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Cloning, expression, and characterization of a highly thermostable family 18 chitinase from *Rhodothermus marinus*

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Abstract A family 18 chitinase gene *chiA* from the thermophile *Rhodothermus marinus* was cloned and expressed in *Escherichia coli*. The gene consisted of an open reading frame of 1,131 nucleotides encoding a protein of 377 amino acids with a calculated molecular weight of 42,341 Da. The deduced ChiA was a non-modular enzyme with one unique glycoside hydrolase family 18 catalytic domain. The catalytic domain exhibited 43% amino acid identity with *Bacillus circulans* chitinase C. Due to poor expression of ChiA, a signal peptide-lacking mutant, *chiAΔsp*, was designed and used subsequently. The optimal temperature and pH for chitinase activity of both ChiA and ChiAΔsp were 70°C and 4.5–5, respectively. The enzyme maintained 100% activity after 16 h incubation at 70°C, with half-lives of 3 h at 90°C and 45 min at 95°C. Results of activity measurements with chromogenic substrates, thin-layer chromatography, and viscosity measurements demonstrated that the chitinase is an endoacting enzyme releasing chitobiose as a major end product, although it acted as an exochitobiohydrolase with chitin oligomers shorter than five residues. The enzyme was fully inhibited by 5 mM $HgCl_2$, but excess ethylenediamine tetraacetic acid relieved completely the inhibition. The enzyme hydrolyzed 73% deacetylated chitosan, offering an attractive alternative for enzymatic production of chitooligosaccharides at high temperature and low pH. Our results show that the *R. marinus* chitinase is the most thermostable family 18 chitinase isolated from Bacteria so far.

Keywords Cloning · Expression · Family 18 chitinase · Highly thermostable · *Rhodothermus marinus* · Thin layer chromatography · Viscosity measurements

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Introduction

Chitin, an insoluble and linear homopolymer of β-linked 1,4-*N*-acetylglucosamine (GlcNAc) is an abundant, renewable natural source present in cell walls of fungi and certain green algae, and it is the major component of the shells and exoskeletons of crustaceans and insects. It is the second most abundant organic compound after cellulose, with an estimated formation rate of about 10^{10}–10^{11} tons/year (Ramaiah et al. 2000). Chitin hydrolysis is performed by a major pathway composed of three separate enzymes: endochitinases (EC 3.2.1.14), which produce multimers of β-*N*-acetylglucosamine; exochitinases or chitobiohydrolases (EC 3.2.1.52), which catalyze the sequential release of soluble dimers starting at the non-reducing end of the polymer; and chitobiases or β-*N*-acetylglucosaminidases (EC 3.2.1.30), which hydrolyze chitobiose into monomers of *N*-acetylglucosamine (Souza et al. 2003).

Chitinases are commonly found in a wide variety of higher plants, in vertebrates, in Bacteria, and in

Archaea (Huber et al. 1995; Tanaka et al. 1999, 2001, 2003; Andronopoulou and Vorgias 2003; Gao et al. 2003); for review, see (Henrissat 1999). Bacteria and Archaea have been shown to produce chitinases for the digestion of chitin and for the utilization of its fragments as carbon and energy sources (Cohen-Kupiec and Chet 1998; Mabuchi and Araki 2001), in marine environments by the hydrolysis of free chitin (Cottrell et al. 1999; Ramaiah et al. 2000), and in soil by the hydrolysis of fungal hyphae (Williamson et al. 2000; de Boer et al. 2001). It is thought that chitin hydrolysis occurs either by a sequential or synergetic action of endochitinases, exochitinases, and chitobiases (Tanaka et al. 2003).

Due to their ecological role and growing interests in biotechnology in particular for chitinous waste recycling, crop protection, and chitooligosaccharide production (Felse and Panda 1999; Patil et al. 2000; Wang and Hwang 2001; Haki and Rakshit 2003), a large number of bacterial chitinases have been isolated, and their respective genes have been cloned and characterized. Yet, few thermostable and hyperthermostable chitinases have been found in microbes. High temperature optima (≥55–60°C) have been reported for chitinases from *Bacillus* species (Takayanagi et al. 1991; Bhushan and Hoondal 1998; Sakai et al. 1998), *Streptomyces thermoviolaceus* (Tsujibo et al. 1993), and *Microbispora* sp. V2 (Nawani et al. 2002). Moreover, the hyperthermophilic Archaea *Thermococcus chitonophagus*, *Thermococcus kodakaraensis*, and *Pyrococcus furiosus* have been recently shown to grow on chitin, and unique hyperthermostable chitinolytic enzymes showing remarkable thermostability and resistance to denaturation have been described (Huber et al. 1995; Tanaka et al. 1999, 2001, 2003; Andronopoulou and Vorgias 2003; Gao et al. 2003).

Rhodothermus marinus is a thermohalophilic, Gram-negative eubacterium originally isolated from a shallow submarine hot spring in Iceland (Alfredsson et al. 1988). It is an obligately aerobic heterotrophic bacterium, with an optimum growth temperature at 65°C and grows in presence of 1–6% w/v of NaCl. *R. marinus* has received increasing interest due to the presence of many thermostable enzymes with potential biotechnological applications such as β-glucanase (Spilliaert et al. 1994), DNA ligase (Thorbjarnardottir et al. 1995), xylanase (Nordberg-Karlsson et al. 1997), cellulase (Halldorsdottir et al. 1998), laminarinase (Krah et al. 1998), endo-(1,4)-β-mannanase (Politz et al. 2000), and α-L-arabinofuranosidase (Gomes et al. 2000). Here, we describe the cloning, overexpression, purification, and biochemical characterization of a family 18 chitinase from *R. marinus*. Based on our study, the *R. marinus* family 18 chitinase offers attractive interests due to its ability to yield valuable chitooligosaccharides from partially deacetylated chitosan. Moreover, our results demonstrate that this enzyme is the most thermostable chitinase from Bacteria reported so far.

Materials and methods

Bacterial strains and culture conditions, DNA, and plasmids

Rhodothermus marinus PRI378 was acquired from the Prokaria strain collection and grown as described (Alfredsson et al. 1988). All DNA manipulations were performed by standard methods as described by Sambrook et al. (1982). Kits were used according to the manufacturers' instructions, unless described otherwise. Isolation of the genomic DNA from *R. marinus* was performed using the Plasmid Maxi kit (Qiagen, Hilden, Germany). Intermediate cloning procedures and sequencing were performed with the pCR4 TA cloning vector (Invitrogen, Carlsbad, Calif., USA) into TOP10 *Escherichia coli* cells (Invitrogen). Heterologous expression was performed using the expression cloning vector pJOE3075 (Wilms et al. 2001) into *E. coli* BL21 (DE3) pRIL cells (Stratagene, La Jolla, Calif., USA). The *E. coli* strains were grown at 37°C on Luria-Bertani (LB) medium supplemented with 100 μg ml^{-1} of ampicillin for pCR4 clones and 100 μg ml^{-1} ampicillin and 34 μg ml^{-1} chloramphenicol for pJOE3075 clones.

Preparation of colloidal chitin

Colloidal chitin was prepared following the protocol established by Hsu and Lockwood (1975). Forty grams unbleached milled chitin (MP Biomedical, Irvine, Calif., USA) were dissolved in 400 ml concentrated hydrochloric acid by stirring for 1 h. The chitin was precipitated by the addition of 2 l of 5°C distilled water (DW). The colloids were collected by filtration and resuspended in 5 l DW. Colloids were then concentrated by centrifugation for 10 min at 10,000 *g* and resuspended into DW. Centrifugation was resumed until the pH reached 4. The final chitin cake was resuspended into 2 l DW and frozen at −80°C. The chitin was then lyophilized and stored at room temperature. About 75% w/w of chitin was recovered.

