Extremophiles
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ORIGINAL PAPER

Molecular analysis of the gene encoding a new chitinase from the marine psychrophilic bacterium *Moritella marina* and biochemical characterization of the recombinant enzyme

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Abstract The marine psychrophilic bacterium *Moritella marina*, isolated from a sample raised from a depth of 1,200 m in the northern Pacific Ocean, secretes several chitinases in response to chitin induction. A gene coding for an extracellular chitinolytic enzyme was cloned and its nucleotide sequence was determined. The chitinase gene consists of an open reading frame of 1,650 nucleotides and encodes a protein of 550 amino acids with a calculated molecular weight of 60.788 kDa, named *Mm*Chi60. *Mm*Chi60 has a modular structure consisting of a glycosyl-hydrolase family 18 N-terminal catalytic region as well as a C-terminal chitin-binding domain (ChBD). The new chitinase was purified to homogeneity from the intracellular fraction of *Escherichia coli*. The optimum pH and temperature of the recombinant *Mm*Chi60 were 5.0 and 28°C, respectively. The mode of action of the new enzyme on N-acetylchitooligomers, chitin polymers, and other substrates was examined, and *Mm*Chi60 was classified as an endochitinase. Thermal unfolding of *Mm*Chi60 was studied using differential scanning microcalorimetry and revealed that the protein unfolds reversibly at 65°C. On the basis of the crystal structure of the chitinase C of *Streptomyces griseus*, a homology-based 3-D model of the ChBD of the *Mm*Chi60 was calculated.

Keywords Cold-adapted · Chitinase · Marine bacterium · *Moritella* · Purification · Biochemical characterization

Introduction

Chitin, a highly insoluble biopolymer, is composed of linear chains of β-(1,4)-linked N-acetyl-D-glucosamine residues that are highly cross-linked by hydrogen bonds, like cellulose. Chitin is abundant in nature, second only to cellulose, as a crucial structural component of the cell walls of fungi and certain green algae, and as a major constituent of the shells, cuticles, and exoskeletons of worms, mollusks, and arthropods, including crustaceans and insects (Muzzarelli 2002). Chitin and its partially deacetylated derivative, chitosan, as well as other derivatives exhibit interesting properties and constitute a valuable raw material for biomedical, agricultural, cosmetics, and innovative biotechnological applications (Muzzarelli 1997; Shigemasa and Minami 1996). In the aquatic biosphere, chitin corresponds to an annual production of about 10^{11} ton (Keyhani and Roseman 1999).

Chitinases (EC 3.2.1.14) hydrolyse the β-1,4-linkages in chitin. The chitinases, so far sequenced or identified, are classified into two different families 18 and 19 within the glycosyl-hydrolases superfamily established by Henrissat and Bairoch (1993) based on the amino acid sequence similarity of their catalytic regions. Family 18 contains chitinases from bacteria, fungi, viruses, animals, and some plant chitinases. On the other hand, family 19 contains plant chitinases and few bacterial chitinases such as *Streptomyces griseus* chitinase C (ChiC) (Ohno et al. 1996). The chitinases of both families do not share amino acid sequence similarity, have various 3-D structures (Perrakis et al. 1994; Terwisscha van Scheltinga et al. 1995) and enzymatic mechanisms, and

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are therefore likely to have evolved from diverse ancestors. Bacterial chitinases generally consist of multiple functional domains such as chitin-binding domain (ChBD) and fibronectin type III-like domain (Fn3 domain) linked to the catalytic domain. The involvement of the ChBD in the degradation of insoluble chitin has been analysed for few bacterial chitinases (Svitil and Kirchman 1998; Watanabe et al. 1994).

The major part of the marine biosphere is characterized by permanent low temperatures (−2–10°C). Psychrophilic microorganisms can be found in marine biosphere and in permanently cold environments. Thus, the chitinases produced by psychrophilic bacteria, responsible for the degradation of the krill chitin, should have high catalytic activities under these low-temperature conditions, and are most often, if not always, associated with high thermosensitivity (Gerday et al. 1997). These properties can be extremely useful for various applications. During the past few years, several psychrophilic enzymes have been reported (Luo et al. 2006; Yaish et al. 2006) and the 3-D structure of some of them has been determined (Van Petegem et al. 2003; Violot et al. 2005). Until recently, few psychrophilic chitinases have been isolated from bacteria (Bendt et al. 2001; Lonhienne et al. 2001; Orikoshi et al. 2003) and fungi (Fenice et al. 1998).

In this work, we report cloning, sequence, and characterization of the gene encoding for a new chitinase, *Mm*Chi60, from the psychrophilic marine bacterium *Moritella marina*. The production, purification, and biochemical characterization of the recombinant enzyme are also described. On the basis of current available data, the model of the ChBD of the protein has been constructed.

Materials and methods

Bacterial strains, plasmids, DNA manipulations, and other materials

The bacterial strain *M. marina* (synonym *Vibrio marinus*) was isolated from a sample raised from a depth of 1,200 m in the northern Pacific Ocean and at a temperature of 3.24°C (ATCC 15381) (Baumann et al. 1984). The bacterial cells were grown at the temperature of 15°C for 48 h in Bacto Marine broth medium 2216 (Difco, USA). Induction of chitinolytic activity was carried out on Marine agar (Difco, USA) containing 1% (w/v) of colloidal chitin (Shimahara and Takiguchi 1988) and in Marine broth medium containing 0.5% (w/v) of colloidal chitin. *Escherichia coli* strains INVaF′ and Top10F′ were purchased from Invitrogen (USA). *E. coli* strain BLR(DE3) was obtained from Novagen (Germany). *Eco*RI cassettes were provided by Takara (Japan). The plasmids pCR 2.1 and pET-11a were obtained from Invitrogen and Novagen, respectively. DNA manipulations were performed by standard methods as described by Sambrook et al. (1989). Chitin and various substrates were purchased from Sigma (Germany). Carboxymethyl-chitin-Remazol Brilliant Violet (CM-chitin-RBV) was obtained from Loewe Biochemica (Germany). Allosamidin was a gift from Professor Shohei Sakuda (University of Tokyo, Japan). The column chromatography media were purchased from Pharmacia (Sweden). Marker proteins and polyacrylamide electrophoresis reagents were provided by Bio-Rad (USA) and Serva (Germany), respectively. All the other chemicals were provided by Sigma (Germany).

