet been able to isolated and purified Ra-ChiC from culture filtrate of *Ralstonia* sp. A-471. To verify whether the gene encoding Ra-ChiC is silent or not in *Ralstonia* sp. A-471, we checked the transcript of Ra-ChiC gene using RT-PCR. The transcription of Ra-ChiA gene

Fig. 9 Time-dependent HPLC profile showing the hydrolysis of (GlcNAc)6 by Ra-ChiC. Five millimolars of (GlcNAc)6 was dissolved in 50 mM sodium acetate buffer, pH 6.0, and several microliters of the enzyme solution was added to 200 μl of the substrate solution. The enzyme reaction was carried out at 37 °C for an appropriate period. HPLC analysis of the hydrolysis products was carried out using a Tosoh TSK-Gel amide-80 column (4.6×250 mm) with 70% acetonitrile as an eluent at a flow rate of 0.7 ml/min and detected with a UV detector

was induced in the presence or absence of colloidal chitin, while that of Ra-ChiC gene was only induced in the presence of colloidal chitin (data not shown).

Discussion

Lysozymes (EC 3.2.2.17) are self-defense enzymes found mainly in egg white, tears, and various other secretions of eukaryotic cells. These enzymes are one of the most structurally well-characterized carbohydrate hydrolases by cleavage of the glycosidic linkage between *N*-acetyl muramic acid and GlcNAc in the bacterial cell walls. G-type lysozyme belongs to glycoside hydrolase family 23, which enzymes are only expressed in goose egg white. A novel G-type lysozyme gene from *Ralstonia* sp. A-471 was cloned, sequenced, and expressed in *E. coli*. From the deduced amino acid sequence, the enzyme was found to possess an N-terminal chitin-binding domain and a linker. In addition, the molecular mass of catalytic domain of Ra-ChiC was 13 kDa, which was smaller than that of the G-type lysozyme (23 kDa). Thus, the *Ralstonia* enzyme was found to be an enzyme with a novel molecular construction (Fig. 2). The active site of G-type lysozymes has a highly conserved Glu residue, which believed to act as a general acid in catalysis (Glu73 in G-type lysozyme) (Grutter et al. 1983; Weaver et al. 1985, 1995). A Glu residue (Glu 141) was also conserved in Ra-ChiC (Fig. 2). The discovery of Ra-ChiC raised the question of evolutionary relationships between the bacterial and goose egg enzymes. *Ralstonia* sp. A-471 studied in this article has been isolated from compost containing soil. *Ralstonia* sp. A-471 could possibly interact with animal eggs in soil, bringing about horizontal gene transfer from goose eggs to the *Ralstonia*. Ra-ChiC cannot hydrolyze the cell wall of *M. lysodeikticus*. It is possible that the *Ralstonia* enzyme lost the lytic activity in the process of evolution.

Matured Ra-ChiC was overexpressed in *E. coli*. It was obtained at 5.69 mg purified enzymes per 1 l of culture broth. Regarding optimum pH, Ra-ChiC had two optimum pH ranges of 6 and 9 toward ethylene glycol chitin. This pH behavior was similar to those of the *Bombyx mori* chitinases and Yam chitinases (Koga et al. 1997; Arakane et al. 2000). Regarding optimum temperature and heat stability, optimum temperature of Ra-ChiC was 55 °C, and stable up to 60 °C. These results were similar to those of Ra-ChiA and Ra-ChiB (Sutrisno et al. 2004; Ueda et al. 2005). It was indicated that Ra-ChiC is also the heat stable enzyme. The addition of Hg^+ inhibited the activity of Ra-ChiC, but other metal ions had almost no effect against the enzyme activity. In addition to the property against metal ions, the enzyme retained considerably high activity when incubated with high concentrations of organic compounds

such as 50% acetonitrile, 50% methanol, and 50% ethanol. From these results, the enzyme will be advantageous for practical applications. The activities towards soluble substrates, ethylene glycol chitin, CM-chitin, and soluble chitin were much higher than towards insoluble substrates, colloidal chitin, and powdered chitin. Ra-ChiC was capable of degrading not only chitin but also chitosans with various degrees of acetylation. This property was also shown in Ra-ChiA and Ra-ChiB.

Ra-ChiC was found to produce α-anomer by hydrolyzing the β-1,4-glycosidic linkage, indicating that the enzyme is an inverter. The G-type lysozymes (GH family 23) are also inverters. Ra-ChiC hydrolyzed (GlcNAc)6 in an endo-splitting manner, which produced almost the same concentration of (GlcNAc)2+(GlcNAc)4 and (GlcNAc)3+(GlcNAc)3 in the early stage of the reaction. (GlcNAc)4 should be rapidly hydrolyzed into (GlcNAc)2+(GlcNAc)2. Hence the (GlcNAc)6 splitting into (GlcNAc)3+(GlcNAc)3 is predominant to that into (GlcNAc)2+(GlcNAc)4 in G-type lysozymes (Honda and Fukamizo 1998; Pooart et al. 2005). The difference in splitting specificity between Ra-ChiC and the G-type lysozyme should be a result of the different subsite structures. The *Ralstonia* enzyme and G-type lysozyme have different modular structures and a different size catalytic domain; such structural differences might cause a difference in the subsite structure. Further experiments are now under way to refine the substrate-binding cleft of this enzyme.

The expression of chitinolytic enzyme genes in *Ralstonia* sp. A-471 was analyzed at the transcription level. The transcription of Ra-ChiA gene was induced in the presence or absence of colloidal chitin in the culture media, while Ra-ChiC gene was only induced in the presence of colloidal chitin. It has been reported that the transcription of chitinase genes in *Streptomyces coelicolor* A3 was induced by colloidal chitin and chitobiose (Saito et al. 2000). It is suggested that, in *Ralstonia*, several chitinases work synergistically to degrade chitin, which produce heterogeneous saccharides. It is assumed that the accumulation of the heterogeneous saccharides was triggered the induction of Ra-ChiC gene transcription. Study on the X-ray structural analysis of Ra-ChiC from *Ralstonia* sp. A-471 is in progress.

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