Recovery of the *R. marinus chiA* gene

Chitinase A from *R. marinus* was identified *in silico* from a local Prokaria genome database. In brief, the database was prepared as follows. DNA was fragmented by nebulization and cloned into pTrueBlue (Stratagene). Plasmids were isolated by high-throughput minipreparation, and sequencing was performed. Contigs were assembled with the Phred–Phrap package (Ewing and Green 1998), and putative open reading frames (ORFs) were identified with the GetORF program from the EMBOSS package (Rice et al. 2000), followed by BLASTP searches (Altschul et al. 1997) against protein sequence databases.

Complete gene retrieval was performed using the Universal GenomeWalker kit (Clontech, BD Biosciences, Franklin Lakes, N.J., USA), with variations of the suggested protocol. Four aliquots of 2.5 μg genomic DNA were digested overnight in four reactions, using in each 80 U of blunt-end restriction enzymes *Eco*RV, *Dra*I, *Pvu*II, and *Stu*I (New England Biolabs, Beverly, Mass., USA) in a total reaction volume of 100 μl. DNA fragments from 100–10,000 bp were isolated and purified from restriction enzymes, using a Qiaquick PCR purification kit (Qiagen) according to the manufacturer's instructions and resuspended into 20 μl TE buffer. For the creation of four genomic DNA libraries, 1.9 μl Genome Adapter Clamp was ligated to 4 μl each restricted DNA mix, using 3 U of T4 DNA ligase (New England Biolabs) in overnight incubation at 16°C in a total volume of 8 μl. The ligation reaction was terminated by incubating at 65°C for 15 min. The mix was eventually resuspended into 72 μl TE buffer for a total volume of 80 μl.

The partial chitinase gene sequence identified in the genome database was used to design gene-specific primers for an inverse PCR-based method (Table 1). First, PCR was carried out using gene-specific primers (GSP1 and GSP2) against the adaptor primer 1, using 1 μl each genome library as the template and 1 U DynaZyme DNA polymerase (Finnzymes, Espoo, Finland) in a PTC225 Peltier Thermal Cycler. The PCR program was as follows: initial denaturation step for 2 min at 95°C, followed by 45 cycles of denaturation at 95°C for 40 s, annealing at 50°C for 40 s, and amplification at 72°C for 90 s, with a further 5 min extension at 72°C. PCR fragments were then purified from excess primers and enzymes, using the Qiaquick PCR purification kit (Qiagen) and resuspended in the identical volume used for the primary amplification reaction. Secondary PCR was carried out using nested gene-specific primers (GSP3 and GSP4) against the adaptor primer 2 and by using the identical PCR program used for the primary amplification (50 μl). All resulting PCR fragments were gel-purified using the GFX gel purification kit (Pharmacia Biotech, Uppsala, Sweden) and cloned into pCR4. Sequencing was performed by PCR amplification of the insert with the forward and reverse M13 primers, followed by enzymatic cleansing of the PCR products, using an ExoSap-it kit (Amersham Biosciences), and sequencing with both forward and reverse M13 primers and the Big Dye Terminator Cycle Sequencing kit on a ABI PRISM 3700 DNA Analyzer (Applied Biosystems, Foster City, Calif., USA). The sequences were analyzed with Sequencher, version 4.0 (GeneCodes, Ann Arbor, Mich., USA) and identified with BLASTX searches (Altschul et al. 1997).

Table 1 Primers used for genome walking and recovery of *chiA* and heterologous expression of *chiA* and *chiAΔsp*

Primers	Sequence 5′ → 3′
Inverse PCR[a]	
GSP1	CGTACAGATAGCCCACCACACG
GSP2	AGCATAACGGGCGGTATCAGC
GSP3	CACGAAAATCCAGCGCCGGCTGCTC
GSP4	TACGAGGCGTCGCTTACGGAGGAG
Expression[b]	
chiA-F-*Nde*I	GCGACGCAT*atg*cgtatcggatggatatgc
chiAΔsp-F-*Nde*I	GCGACGCAT*ATG*AAGcagccggcgctggattttcg
chiA-R-*Bam*HI	CGC GGATCC*tca*gctctcttctctgcattgc

[a]*GSP* Gene-specific primer. Primers GSP1 and GSP3 were used to recover the 5′ end of the gene, whereas primers GSP2 and GSP4 were used to recover the 3′ end of the gene

[b]*F* Forward primer, *R* reverse primer. Restriction sites of *Nde*I and *Bam*HI are *underlined*, respectively. True or artificial start codon ATG and stop codon TCA (UGA) are indicated in *italics*. The original sequence of *chiA* is in *lower case letters*

Expression cloning of *chiA* and signal peptide-lacking mutant chiAΔsp

The full gene and truncated version of the *chiA* gene were amplified using the primers listed in Table 1. Both forward primers were designed with a *Nde*I restriction site in frame, with the initiation codon ATG. The reverse primer was designed with a stop codon TGA, followed by an in-frame *Bam*HI-compatible restriction site. The full version of *chiA* was amplified using the forward *chiA*-F-*Nde*I and reverse *chiA*-R-*Bam*HI primers. The truncated version of the gene *chiAΔsp*, which lacked a potential signal peptide, was amplified using *chiAΔsp*-F-*Nde*I against the same reverse primer as *chiA*. The forward primer *chiAΔsp*-F-*Nde*I contained an artificial start codon ATG preceding the rest of the gene. Gene amplification was performed with genomic DNA from *R. marinus* and 2 U DynaZyme EXT DNA polymerase (Finnzymes) with the following program: initial denaturation at 95°C for 5 min, followed by seven cycles of denaturation at 94°C for 50 s, annealing at 50°C for 50 s, and extension at 72°C for 3 min, then 23 cycles of denaturation at 94°C for 50 s, annealing 55°C for 50 s, and extension at 72°C for 3 min, and final extension was done at 72°C for 25 min. PCR amplicons were gel-purified by using the GFX PCR purification kit and subcloned into pCR4 designated pCR4-ChiA and pCR4-ChiAΔsp, respectively. Amplification fidelity was assessed by sequencing of the plasmids, using both forward and reverse M13 primers, as described here above. Both pCR4-ChiA and pCR4-ChiAΔsp plasmids were purified by minipreparation (Qiagen) and digested overnight with *Nde*I and *Bam*HI. The genes were ligated into *Nde*I- and *Bam*HI-predigested pJOE3075, using the T4 DNA ligase (New England Biolabs). The resulting plasmids were designated pChiA and pChiAΔsp, respectively. Ligated plasmids were transformed into chemically competent TOP10 *E. coli* cells and selected on LB plates supplemented with ampicillin and chloramphenicol. Ligation efficiency was assessed by PCR, using both the plasmid-specific and gene-specific primers listed in Table 1. pChiA and pChiAΔsp plasmids were eventually purified by minipreparation, transformed by electroporation into BL21 (DE3) pRIL *E. coli* cells, and selected on LB plates supplemented with ampicillin and chloramphenicol.