Cloning of *Mmchi60*

In order to detect and isolate a chitinase gene from *M. marina*, an amino acid sequence alignment of several psychrophilic bacterial chitinases was carried out and a number of highly conserved regions were detected (data not shown). This information was compiled to design degenerate primers (Table 1), which were used to amplify a chitinase gene from *M. marina*. PCR amplifications were performed using Platinum *Taq* DNA Polymerase High Fidelity (Invitrogen) for 35 cycles, consisting of 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min. Three DNA fragments were isolated from *M. marina* chromosomal DNA. The 1.5-kbp fragment was amplified from the chromosomal DNA, and the 170 and 340 bp fragments were amplified from a genomic library, which was constructed using *Eco*RI cassettes. All three fragments

Table 1 PCR primers used for amplification of DNA fragments encoding respective domains of the *Mmchi60* gene

Primers	5′-sequence-3′
1-Forward	GGTGTCGTCGTGGGTTACTGGCATAATT
2-Reverse	TTAATGCTTAGTCCACGCATCTTGCCA
3-Direct	GGTGAAGTTGATGCTAATGTCGTTGGTG
4-Reverse	TTGGTAGCCACGACCATCACACCAATTATG
5-*Eco*RI cassette	GTAATACGACTCACTATAGGGC
6-*Nde*I-forward	GCTCATATGAAGCTTAAATCGATACTTTCAGCGGC
7-*Bam*HI-reverse	AAAGGATCCTTACTAACGCCAAACTCCCCATTCGC

composed the open reading frame of a chitinase gene from *M. marina* and parts beyond its 3′ and 5′ ends. The amplification of the entire *Mmchi60* gene, 1,653 bp long, including an *Nde*I and *Bam*HI sites at the N-end and C-end, respectively, was obtained from the chromosomal DNA of *M. marina*. The final product was inserted into the plasmid vector pCR 2.1. The pCR2.1-*Mmchi60* clone was verified by DNA sequencing. The *Mmchi60* gene was further cloned into the T7 expression vector pET-11a by ligating the *Nde*I–*Bam*HI fragment of the clone pCR2.1-*Mmchi60* to *Nde*I–*Bam*HI-digested pET-11a vector. Ligated plasmids were used to transform *E. coli* BLR(DE3) host cells.

Production and purification of recombinant *Mm*Chi60

Induction kinetics of *E. coli* BLR(DE3) host cells harboring the pET-11a-*Mmchi60* plasmid were performed in Luria-Bertani medium containing 100 μg/ml of ampicillin at 37°C. The culture was induced with 0.5 mM iso-propyl-thio-galactoside at the mid-exponential growth phase and further incubated at 18°C. Samples were withdrawn at various time points. For large-scale production, the induction time was 3 h. The produced bacterial cells in 3.5 g quantities were either used immediately or kept frozen until needed. All extraction and purification procedures were performed at 4°C, except otherwise specified. Cells were harvested by centrifugation at 8,000×*g*, washed, and suspended (10 ml/g) in 20 mM Na phosphate buffer (pH 8.0), 100 mM NaCl, 1 mM EDTA, 0.5% (v/v) Triton X-100, and 0.5 mM phenyl-methane-sulphonyl-fluoride (PMSF) The cells were disrupted by sonication, and the lysate was centrifuged at 12,000×*g* for 1 h. The clear supernatant was further fractionated with solid ammonium sulphate. *Mm*Chi60 activity was detected in the fraction of 40–60% saturation with ammonium sulphate. This protein fraction was dissolved in 20 mM Na phosphate buffer (pH 8.0) and 0.5 M ammonium sulphate, and directly applied on a 10-ml Phenyl-Sepharose CL-6B column (Pharmacia, Sweden), previously equilibrated in the same buffer. The column was washed with the same buffer and bound proteins were eluted with a 100-ml descending gradient of ammonium sulphate between 0.5 and 0 M. *Mm*Chi60 was eluted at about 0.1 M ammonium sulphate. *Mm*Chi60 pooled fractions were diluted five times with 20 mM Na phosphate buffer (pH 8.0) and directly applied on a 10-ml Q-Sepharose Fast Flow column (Pharmacia), previously equilibrated in the same buffer. Bound proteins were eluted using a 100-ml linear ascending gradient between 0 and 0.5 M NaCl. Pure *Mm*Chi60 was eluted at about 0.45 M NaCl. Enzymatically active and pure *Mm*Chi60 fractions were pooled and stored at 4°C. Under these conditions, the purified enzyme was stable for at least 3 months. Gel filtration column chromatography of pure *Mm*Chi60 was performed using a Sephacryl S-100 column (2.5 cm × 80 cm, Pharmacia) in 20 mM Na phosphate and 100 mM NaCl (pH 8.0).

Enzyme assays and kinetic parameters

Chitinase activity was measured using p-nitrophenyl-*β*-1,4-*N*,*N*′-diacetyl-chitobiose [pNP-$(NAG)_2$] (Sigma) as a substrate. In standard activity assay, 1 μg *Mm*Chi60 was incubated with 20 mM Na acetate (pH 5.0) and 20 μM pNP-$(NAG)_2$ at 28°C for 10 min. The reaction was terminated by the addition of 1 M glycine-NaOH (pH 10.5), and the release of *p*-nitrophenol was monitored at 405 nm. Enzyme activity was calculated on the basis of an extinction coefficient for *p*-nitrophenol of 18,500 M/cm. One unit of chitinase activity was defined as the amount of the enzyme, which produces 1 μmol of *p*-nitrophenol/min.

The optimum temperature for *Mm*Chi60 activity was measured in the standard activity assay at temperatures ranging from 0 to 60°C. The optimum pH was measured in the standard activity assay at a pH range of 3–10.