Standard assay of ChiA and ChiAΔsp activity

Chitinase activity was routinely determined by measuring the release of *p*-nitrophenol from *p*-nitrophenyl-*β*-D-*N*,*N*′-diacetyl chitobioside (pNP-GlcNAc$_2$, Fluka). Chitinase assay was conducted in 100 mM KH_2PO_4/K_2HOPO_4 (pH 5) with 0.9 mM pNP-GlcNAc$_2$ at 70°C for 10 min in a total reaction volume of 50 µl. Potassium phosphate buffer was adjusted to achieve correct pH at 70°C, according to Beynon and Easterby (1996). The reaction was stopped with 100 µl of 1 M sodium carbonate, and the amount of released *p*-nitrophenol was measured in a Sunrise Remote plate reader (Tecan, Maennedorf, Switzerland) at 405 nm (molar extinction coefficient ϵ_{405} = 18,500 M^{-1} cm^{-1}) using 125 µl of the total reaction volume. One unit of chitinase activity was defined as the amount of enzyme that produces 1 µmol of *p*-nitrophenol per minute under the standard assay conditions. All incubations were performed in a PTC-225 Peltier Thermal Cycler to ensure optimum incubation timing and to avoid evaporation. All chitinase activity values are provided as the mean of three independent replications, and standard deviations were equal or less than 5% of the means.

Purification of recombinant ChiAΔsp

Recombinant ChiAΔsp expression was conducted in a 10-l batch reactor, using a mAT salt medium (Ramchuran et al. 2002) supplemented with 10 g l^{-1} glucose and 100 mg ml^{-1} ampicillin and 34 mg ml^{-1} chloramphenicol. *E. coli* cells harboring pChiAΔsp were induced for overexpression with 0.2% (w/v) L-rhamnose at the mid-exponential growth phase. Cultivation was conducted for 5 h at 32°C, maintaining a pO_2 of 40% and neutral pH until the cell mass reached stationary phase. Cells were harvested by centrifugation at 10,000 *g* for 20 min at 4°C, the pellet resuspended in DW at a final concentration of 0.2% w/v and disrupted by sonication. The soluble fraction of 80 g of cells was attained by centrifugation at 4°C, first at 8,000 *g* for 10 min then at 17,000 *g* for 15 min. The supernatant was incubated at 70°C for 20 min with gentle shaking every 2 min and centrifuged at 4°C first at 20,000 *g* for 20 min then at 25,000 *g* for 15 min to obtain heat-stable crude extract. The supernatants were buffered to 5 mM KH_2PO_4/K_2HPO_4 (pH 7) + 600 mM ammonium sulfate [$(NH_4)_2SO_4$]. The pH of the solution was corrected with sterile solution of 5 M KOH and filtered through a 0.22-µm filter. The buffered crude extract was then applied to a 50-ml HiTrap High Performance column (hydrophobic interaction) connected to a FPLC Äkta Protein Purifier (Pharmacia Biotech). ChiAΔsp was eluted using two steps of decreasing concentrations of $(NH_4)_2SO_4$ (480 and 210 mM) with a flow rate of 5 ml min^{-1} and by collecting 10-ml fractions. Positive fractions were identified by standard chitinase assay (pNP-GlcNAc$_2$), pooled, and stored at 4°C. The column was furthermore washed with five column volumes of DW to remove tightly bound protein. ChiAΔsp was eluted in the fractions eluted with 210 mM $(NH_4)_2SO_4$. The second purification step was performed on a 6-ml Resource S (cation exchange interaction) column (Pharmacia Biotech). Elution was performed by desalting the sample by dilution (ten times) and re-buffering with 100 mM potassium acetate (K-Ac), pH 4.5. Separation was conducted using two increasing steps of 100 mM K-Ac (pH 4.5), 1 M NaCl (8 and 11%) at a flow rate of 6 ml min^{-1} and collecting 6-ml fractions. Positive fractions were identified as described previously. ChiAΔsp was detected in the fractions eluted by 11% of salt.

Protein concentration was determined by the Bio-Rad protein assay system (Bradford 1976; Bio-Rad, Hercules, Calif., USA) with bovine serum albumin as a standard. Protein purity was assessed by SDS-PAGE fractionation of each sample as described by Laemmli (1970), using 5% stacking and 12.5% resolving gels and a broad range protein standard (New England Biolabs). Proteins were visualized by Coomassie Brilliant Blue staining as described by Wong et al. (2000).

Characterization of the *R. marinus* family 18 chitinase A

Optimal pH and temperature for activity and temperature stability

Optimal pH (pH_{opt}) for ChiA and ChiAΔsp was determined by performing the standard activity assay at 70°C and varying the pH values of the reaction mix between 3.5 and 10.0, with increments of 0.5 pH units. The following buffers used were all designed for use at 70°C: 100 mM K-Ac for pH 3.5–6.0, 100 mM KH_2PO_4/K_2HPO_4 for pH 6.5–7.5, and 100 mM tricine for pH 8.0–10.0. Optimal temperature (T_{opt}) was measured by using standard chitinase assay at temperatures between 20 and 100°C for 10 min incubation and with 10°C intervals. Temperature stability was examined by preincubation of 40 of enzyme solution for periods of time of 0.5, 1, 2, 4, 8, and 16 h, and at the following temperatures: 70, 80, 85, 90, and 95°C. Residual activity was measured using standard chitinase assay at 70°C.

Determination of pattern of chitinolytic activity and substrate specificity on artificial and natural substrates

The chromogenic derivatives *p*-nitrophenyl-*β*-D-*N*-acetyl glucosaminide (pNP-GlcNAc, Fluka), pNP-GlcNAc$_2$, and *p*-nitrophenyl-*β*-D-*N*,*N*′,*N*″-triacetyl chitotrioside (pNP-GlcNAc$_3$, Sigma) were used as substrate for the preliminary determination of *β*-D-acetylglucosaminidase, exochitobiosidase, and endochitinase activities respectively. pNP-GlcNAc$_2$ was replaced by pNP-GlcNAc and pNP-GlcNAc$_3$ in standard chitinase assay (0.9 mM substrate) at 70°C for 10 min and also overnight.

The enzyme catalytic activity pattern was furthermore studied by analyzing the reaction product with various chitin oligosaccharides by silica gel thin-layer chromatography (TLC). Following oligomers were used: *N*-acetyl-glucosamine ($GlcNAc_1$, Sigma), *N*,*N*′-diacetyl chitobiose ($GlcNAc_2$, Fluka), *N*,*N*′,*N*″-triacetyl chitotriose ($GlcNAc_3$, Sigma), *N*,*N*′,*N*″,*N*‴-tetraacetyl chitotetraose ($GlcNAc_4$, Sigma), *N*,*N*′,*N*″,*N*‴,*N*⁗-pentaacetyl chitopentaose ($GlcNAc_5$, Seikagaku America, Rockville, Md., USA), *N*,*N*′,*N*″,*N*‴,*N*⁗,*N*⁗′-hexaacetyl chitohexaose ($GlcNAc_6$, Sigma), *N*,*N*′,*N*″,*N*‴,*N*⁗,*N*⁗′,*N*⁗″-heptaacetyl chitoheptaose ($GlcNac_7$, IsoSep, Tullinge, Sweden), and *N*,*N*′,*N*″,*N*‴, *N*⁗, *N*⁗′,*N*⁗″,*N*⁗‴-octaacetyl chitooctaose ($GlcNAc_8$, IsoSep). Reaction mixtures contained 2.1 mM each oligosaccharide in 100 mM KH_2PO_4/K_2HPO_4 (pH 5) in a final volume of 50 µl and were incubated at 70°C for the following times: 5, 10, 20, 40, and 80 min. Colloidal chitin and crude chitin substrates [unbleached milled chitin and raw chitin flakes (Sigma)] were used with concentration of 1 and 5% (w/v) under identical conditions in final volume of 1 ml. Aliquots of 2 µl of the reaction mixtures were analyzed on a silica gel plate (Kieselgel 60, Merck, Berlin, Germany) with a 2-propanol:acetone:25% ammonia:water running phase (2:2:1:1 v/v/v/v). The products were detected by applying an aniline/diphenylamine reagent solution (4 ml aniline, 4 g diphenylamine, 200 ml acetone, and 30 ml 85% phosphoric acid) and baking the plate at 140°C for 5 min. Released products were compared to standards composed of chitin oligomers of known sizes.