The substrate specificity of *Mm*Chi60 was determined using synthetic and natural substrates (Sigma) (Table 3). Concerning the natural substrates, the amount of the reducing sugar from the action of *Mm*Chi60 was quantified according to a modification of the Schales procedure (Imoto and Yagishita 1971). The *Mm*Chi60 activity on synthetic substrates was measured under the standard activity assay.

The thermal stability of the *Mm*Chi60 was measured by incubating the purified enzyme with 20 mM Na phosphate buffer (pH 8.0) at temperatures ranging from 0 to 60°C for a time period of up to 96 h. Similarly, the pH stability was determined by pre-treating the enzyme with various pH buffers at 4°C for a time period of up to 10 days. The effect of allosamidin (0.1–10 μM final concentration), urea (0.5–5 M final concentration), EDTA, dithiothreitol (DTT), iodoacetamide (IAAM), and PMSF (1–10 mM final concentration), as well as bivalent cations (Fe^{2+}, Mg^{2+}, Zn^{2+}, Co^{2+}, Mn^{2+}, Ca^{2+}, Sr^{2+}, Ni^{2+}, 1–10 mM final concentration) on the *Mm*Chi60 activity was examined. The enzyme was pre-treated with each reagent mentioned above for 30 min at room temperature, and 20 μM pNP-$(NAG)_2$ was subsequently added. The residual activity was determined under the standard activity assay.

The values of kinetic constants K_m, V_{max}, and k_{cat} were determined from the respective Lineweaver–Burk plots.

Analysis of hydrolysis products of various chitinous polymers

Hydrolysis products arising from the action of *Mm*Chi60 were determined using colloidal chitin and synthetic substrates. Oligosaccharides derived from chitin are *β*-(1,4)-linked oligomers of NAG and are designated: $(NAG)_2$ for

β-1,4-*N*,*N*′-diacetyl-chitobiose; $(NAG)_3$, $(NAG)_4$, $(NAG)_5$, and $(NAG)_6$ for the corresponding chitotriose, chitotetraose, chitopentaose, and chitohexaose. *Mm*Chi60 (1 µg/ml) was incubated with 1 mg/ml of synthetic oligosaccharides and 20 mM Na acetate (pH 5.0) at 28°C for 2 h. In the case of colloidal chitin, 10 µg/ml of *Mm*Chi60 was incubated with 1 mg/ml of substrate and 20 mM Na acetate (pH 5.0) at 28°C for 12 h. The hydrolytic products were analysed by high performance liquid chromatography (HPLC) (reverse phase, aminopropyl-silica column, μ-Bondapak, 125A, Waters). The sugars were isocratically eluted with 75% acetonitrile in double distilled H_2O at a rate of 3 ml/min and at room temperature. The separated products were detected by refractive index at 210 nm and compared to standard chitooligosaccharides.

Polyacrylamide gel electrophoresis, zymograms, and protein determination

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (1970). Non-denaturing polyacrylamide gels were prepared using the Davis method (Davis 1964). Precision Plus protein standards were used as molecular weight marker (Bio-Rad, USA). The chitinolytic activity was also detected using a zymogram technique in SDS-PAGE containing 0.7 mg/ml CM-chitin-RBV (McGrew and Green 1990). Protein concentration was determined using the Coomassie dye binding assay (Pierce, USA) according to Bradford (1976), using bovine serum albumin (Pierce) as standard. The protein concentration of the purified protein was determined using the extinction coefficient for *Mm*Chi60 of 106,355 M/cm at 280 nm (Pace et al. 1995; Gill and von Hippel 1989).

Western blotting

*Mm*Chi60 purified from *E. coli* and proteins in the culture supernatant of *M. marina* grown on colloidal chitin was separated by SDS-PAGE and transferred onto a poly vinylidene di-fluoride (PVDF) membrane (Amersham, UK). The membrane was incubated for 1 h at room temperature with anti-*Mm*Chi60 polyclonal rabbit anti-serum diluted to 1:1,000 with phosphate-buffered saline containing 10% skim milk. Immunoreactive proteins were detected using peroxidase-conjugated goat anti-rabbit immunoglobulins (Amersharm) and enhanced chemiluminescence reagents (Amersharm).

Differential scanning microcalorimetry

The VP DSC calorimeter was employed (Microcal, Northampton, MA, USA). A typical DSC experiment consisted of a temperature scan at a heating rate 1.5°C/min, followed by a second temperature scan at the same heating rate after cooling, to probe the reversibility of the thermal transition. Whenever needed, the difference in the heat capacity between the initial and final states was modeled by a sigmoidal chemical baseline. Protein concentration was 2 mg/ml for *Mm*Chi60, and the buffer used was 50 mM Na phosphate (pH 8.0). The calorimetric data were analysed via non-linear least squares fitting procedures of the ORIGIN 6.1 software.

N-terminal amino acid sequencing and bioinformatica analysis

The N-terminal amino acid sequence was determined by stepwise Edman degradation (Niall 1973) in an automated protein sequencer (Applied Biosystems, USA), using an electroblotted protein sample on PVDF membrane (Amersham).

Bioinformatica analysis of *Mm*Chi60 was carried out using the service facilities of the EBI (http://www.ebi.uk/tools) and PD server (http://www.expasy.ch/tools).

Nucleotide sequencing and accession number

Nucleotide sequencing was carried out by the DNA sequencing service of the VBC Genomics Company (Austria). The nucleotide sequence of *Mmchi60* has been deposited and assigned accession number AM691849 in the EMBL/GenBank/DDBJ database.

Results

Growth of *M. marina* on chitin

To detect chitinolytic activity of *M. marina*, individual colonies of this strain were streaked on Marine agar plates supplemented with 1% colloidal chitin. The plates were incubated at 4°C, and after 2 days, the formation of halos around bacterial colonies was detected as a result of chitin degradation (data not shown).