Substrate specificity was studied by incubating ChiAΔsp with 1% w/v (final concentration) of xylan solution (birchwood β-1,4 xylan, Sigma), carboxymethylcellulose solution (CMC, MP Biochemicals), glycol chitosan solution (Sigma) for 16 h at 70°C in 100 mM KH_2PO_4/K_2HPO_4 (pH 5). In addition, *p*-nitrophenyl synthetic compounds (Sigma) were assayed for activity: *p*-nitrophenyl-xylobioside, *p*-nitrophenyl-galactopyranoside, *p*-nitrophenyl-cellobioside, *p*-nitrophenyl-mannopyranoside, and *p*-nitrophenyl-glucopyranoside. All *p*-nitrophenyl substrates replaced pNP-NAc_2 in chitinase assay using 0.9 mM (final concentration) at 70°C for 2 h in 100 mM KH_2PO_4/K_2HPO_4 (pH 5).

Analysis of viscosity of hydrolyzed chitosan solutions

Partially deacetylated chitosan polymer solutions (degree of deacetylation DDA: DDA 73% and DDA 99%) were provided by Primex (Siglufjordur, Iceland). One percent solutions (w/v) were prepared by soaking 5 g chitosan flakes in 400 ml DW for 1 h with constant stirring. Chitosan was then dissolved with 4 ml 100% acetic acid. Stirring was resumed for 1 h, followed by incubation at 4°C overnight to complete the dissolution of residual chitosan flakes. After warming to room temperature, pH was adjusted to 4.5 with drops of NaOH, and the final volume was completed to 500 ml with DW. The changes in viscosity of the two chitosan solutions hydrolyzed by ChiAΔsp was measured at room temperature (22–23°C) with a DV-II + PRO Digital Viscometer (Brookfield Engineering Laboratories, Middleboro, Mass., USA). One unit of purified ChiAΔsp was added to 120 ml 1% chitosan solution, and the mix was thoroughly stirred. Viscosity was measured online every minute over 16 h, and data were stored using the Wingather data collection software (Brookfield Engineering Laboratories). For TLC analyses, 10 U ChiAΔsp was incubated at 65°C in 150 ml 1% DDA 73%, and DDA 99% chitosans, and aliquots were taken at designated time points. TLC analyses were run as described above.

Enzyme kinetics

Using purified enzyme, kinetics experiments were performed using the artificial substrate pNP-$GlcNAc_2$ by varying the substrate concentration in a standard activity test from 72–0.96 mM and incubating the reaction at 70°C in 100 mM KH_2PO_4/K_2HPO_4 (pH 5). The Michaelis–Menten constant (K_M) and maximal velocity (V_{max}) were determined using Lineweaver–Burke and Eadie–Hofstee plots (Price and Stevens 1989). Characteristic constants K_{cat} and the catalytic efficiency ratio K_{cat}/K_M were deduced from the obtained K_M and V_{max} values when K_{cat} was calculated by using the molecular weight of the artificial substrate.

Effects of metal divalent ions and ethylenediamine tetraacetic acid

The effect of divalent cations on the activity of the enzyme was determined by testing pure enzyme with 1 mM salt solution of $HgCl_2$, $CoCl_2$, $FeCl_2$, $CuCl_2$, $MnCl_2$, $MgCl_2$, $NiCl_2$ and $ZnCl_2$, and 50 mM ethylenediamine tetraacetic acid [(EDTA) Merck]. Additionally, the effect of $HgCl_2$ was tested at 1, 5, and 10 mM in presence and absence of 50 mM EDTA. The chitinase activity was studied under standard chitinase assay conditions.

Nucleotide sequence accession number

The nucleotide sequence of the *chiA* gene is available in the GenBank database under accession number AY706992.

Results

Cloning and sequence analysis of the *Rhodothermus marinus chiA* gene

Data analysis of the partially sequenced genome of *Rhodothermus marinus* revealed one partial ORF (1,021 bp) showing high sequence similarities with

known chitinase genes. The putative *R. marinus* chitinase gene was designated *chiA*. Genome walking towards both 5′ and 3′ ends of the *chiA* gene revealed a complete ORF of 1,131 bp after one round of inverse PCR-based method (Fig. 1). The ORF encodes a protein of 377 amino acids, with a calculated molecular mass of 42,341 Da and a theoretical isoelectric point (pI) of 5.13. This ORF was shown to have a G + C content of 62.9%, which is in accordance with the 64.4% value found for the *R. marinus* genome (Alfredsson et al. 1988). Analysis of nucleotide sequence showed that the speculated initiation codon was ATG, and termination of the transcription was encoded by a TGA codon. The putative promoter sequence, ribosome-binding sites, and terminator sequence could not be identified as little is known about *R. marinus* translation and transcription mechanisms. Nevertheless, further analysis of the upstream region of the gene showed the presence of putative genes oriented in the inverse direction of *chiA*, suggesting that the gene was not located in a polycistronic operon (data not shown).

Amino acid sequence of ChiA

Analysis of the deduced N-terminal amino acid sequence with the SignalIP program (Nielsen et al. 1997) showed that the protein contained a putative signal peptide of 19 amino acids. The truncated version ChiA, designated ChiAΔsp, was thus 358 amino acids, with a calculated mass of 40,137 Da and a deduced pI of 5.04. Comparison with closely related sequences from GenBank and SWISSPROT databases identified by BLASTP searches showed that ChiAΔsp was a non-modular enzyme composed of one unique domain classified as family 18 glycoside hydrolase. No other modules were found, such as chitin-binding domain or fibronectin III-like domain both often associated with the catalytic domain in family 18 chitinases (Henrissat 1999). The putative mature peptide sequence shared highest sequence similarity to the catalytic domains of chitinase ChiC (sequence identity, 43%) of *Bacillus circulans* (GenBank accession number, BAA13974), chitinase (40.3%) from *B. ehimensis* (BAC76694), endochitinase (37.8%) from *Clostridium thermocellum* (CAA93150), ChiA (35.2%) from *T. kodakaraensis* KOD1 (T43916), and chitinase (35.2%) from *Halobacterium* sp. NRC-1 (AAG19274). As shown on Fig. 2, the core of the putative mature sequence exhibited significant high sequence conservation with those of many chitinases. The aspartic acid Asp^{131} and glutamic acid Glu^{135} and from *R. marinus* amino acid sequence were aligned with Asp^{311} and Glu^{315} of the *S. marcescens* chitinase A that have been proven essential in the acid–base catalysis of family 18 chitinases (Watanabe et al. 1993; van Aalten et al. 2001). Specific amino acid motifs have been previously shown to be highly conserved among microbial chitinases and therefore could be used to classify the respective catalytic domains into three groups (groups A, B, and C) (Watanabe et al. 1993; Mabuchi and Araki 2001). The chitinase from *R. marinus* contained a catalytic domain from group A due to the presence of the two motifs GGWTWS and FDGxDxDWEYP specific to that group (Fig. 2).