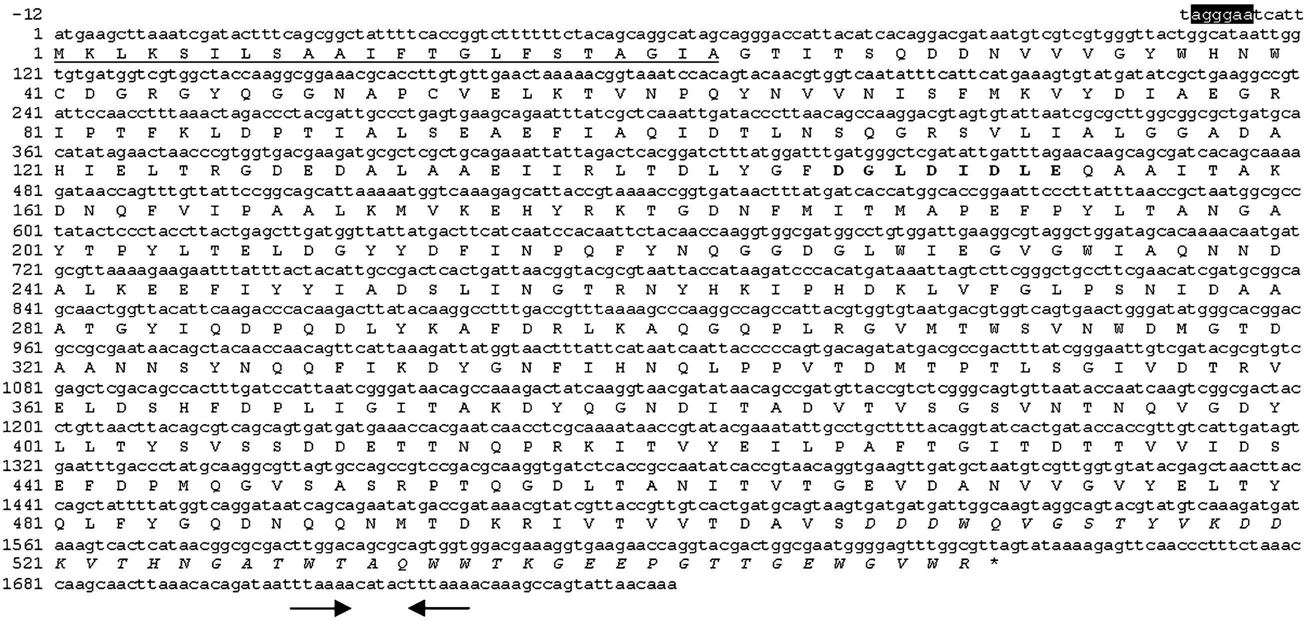
Cloning and nucleotide sequence of *Mmchi60* gene

Three fragments (1.5 kbp, 170 bp, and 340 bp) of a chitinase gene were amplified using degenerate primers (Table 1), chromosomal DNA, and a genomic library of *M. marina*. Sequencing analysis revealed overlapping regions with all three fragments, indicating that all fragments carried the same chitinase gene of *M. marina*.

The *Mmchi60* gene consists of 1,653 nucleotides encoding a protein of 550 amino acids with a calculated molecular weight of 60.788 kDa. The open reading frame contains a putative initiation codon ATG that is preceded at a spacing of 6 bp by a potential ribosome-binding sequence (5′-AGGGAA-3′) homologous to the consensus Shine–Dalgarno sequence (Shine and Dalgarno 1975) and a stop codon TAG. Downstream of the stop codon are short inverted repeats, which are possible transcription terminators. The N-terminal region of *Mm*Chi60 protein exhibited a predicted signal peptide consisting of 22 amino acids (Fig. 1).

Domain structure of *Mm*Chi60

The BLAST analysis programme was used to compare *Mm*Chi60 with proteins in databases. The best identity (86%) was detected with chitinase A of *Moritella* sp. PE36 (accession number ZP01898665) and a high similarity with a number of other prokaryotic chitinases has also been detected. Prediction of the functional domains of the protein revealed an N-terminal region (110–154 amino acid residues) corresponding to the catalytic region of family 18 of glycosyl-hydrolases and a C-terminal region (507–550 amino acid residues) corresponding to the ChBD of chitinases. The amino acid sequence of these domains was compared with the respective regions of bacterial chitinases using the CLUSTAL W programme. The N-terminal catalytic region of *Mm*Chi60 showed sequence identity to the family 18 catalytic regions identified in some bacterial chitinases (Fig. 2). This catalytic region showed sequence identities of 93% to *Moritella* sp. PE36 chitinase A