Overexpression and purification of the recombinant signal-peptide lacking mutant ChiAΔsp

Both wild-type and mutant *R. marinus* chitinase genes were cloned into the expression vector pJOE3075, which possesses a tightly regulated rhamnose promoter (Wilms

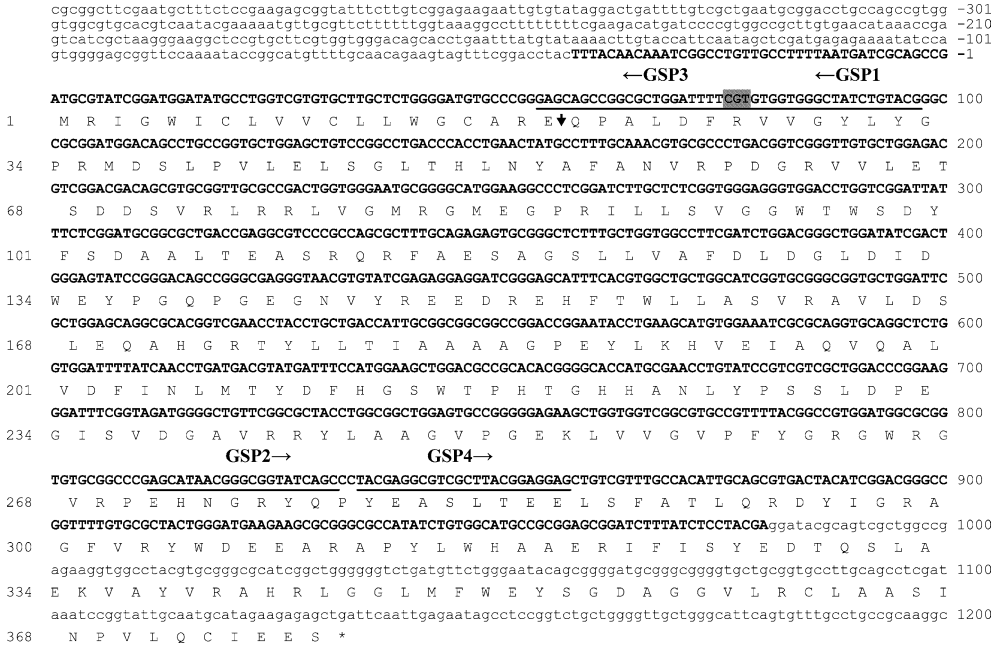

Fig. 1 The nucleotide sequence of *chiA* gene and its deduced amino acid sequence. The sequence identified primarily by analysis of the partial genome database is indicated in *boldface*. Target sequence for the gene specific primers GSP1, GSP2, GSP3, and GSP4 used for genome walking are *underlined* and *oriented*. The *shaded segment* of GSP1 and GSP3 displays a sequence overlap between the two primers. The signal peptide-cleavage site is shown by an *arrow* and the stop codon by an *asterisk*. Amino acid numbering is labeled on the *left*, and nucleotide numbering is indicated on the *right*

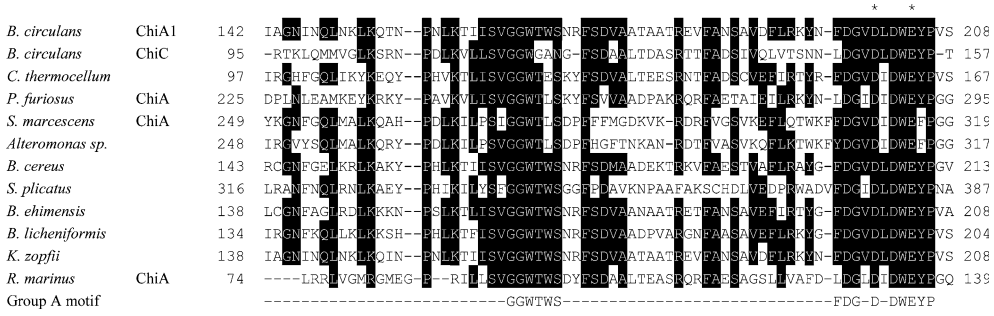

Fig. 2 Sequence comparison of the core region of the catalytic domain in *Rhodothermus marinus* chitinase A with those in other microbial chitinases. Highly conserved amino acids are *highlighted in black*. The essential Asp and Glu responsible for catalysis are indicated with *asterisks* (Watanabe et al. 1993; van Aalten et al. 2001). Sequence fragments displaying the typical motifs from the defined group A catalytic domain (Watanabe et al. 1993; Mabuchi and Araki 2001) include *Bacillus circulans* chitinases A1 and C, *Clostridium thermocellum* endochitinase, *Thermococcus kodakaraensis* chitinase A, *Serratia marcescens* chitinase A, *B. cereus* chitinase B, and chitinases from *Alteromonas* sp., *Streptomyces plicatus*, *B. ehimensis*, *B. licheniformis* and *Kurthia zopfii*. The respective GenBank accession numbers are listed in "Materials and methods." *Numbers* refer to the amino acid residues at the beginning and the end of the respective line. Sequences are numbered from the start Met (1) for each enzyme

et al. 2001). The primer used for the cloning of the signal peptide-lacking mutant *chiAΔsp* was designed with an ATG initiation codon identical to the one found in *chiA*. Expression of the recombinant ChiA and ChiAΔsp in 30-ml cultures showed detectable chitinase activities by standard chitinase assay. However, the activity test also revealed three- to fivefold higher expression levels for the ChiAΔsp mutant (data not shown). Partial characterization of both recombinant proteins showed identical T_{opt} and pH_{opt} values, suggesting that no biochemical properties were significantly altered by the suppression of the putative signal peptide (data not shown). Overexpression of ChiAΔsp was performed in a batch fermentor to guarantee higher production yields. Fermentation was conducted over 5 h, after induction with 0.2% L-rhamnose, until the cell density stabilized at $OD_{600} = 13.5$. About 236 g of cells were recovered after centrifugation and 80 g thereof were disrupted by sonication. After heat treatment of the supernatant from the sonicated cells (denaturation of thermosensitive *Escherichia coli* proteins), the recombinant chitinase was purified by automated protein chromatography (Table 2). Analysis of each fraction by SDS-PAGE showed the purification of an apparent monomeric enzyme with a deduced molecular weight of 39 kDa, in agreement with the one calculated for ChiAΔsp (40,137 Da). ChiAΔsp was expected to be >95% pure after ion exchange purification by analysis on SDS-PAGE and was used as such for the enzyme characterization (Fig. 3).

Characterization of the purified chitinase ChiAΔsp

The optimal values of T_{opt} and pH_{opt} found for both ChiA and ChiAΔsp were 70°C and pH 4.5–5, respectively (Fig. 4). The protein retained over 50% activity between pH 4 and 6.5 and between 60 and 80°C (as deduced from Figs. 4a, b). The thermal stability was examined after maintaining ChiAΔsp at temperatures higher than 70°C for 30 min, 1, 2, 4, 8 and 16 h in 100 mM KH_2PO_4/K_2HPO_4 (pH 5) and measurement of the residual activity. The enzyme showed no decrease in activity for temperatures lower than 70°C. As shown on Fig. 4c, the chitinase was highly thermostable, maintaining over 80% activity after 16 h at 80°C, and ChiAΔsp had half-lives of 3 h and 45 min at 90 and 95°C, respectively.