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  -12                                                                                                             tagggaatcatt
    1 atgaagcttaaatcgatactttcagcggctattttcaccggtctttttctacagcaggcatagcagggaccattacatcacaggacgataatgtcgtcgtgggttactggcataattgg
    1 M  K  L  K  S  I  L  S  A  A  I  F  T  G  L  F  S  T  A  G  I  A  G  T  I  T  S  Q  D  D  N  V  V  V  G  Y  W  H  N  W
  121 tgtgatggtcgtggctaccaaggcggaaacgcaccttgtgttgaactaaaaacggtaaatccacagtacaacgtggtcaatatttcattcatgaaagtgtatgatatcgctgaaggccgt
   41 C  D  G  R  G  Y  Q  G  G  N  A  P  C  V  E  L  K  T  V  N  P  Q  Y  N  V  V  N  I  S  F  M  K  V  Y  D  I  A  E  G  R
  241 attccaacctttaaactagaccctacgattgccctgagtgaagcagaatttatcgctcaaattgatacccttaacagccaaggacgtagtgtattaatcgcgcttggcggcgctgatgca
   81 I  P  T  F  K  L  D  P  T  I  A  L  S  E  A  E  F  I  A  Q  I  D  T  L  N  S  Q  G  R  S  V  L  I  A  L  G  G  A  D  A
  361 catatagaactaacccgtggtgacgaagatgcgctcgctgcagaaattattagactcacggatctttatggatttgatgggctcgatattgatttagaacaagcagcgatcacagcaaaa
  121 H  I  E  L  T  R  G  D  E  D  A  L  A  A  E  I  I  R  L  T  D  L  Y  G  F  D  G  L  D  I  D  L  E  Q  A  A  I  T  A  K
  481 gataaccagtttgttattccggcagcattaaaaatggtcaaagagcattaccgtaaaaccggtgataactttatgatcaccatggcaccggaattcccttatttaaccgctaatggcgcc
  161 D  N  Q  F  V  I  P  A  A  L  K  M  V  K  E  H  Y  R  K  T  G  D  N  F  M  I  T  M  A  P  E  F  P  Y  L  T  A  N  G  A
  601 tatactccctaccttactgagcttgatggttattatgacttcatcaatccacaattctacaaccaaggtggcgatggcctgtggattgaaggcgtaggctggatagcacaaaacaatgat
  201 Y  T  P  Y  L  T  E  L  D  G  Y  Y  D  F  I  N  P  Q  F  Y  N  Q  G  G  D  G  L  W  I  E  G  V  G  W  I  A  Q  N  N  D
  721 gcgttaaaagaagaatttatttactacattgccgactcactgattaacggtacgcgtaattaccataagatcccacatgataaattagtcttcgggctgccttcgaacatcgatgcggca
  241 A  L  K  E  E  F  I  Y  Y  I  A  D  S  L  I  N  G  T  R  N  Y  H  K  I  P  H  D  K  L  V  F  G  L  P  S  N  I  D  A  A
  841 gcaactggttacattcaagacccacaagacttatacaaggcctttgaccgtttaaaagcccaaggccagccattacgtggtgtaatgacgtggtcagtgaactgggatatgggcacggac
  281 A  T  G  Y  I  Q  D  P  Q  D  L  Y  K  A  F  D  R  L  K  A  Q  G  Q  P  L  R  G  V  M  T  W  S  V  N  W  D  M  G  T  D
  961 gccgcgaataacagctacaaccaacagttcattaaagattatggtaactttattcataatcaattacccccagtgacagatatgacgccgactttatcgggaattgtcgatacgcgtgtc
  321 A  A  N  N  S  Y  N  Q  Q  F  I  K  D  Y  G  N  F  I  H  N  Q  L  P  P  V  T  D  M  T  P  T  L  S  G  I  V  D  T  R  V
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  361 E  L  D  S  H  F  D  P  L  I  G  I  T  A  K  D  Y  Q  G  N  D  I  T  A  D  V  T  V  S  G  S  V  N  T  N  Q  V  G  D  Y
 1201 ctgttaacttacagcgtcagcagtgatgatgaaaccacgaatcaacctcgcaaaataaccgtatacgaaatattgcctgcttttacaggtatcactgataccaccgttgtcattgatagt
  401 L  L  T  Y  S  V  S  S  D  D  E  T  T  N  Q  P  R  K  I  T  V  Y  E  I  L  P  A  F  T  G  I  T  D  T  T  V  V  I  D  S
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  441 E  F  D  P  M  Q  G  V  S  A  S  R  P  T  Q  G  D  L  T  A  N  I  T  V  T  G  E  V  D  A  N  V  V  G  V  Y  E  L  T  Y
 1441 cagctattttatggtcaggataatcagcagaatatgaccgataaacgtatcgttaccgttgtcactgatgcagtaagtgatgatgattggcaagtaggcagtacgtatgtcaaagatgat
  481 Q  L  F  Y  G  Q  D  N  Q  Q  N  M  T  D  K  R  I  V  T  V  V  T  D  A  V  S  D  D  D  W  Q  V  G  S  T  Y  V  K  D  D
 1561 aaagtcactcataacggcgcgacttggacagcgcagtggtggacgaaaggtgaagaaccaggtacgactggcgaatggggagtttggcgttagtataaaagagttcaaccctttctaaac
  521 K  V  T  H  N  G  A  T  W  T  A  Q  W  W  T  K  G  E  E  P  G  T  T  G  E  W  G  V  W  R  *
 1681 caagcaacttaaacacagataatttaaaacatactttaaaacaaagccagtattaacaaa
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Fig. 1 Nucleotide (*upper*) and deduced amino acid sequence (*lower*) of the *Mmchi60* gene and its flanking region. The Shine–Dalgarno (SD) sequence is *shaded*. The signal peptide is *underlined*. The amino acid residues $F_{145}DGLDIDLE_{153}$ are essential for chitinase activity ("Prosite signature" for family 18 of glycosyl-hydrolases). The chitin-binding domain homologs are in *italics*. The stop codon is indicated by an *asterisk*. *Horizontal arrows* indicate inverted sequences, which are possible transcription terminators

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MmChi60     110 SVLIALGG---ADAHIELTRGDE--DALAAEIIRLTDLYG-FDGLDIDLEQ 154
MPE36ChiA   110 SVLLALGG---ADAHIALTRGDE--DALAAEIIRLTDRYG-FDGLDIDLEQ 154
VhChiA      114 SVLLALGG---ADAHVELETGDE--RAFADEIIRLTERYG-FDGLDIDLEQ 158
SmChiC       98 AVLISLGG---ADAHIELKTGDE--DKLKDEIIRLVEVYG-FDGLDIDLEQ 142
PachiC      100 AVLISLGG---ADAHIELHAGQE--QALAAEIVRLVETYG-FDGLDIDLEQ 144
BcChiD      258 KVLISMGG---ANGRIELTDATKKRQQFEDSLKSIISTYG-FNGLDIDLEG 304
BcChiA1     156 KTIISVGGWTWSNRFSDVAATAATREVFANSAVDFLRKYN-FDGVDLDWEY 205
SmChiA      265 KILPSIGGWTLSDPFFFMGDKVK-RDRFVGSVKEFLQTWKFFDGVDIDWEF 316
                                                                *
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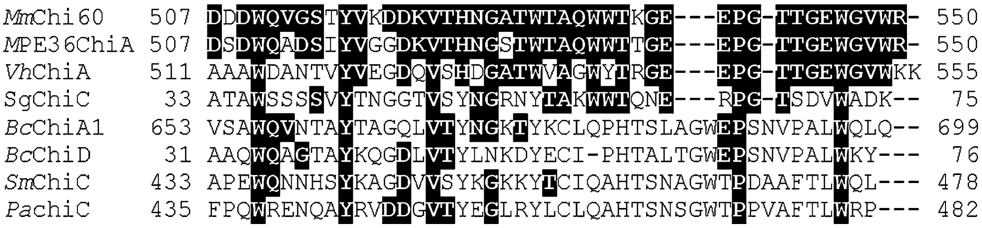
Fig. 2 Amino acid sequence alignment of family 18 catalytic regions of *Mm*Chi60 and several bacterial chitinases. *MPE36 Moritella* sp. PE36, *VhChiA Vibrio harveyi* chitinase A, *SmChiC Serratia marcescens* chitinase C, *PaChiC Pseudomonas aeruginosa* chitinase C, *BcChiD Bacillus circulans* chitinase D, *BcChiA1 B. circulans* chitinase A1, *SmChiA S. marcescens* chitinase A. A *black background* indicates the amino acid residues that are identical with those of *Mm*Chi60. Glu residue, identified as a proton donor, is marked with an *asterisk*. The alignment was obtained using the CLUSTAL W programme