Substrate specificity and catalytic pattern

The hydrolyses of pNP-GlcNAc, pNP-$GlcNAc_2$, and pNP-$GlcNAc_3$ were performed to investigate the catalytic mode of action of the truncated chitinase. No release of chromogenic residues from pNP-GlcNAc and pNP-$GlcNAc_3$ was detected after 16-h incubations,

Table 2 Purification of the *Rhodothermus marinus* recombinant chitinase ChiAΔsp (very low expression yields were obtained from pChiAΔsp, and therefore, 80 g of cells were processed for the purification of ChiAΔsp)

Purification steps	Total protein (mg)	Total activity (units)	Specific activity (units mg^{-1})	Recovery from original (%)	Purification factor (fold)
Sonication[a]	5,898	62.4	0.011	100	–
Heat precipitation	715	91.6	0.12	147[b]	11
Hydrophobic separation	25.2	26.6	1.05	43	99
Ion exchange separation	1.7	12.5	7.6	20	719

[a]Clarified soluble extracts obtained after centrifugation
[b]Thermal activation of the enzyme after heat treatment might explain higher recovery yields

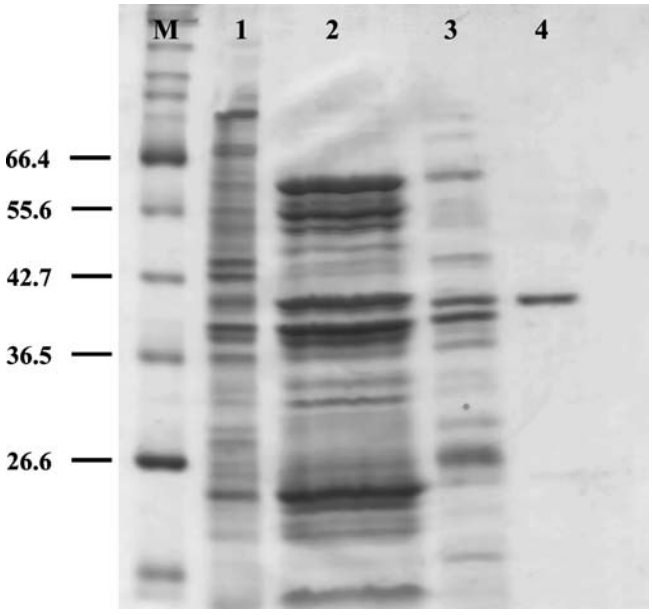

Fig. 3 Purification of ChiAΔsp followed by analysis on 12.5% SDS-PAGE stained by Coomassie Brilliant Blue. *M* Molecular mass markers (kDa), *lane 1* supernatant of sonication from crude extract (dilution 1:20), *lane 2* heat treatment of the sonicated sample (dilution 1:1), *lane 3* positive fractions of hydrophobic chromatography (dilution 1:1), *lane 4* positive fractions of cation exchange chromatography (dilution 1:1). The chitinase band appears >95% pure in lane 4

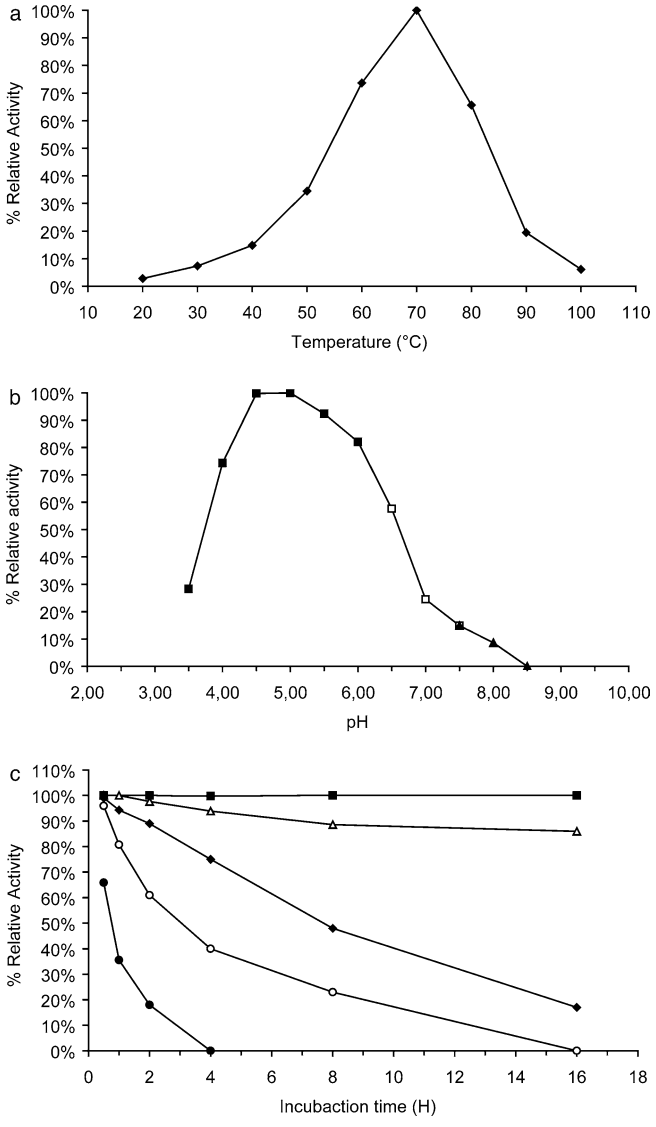

Fig. 4 Determination of physicochemical properties of ChiAΔsp under standard conditions. **a** Optimum temperature (T_{opt}) of ChiAΔsp. Experiments were conducted at pH 5, with temperature increments of 10°C. Optimum activity was found 100% at $T_{opt} = 70$°C. **b** Optimum pH (pH_{opt}) of ChiAΔsp. Experiments were conducted at 70°C. Three buffers (100 mM) were used, depending on the desired pH range: potassium acetate from pH 3.5–pH 6.5 (*filled square*), potassium phosphate from pH 6.5–7.5 (*open squares*), and tricine from pH 7.5–pH 9.5 (*filled triangles*). Optimum activity was found 100% at $pH_{opt} = 4.5$–5. **c** Thermal stability of ChiAΔsp. The enzyme was preheated at pH 5 for up to 16 h. The residual activity was measured under standard conditions for incubations at 70°C (*filled squares*), 80°C (*open triangles*), 85°C (*filled diamonds*), 90°C (*open circles*), and 95°C (*filled circle*)

suggesting that only GlcNAc-GlcNAc and GlcNAc-pNP were obtained as final products. Analysis of the enzymatic hydrolysis of natural chitin oligosaccharides $GlcNAc_{2\text{-}8}$, using 5 mU of ChiAΔsp, by TLC confirmed the results obtained with the small chromogenic substrates (Fig. 5). Incubation of ChiAΔsp with $GlcNAc_2$ for up to 80 min did not yield hydrolysis of the substrate (data not shown). The final products for chitin trimers, pentamers, and heptamers ($GlcNAc_3$, $GlcNAc_5$, and $GlcNAc_7$) were dimers and monomers. However, analysis of the hydrolysis of chitin hexamers and chitin octamers showed the formation of intermediate byproducts of three or five residues (Fig. 5), suggesting that ChiAΔsp has an endoacting catalytic activity that may be directly related to the size of the substrate. The viscosity of 1% chitosan acetate solutions was used as a tool to determine the catalytic activity of ChiAΔsp on chitosan copolymers. The enzyme was very active at 60°C, and the viscosity of the chitosan solutions dropped dramatically within less than a minute (data not shown). The measurements at room temperature showed a rapid decrease in viscosity for the DDA 73% chitosan, whereas low decrease in viscosity was observed for the DDA 99% chitosan (Fig. 6). Hydrolysis of both substrates followed by TLC showed that a large variety of additional compounds to $GlcNAc_{1\text{-}6}$ were detected at each time points for the DDA 73% solution (Fig. 7, lanes a, c, d, f, g) and are thought to be GlcN oligomers. Analysis of the hydrolysis of the DDA 99% by TLC showed no detectable products, even after an overnight incubation. The exact nature of the GlcN oligomers (i.e., acetylation pattern) remained undetermined. Hydrolysis of colloidal and crude chitin substrates yielded dimers and monomers after 16 h incubation and also small fractions of tetramers (data not shown). Chromatography on TLC revealed that no intermediate products could be detected from the first minutes (≤ 5 min) to the first hours (≤ 8 h) of incubation (data not shown).

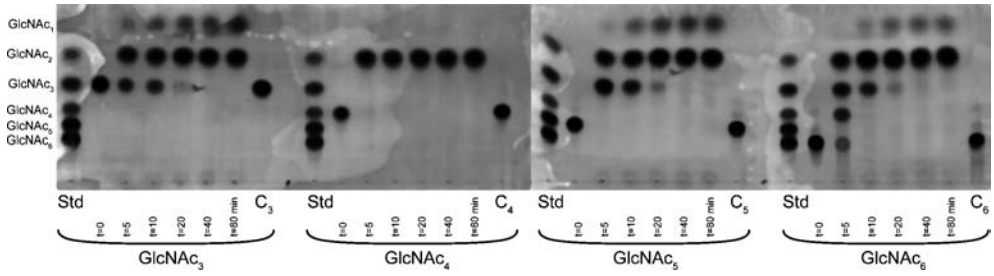

Fig. 5 Thin layer chromatography (TLC) analysis of the hydrolysis products by ChiAΔsp from the chitin oligosaccharides $GlcNAc_3$, $GlcNAc_4$, $GlcNAc_5$, and $GlcNAc_6$. ChiAΔsp was incubated for 5, 10, 20, 40, and 80 min in presence of 2.1 mM of substrates at 70°C. *Lanes* C_3, C_4, C_5, C_6 are $GlcNAc_{3\text{-}6}$ controls without enzyme; *Std* standards of $GlcNAc_{1\text{-}6}$, as labeled

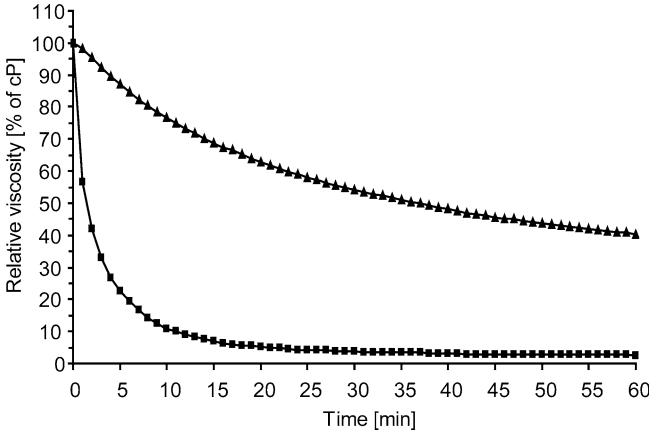

Fig. 6 Relative viscosity of 1% chitosan acetate solutions hydrolyzed with 1 U ChiAΔsp at room temperature. *Filled square* DDA 73% chitosan, *filled circle* DDA 99% chitosan

Accumulation of $GlcNAc_4$ with both soluble and insoluble chitin substrates was associated with a transglycosylation activity of ChiAΔsp catalyzing the formation of dimers into tetramers (Fig. 7, lane e). Yet, experiments to reproduce transglycosylation per se by using 2.1 mM $GlcNAc_2$ in 100 mM KH_2PO_4/K_2HPO_4 (pH 5) in an overnight reaction proved unsuccessful on TLC. ChiAΔsp showed no activity against xylan, CMC, and surprisingly, glycol chitosan. Moreover, ChiAΔsp was found unable to hydrolyze the chromogenic derivates (*p*-nitrophenyl) of xylobiose, galactopyranose, cellobiose, mannopyranose, and glucopyranose.

Kinetics of ChiAΔsp

On the basis of Lineweaver–Burke calculations, K_M was found to be 3.01 mM^{-1}, and the V_{max} value was 3.2 U min^{-1} on $pNPGlcNAc_2$. The Eadie–Hofstee calculation gave in accordance 3.43 U min^{-1} for V_{max} and 3.19 mM^{-1} for K_M. Using Lineweaver–Burke calculations, K_{cat} indicated a low enzyme turnover of 2.4 s^{-1}. The catalytic efficiency ratio K_{cat}/K_M ratio was thus 0.8 mM^{-1} s^{-1}. Kinetic parameters could not be determined for natural oligomers ($GlcNAc_{3\text{-}6}$), as it was found that more than >50% of the total substrate concentration was degraded during the reaction with ChiAΔsp under our conditions therefore skewing the measurements.

Effect of divalent cations and EDTA on enzyme activity

The residual activity obtained after conducting a standard chitinase activity test in the presence of 1 mM of divalent cation salts is listed in Table 3. Many chitinases are completely inhibited by 1 mM Hg^{2+} (Wang and Chang 1997; Xia et al. 2001; Nawani et al. 2002), but ChiAΔsp showed 23% of residual activity. No other cation had an influence on the enzyme activity. EDTA did not have any negative or positive effect in presence as well as absence of divalent ions, which clearly demonstrated that the enzyme was not dependent on divalent cations for activity. Concentrations >5 mM of Hg^{2+} resulted in complete inhibition of chitinase activity, but the addition of large excess of EDTA to mercury ions relieved integrally the inhibition.

Discussion

The *Rhodothermus marinus* recombinant chitinase ChiAΔsp hydrolyzed short oligomers (≤ five units) releasing chitobiose, therefore indicating that it was an exoacting chitobiosidase. The detection of chitin trimers by TLC analysis in the early stages of hydrolysis of $GlcNAc_{6\text{-}8}$ yet suggested that ChiAΔsp could, to a certain extent, also hydrolyze glycosidic bonds inside chitin chains, as reported previously for other exochitinases (Tanaka et al. 2001; Xia et al. 2001; Gao et al. 2003). Endoactivity of ChiAΔsp was then confirmed by using chitosan as substrate. The viscosity of chitosan solutions reflects the average molecular weight of the chitosan copolymers. A few breaks inside the chains (endoacting chitinase) will cause a rapid decrease in viscosity, whereas progressive degradation from either ends (exoacting chitinase) will show little changes in viscosity (Christodoulou et al. 2001). As shown in Fig. 6, the dramatic changes in viscosity from the DDA 73% chitosan clearly indicated that hydrolysis occurred inside the polymers and therefore proved that ChiAΔsp was an endoacting chitinase on chitopolysaccharides. The nature of glycosidic bonds hydrolyzed, i.e., whether ChiAΔsp would cut uniquely GlcNAc-GlcNAc,Glc-

Table 3 The effect of divalent cations and ethylenediamine tetraacetic acid (*EDTA*) on chitinase activity. The enzyme was incubated with 1 mM salts or 10 mM EDTA in a standard chitinase activity assay. The enzyme without additive was taken as 100%

Salts (1 mM)	Relative activity (%)
Co^{2+}	106
Fe^{2+}	100
Cu^{2+}	96
Ca^{2+}	103
Mn^{2+}	106
Mg^{2+}	104
Ni^{2+}	106
Zn^{2+}	106
EDTA	95
Hg^{2+}	23
Hg^{2+} (5 mM)	2
Hg^{2+} (10 mM)	1
5 mM Hg^{2+} + 50 mM EDTA	100