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MmChi60    507 DDDWQVGSTYVKDDKVTHNGATWTAQWWTKGE---EPG-TTGEWGVWR- 550
MPE36ChiA  507 DSDWQADSIYVGGDKVTHNGSTWTAQWWTTGE---EPG-TTGEWGVWR- 550
VhChiA     511 AAAWDANTVYVEGDQVSHDGATWVAGWYTRGE---EPG-TTGEWGVWKK 555
SgChiC      33 ATAWSSSSVYTNGGTVSYNGRNYTAKWWTQNE---RPG-TSDVWADK--  75
BcChiA1    653 VSAWQVNTAYTAGQLVTYNGKTYKCLQPHTSLAGWEPSNVPALWQLQ-- 699
BcChiD      31 AAQWQAGTAYKQGDLVTYLNKDYECI-PHTALTGWEPSNVPALWKY---  76
SmChiC     433 APEWQNNHSYKAGDVVSYKGKKYTCIQAHTSNAGWTPDAAFTLWQL--- 478
PachiC     435 FPQWRENQAYRVDDGVTYEGLRYLCLQAHTSNSGWTPPVAFTLWRP--- 482
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Fig. 3 Amino acid sequence alignment of chitin-binding domains of *Mm*Chi60 and several bacterial chitinases. *MPE36 Moritella* sp. PE36, *VhChiA V. harveyi* chitinase A, *SgChiC S. griseus* chitinase C, *BcChiA1 B. circulans* WL-12 chitinase A1, *BcChiD B. circulans* WL-12 chitinase D, *SmChiC S. marcescens* chitinase C, *PaChiC P. aeruginosa* chitinase C. A *black background* indicates the amino acid residues identical to those of *Mm*Chi60. The alignment was obtained using the CLUSTAL W programme

(accession number ZP01898665), 80% to *V. harveyi* chitinase A (U81496) (Svitil and Kirchman 1998), 78% to *Serratia marcescens* ChiC (L41660) (Gal et al. 1998), 78% to *Pseudomonas aeruginosa* ChiC (AF279793) (Folders et al. 2001), 44% to *Bacillus circulans* chitinase D (D10594) (Watanabe et al. 1992), 24% to *B. circulans* chitinase A1 (M57601) (Watanabe et al. 1990), and 29% to *S. marcescens* chitinase A (X03657) (Jones et al. 1986).

The C-terminal ChBD of *Mm*Chi60 displayed sequence similarity to ChBDs identified in some bacterial chitinases (Fig. 3). This ChBD showed sequence identities of 84% to *Moritella* sp. PE36 chitinase A (accession number ZP01898665), 59% to *V. harveyi* chitinase A (U81496) (Svitil and Kirchman 1998), 36% to *S. griseus* ChiC (AB009289) (Ohno et al. 1996), 27% to *B. circulans* chitinase A1 (M57601) (Watanabe et al. 1990), 23% to *B. circulans* WL-12 chitinase D (D10594) (Watanabe et al. 1992), 20% to *S. marcescens* ChiC (L41660) (Gal et al. 1998), and 20% to *P. aeruginosa* ChiC (AF279793) (Folders et al. 2001).

Purification and N-terminal sequencing of *Mm*Chi60

The enzyme was produced intracellularly in a soluble form and purified to homogeneity from 3.5 g of *E. coli* BLR(DE3) cells harboring the pET-11a-*Mmchi60* plasmid employing a fractionation step with ammonium sulphate and two chromatographic steps, a hydrophobic on Phenyl-Sepharose and an anion exchange on Q-Sepharose. The final product was a protein single band on SDS-PAGE (Fig. 4). The molecular weight of *Mm*Chi60 was estimated to be 60 kDa, which is highly consistent with the value (60.788 kDa) calculated from the deduced amino acid sequence. Table 2 summarizes the entire purification procedure, where the purification factor was 25-fold and the total recovery was 48%. The purification procedure of the recombinant *Mm*Chi60 was very efficient and reproducible. A weight of 10 mg of pure protein can be produced from 3.5 g cells within 2 days. Analysis of purified *Mm*Chi60 by native PAGE at pH 8.8 and zymogram (Fig. 7) revealed one single band. Gel filtration chromatography of purified *Mm*Chi60 showed a single peak at the area of 60 kDa, clearly proving that the enzyme exists as a monomer. The isoelectric point of the *Mm*Chi60 was predicted to be 4.32.