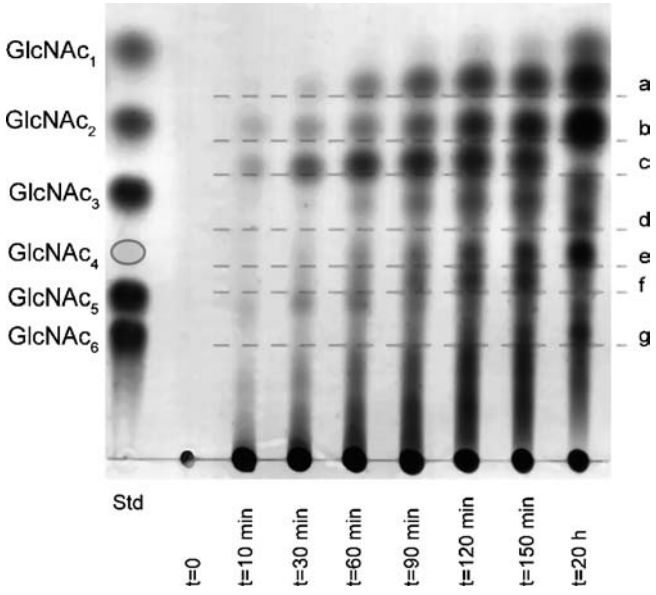

Fig. 7 TLC analysis of the hydrolysis of the DDA 73% chitosan solution by ChiAΔsp. Ten units of enzyme were incubated at 65°C with 150 ml 1% substrate from 10 min to 20 h. Aliquots were taken at indicated times. *Lanes a–g* stress the evolution of intermediate and end products throughout the experiment. The major product obtained was chitin dimers $GlcNAc_2$ (*lane b*). *Lanes a*, *c*, *d*, and *f* show the accumulation of various chitooligosaccharides, i.e., homologues of GlcNAc and GlcN, where the amine group (GlcN) interferes with the clear distribution of chitin oligosaccharides ($GlcNAc_n$). *Lane e* shows the progressive formation of chitin tetramers due to transglycosyl activity of ChiAΔsp. *Std* standards of $GlcNAc_{1\text{-}6}$

NAc-GlcN, or GlcN-GlcNAc bonds, as well as the acetylation patterns of the target substrates recognized by ChiAΔsp, could not be identified. However, the slow changes observed for the DDA 99% chitosan strongly suggested that ChiAΔsp is not able to hydrolyze fully deacetylated chitosan polymers (GlcN-GlcN bonds). Additionally, although chitin dimers and monomers were obtained after overnight incubation, pure endochitinase activity on native crystalline and colloidal chitin was not detected. Those substrates are not easily accessable due to their highly compact structure, and only protruding chitin fibrils may have been hydrolyzed (Tanaka et al. 2001). The absence of a chitin-binding domain, which has been proposed as mediating the nonhydrolytic disruption of crystalline chitin (Tanaka et al. 2001; Andronopoulou and Vorgias 2003) is likely to be responsible for a lesser activity towards those substrates.

Chitinases from hyperthermophilic Archaea show very high stability at temperatures above 100°C (Tanaka et al. 2001; Andronopoulou and Vorgias 2003; Gao et al. 2003), but thermostable bacterial chitinases show significantly lesser resistance to high temperatures. Chitinases from *Bacillus* species have in particular showed increased T_{opt} and thermostability compared to other bacterial chitinases. Bhushan and Hoondal (1998) reported a *Bacillus* sp. chitinase having a T_{opt} of 55°C and a half-life of 20 min at 90°C. They also showed that immobilization of the enzyme enhanced the thermostability dramatically (Bhushan 2000). Similarly, three thermostable endochitinases from *Bacillus* strain MH-1 were having a T_{opt} between 65 and 75°C and had respective half-lives of 10 min at 85, 75, and 70°C (Sakai et al. 1998). For comparison, the *R. marinus* chitinase retained 100% of activity after 16 h incubation at T_{opt} (70°C), and although the thermal stability dropped with heat, ChiAΔsp still maintained half-lives of 3 h at 90°C and 45 min at 95°C. Our enzyme is thus the most thermostable bacterial chitinase so far.

Chitinases are generally classified into two major groups: (1) endochitinases which randomly hydrolyze GlcNAc-GlcNAc bonds along the chitin chain and (2) exochitinases. Exochitinases can furthermore be grouped in two categories: exochitobiosidases, which hydrolyze chitin by the sequential release of $GlcNAc_2$ units from the non-reducing end and *β-N*-acetylglucosaminidases, which cleave chitin oligomers (≥2 GlcNAc residues) into GlcNAc from the non-reducing end. However, in many instances, the catalytic activity of endoacting chitinases varies from one organism to the next. Andronopoulou and Vorgias (2003) described an endoacting thermostable chitinase from the archaeon *Thermococcus chitonophagus*, which, like ChiAΔsp, yields chitin dimers from chitooligosaccharides larger than three chitin units. Another archaeal endochitinase from *Pyrococcus furiosus* (recombinant catalytic domain) was found to be not only limited by the size of the substrate, i.e., polymers with more than four chitin units, but also yielded glucosamine monomers from those oligomers (Gao et al. 2003). The choice of substrates, i.e., chromogenic substrates pNP-GlcNAc, pNP-$GlcNAc_2$, pNP-$GlcNAc_3$, colloidal chitin, or chitosan, thus influences the identification of the catalytic activity, and many exochitinases identified uniquely by their ability to degrade pNP-$GlcNAc_2$ may very likely have also endochitinase activities on larger substrates (Howard et al. 2003). The classification of chitinases into three unique groups, i.e., endo- and exochitinases and

β-*N*-acetylglucosaminidases, appears too restrictive due to the diversity of the respective catalytic behaviors and classification as family 18 chitinases (or families 3, 20, or 84 for β-*N*-acetylglucosaminidases, for review see Henrissat 1999) seems then more appropriate although less informative.

About 20 strains of *R. marinus* sampled from various geothermal environments in Iceland were showed to grow on minimal medium supplemented with 1% chitosan (unpublished data). The growth of *R. marinus* on chitosan was confirmed by the detection of a β-*N*-acetylglucosaminidase gene in its genome (data not shown), suggesting that the β-*N*-acetylglucosaminidase action is combined with the chitinase for the complete hydrolysis of chitinous substrates in nature and leads to the uptake of GlcNAc. Moreover, the presence of the signal peptide implies then that ChiA is secreted into the extracellular space.

Chitinases have a broad range of industrial applications such as biocontrol against plant pathogenic fungi and insects, single-cell protein production by hydrolysis of yeast material, production of fungal protoplasts, and most particularly, the production of chitooligosaccharides (Patil et al. 2000; Sutrisno et al. 2004). The *R. marinus* chitinase was shown to be active on various types of chitin and chitosan polymers releasing a high diversity of chitodextrins at the early stages of the reaction. Our enzyme was active in the preferred lower temperature range (45–65°C) required for the industrial processing of chitin, and it showed best activity at low pH, which is beneficial for the solubility of chitosan copolymers (Sutrisno et al. 2004). Due to its high versatility regarding pH range, temperature range (from room temperature to 85°C), thermostability, and substrate specificity towards chitosan copolymers, the *R. marinus* family 18 chitinase seems thus to be a highly attractive enzyme for the production of chitooligosaccharides, and more generally for biotechnological applications.

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