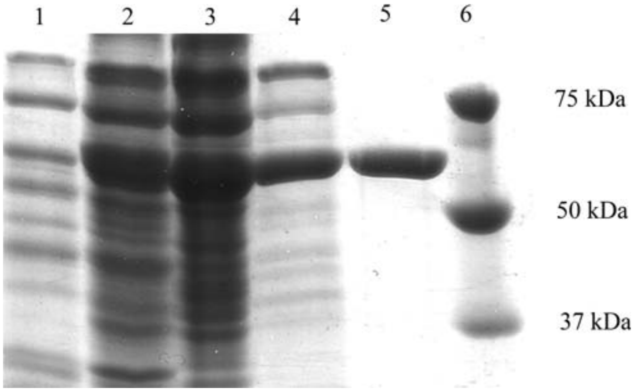

Fig. 4 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis analysis of the fractions identified through production and purification of *Mm*Chi60. *Lane 1* clarified soluble protein extract of *E. coli* BLR(DE3)-pET-11a-*Mmchi60* cells before induction of *Mm*Chi60. *Lane 2* clarified soluble protein extract of *E. coli* BLR(DE3)-pET-11a-*Mmchi60* cells after induction of *Mm*Chi60. *Lane 3 Mm*Chi60 fraction after ammonium sulphate fractionation. *Lane 4 Mm*Chi60 fraction eluted from phenyl-sepharose column. *Lane 5* purified *Mm*Chi60 fraction eluted from Q-Sepharose fast flow column, *Lane 6* molecular weight protein markers from Bio-Rad. The gel was stained using Coomassie brilliant blue R-250

The first 18 amino acids from the N-terminus of *Mm*Chi60 were analysed as described in the experimental part. The MKLKSILSAAIFTGLFST sequence was determined and showed 100% identity with the first 18 deduced amino acids of *Mm*Chi60. These results indicate that the signal peptide of *Mm*Chi60 is not processed by *E. coli*.

Enzymatic characterization of *Mm*Chi60

Maximal activity of *Mm*Chi60 was measured at 28°C and pH 5.0 using pNP-$(NAG)_2$ as a substrate at the standard assay conditions (Fig. 5). The enzyme maintained 100% of its initial activity at temperatures ranging from 0 to 30°C

Table 2 Purification scheme of *Mm*Chi60 using 3.5 g *E. coli* BLR(DE3) cells harboring the pET-11a-*Mmchi60* plasmid

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Protein yield (%)	Enzyme yield (%)	Purification factor (fold)
Total protein soluble extract	511	10	0.019	100	100	1
Fractionation with ammonium sulphate, 40–60% saturation	224	7	0.03	44	70	1.6
Phenyl-Sepharose CL-6B	17	6	0.36	3.3	66	19
Q-Sepharose Fast Flow	10	4.8	0.48	2	48	25

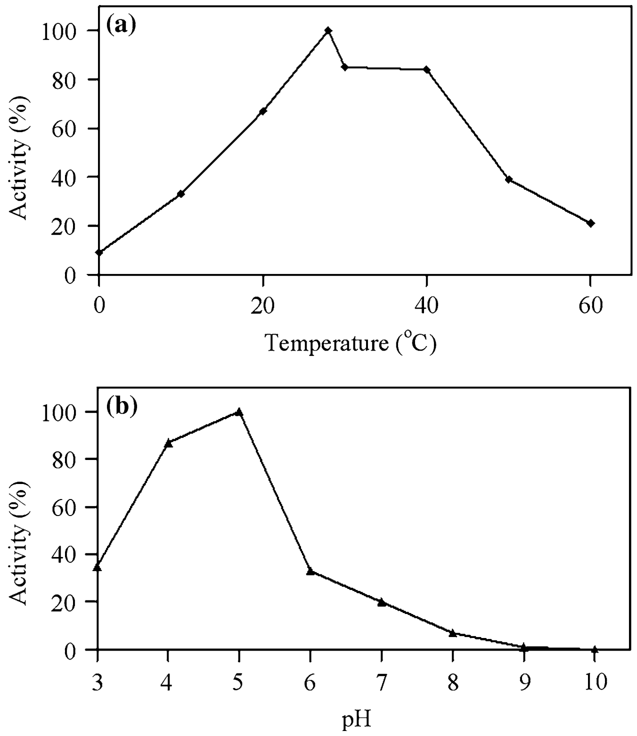

Fig. 5 Enzymatic parameters of *Mm*Chi60. **a** The dependency of *Mm*Chi60 activity on temperature. Purified *Mm*Chi60, 1 µg, was incubated with 20 µM pNP-(NAG)$_2$ and 20 mM Na acetate (pH 5.0) at the indicated temperatures for 10 min. **b** The dependency of *Mm*Chi60 activity on pH. Purified *Mm*Chi60, 1 µg, was incubated with 20 µM pNP-(NAG)$_2$ at the indicated pH at 28°C for 10 min. For certain pH range, various buffers were used: 20 mM Na citrate for pH range 3.0–4.0, 20 mM Na acetate for pH 5.0, 20 mM Na phosphate for pH 6.0–8.0, and 20 mM glycine-NaOH for pH 9.0–10.0

for 72 h. At 40, 50, and 60°C, *Mm*Chi60 had a half-life of 72 h, 5 h, and 10 min, respectively (Fig. 6a). *Mm*Chi60 presented broad pH stability maintaining over 60% activity after 10 days at a pH range of 4–10 at 4°C. No significant loss of activity occurred after incubation for 10 days at pH 8.0, 9.0, and 10.0. At pH 3.0, the enzyme appeared rather unstable, losing 80% of its initial activity after 3 days (Fig. 6b). Similar results were obtained at 18° and 28°C. No influence on the enzymatic activity of *Mm*Chi60 was detected by the effect of various bivalent cations and EDTA as well as by the reducing agent DTT and the alkylating reagent IAAM. PMSF did not influence *Mm*Chi60 activity. Over 70% of the initial activity was maintained in the presence of 5 M urea. *Mm*Chi60 activity was abolished by the addition of allosamidin. The enzyme lost half of its initial activity at 0.5 µM of allosamidin.

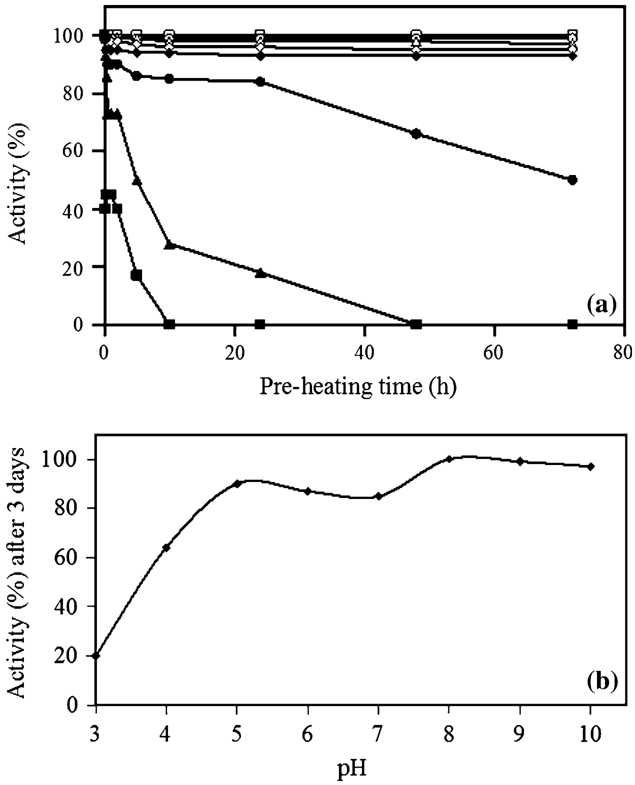

Fig. 6 Enzymatic stability of *Mm*Chi60. **a** Thermal stability of *Mm*Chi60. Purified enzyme, 1 µg, was pre-treated at the indicated temperatures in 20 mM Na phosphate buffer (pH 8.0) for 96 h. At various time intervals, samples were withdrawn and cooled on ice. The residual chitinolytic activity was measured according to the standard assay conditions and expressed as the percentage of the initial activity. *Open square* indicates 0°C, *open circle* indicates 5°C, *open triangule* indicates 10°C, *open diamond* indicates 18°C, *dark filled diamond* indicates 30°C, *dark filled circle* indicates 40°C, *dark filled triangle* indicates 50°C, and *dark filled square* indicates 60°C. **b** pH stability of *Mm*Chi60. Purified enzyme, 1 µg, was incubated at the indicated pH at 4°C for 3 days. The residual chitinolytic activity was measured according to the standard assay conditions and expressed as the percentage of the initial activity

The ability of *Mm*Chi60 to hydrolyse various carbohydrates was examined, and the results are summarized in Table 3. Concerning the natural substrates, *Mm*Chi60 hydrolysed colloidal chitin and powdered chitosan, but not powdered cellulose. Unlike pNP-$(NAG)_2$, the other pNP-synthetic substrates were unhydrolysed.

The hydrolysis pattern of colloidal chitin and NAG-oligomers was analysed by HPLC. The colloidal chitin was hydrolysed to multiple oligomers such as (NAG), $(NAG)_2$, $(NAG)_3$, and $(NAG)_4$. The synthetic substrates $(NAG)_{3\text{-}6}$ were hydrolysed to $(NAG)_2$, but also NAG and $(NAG)_3$. Incubation with $(NAG)_2$ did not yield any products.

Table 3 Substrate specificity of *Mm*Chi60

Substrate	Sp act (unit/mg of protein)
Colloidal chitin	16×10^{-2}
Powdered chitosan	15×10^{-2}
Powdered cellulose	ND
pNP-N-acetyl-D-glucosamine	ND
pNP-*β*-1,4-*N*,*N*′-diacetyl-chitobiose	91×10^{-2}
pNP-*β*-D-galactosaminide	ND
pNP-*β*-D-maltoside	ND
pNP-*β*-D-mannopyranoside	ND
pNP-*β*-D-cellobioside	ND
pNP-*β*-D-lactopyranoside	ND
pNP-*β*-D-galactopyranoside	ND
pNP-*β*-D-galactosaminide	ND

Reaction mixtures containing the natural substrates of colloidal chitin, powdered chitosan, and powdered cellulose in 1 mg/ml as well as 10 µg/ml of *Mm*Chi60 and 20 mM Na acetate (pH 5.0) were incubated at 28°C for 12 h. The amount of reducing sugar was quantified according to a modification of the Schales procedure (adapted from Imoto and Yagishita 1971). The synthetic p-nitrophenyl-linked (pNP) substrates were used in 20 µM final concentration. The *Mm*Chi60 activity was measured under the standard activity assay

ND not detected

Table 4 Calculation of values of kinetic constants K_m, V_{max}, k_{cat}, and k_{cat}/K_m of *Mm*Chi60

	V_{max} (µM/min)	K_m (µM)	k_{cat} (s)	k_{cat}/K_m (s/µM)
0°C	1	101	0.793	7.85×10^{-3}
5°C	5.46	500	4.33	8.66×10^{-3}
10°C	6.89	370	5.46	14.75×10^{-3}
15°C	9.23	416	7.33	17.97×10^{-3}
20°C	14.97	500	11.88	23.60×10^{-3}
28°C	24.63	555	19.54	35.20×10^{-3}

Steady-state enzyme kinetics were performed by incubating 1 µg of *Mm*Chi60 with pNP-$(NAG)_2$ (0–2.5 mM) and 20 mM Na acetate (pH 5.0) in a temperature range of 0–28°C. The values of kinetic constants K_m, V_{max}, and k_{cat} were determined from the respective Lineweaver–Burk plots

On the basis of the respective Lineweaver–Burk plot, the values of kinetic constants K_m and V_{max}, and subsequently k_{cat} and k_{cat}/K_m of *Mm*Chi60 were calculated at temperatures ranging from 0 to 28°C and for pNP-$(NAG)_2$ as summarized in Table 4. This enzyme showed relatively high turnover rates (k_{cat}) and catalytic efficiencies (k_{cat}/K_m) at low temperatures. The values of K_m constant were found to be increased.

Identification of *Mm*Chi60 in *M. marina*

The bacterial strain was grown in Marine broth medium containing 0.5% (w/v) of colloidal chitin at 18°C for 48 h. SDS-PAGE and zymogram analysis indicated that several proteins with chitinase activity were produced in the culture supernatant of *M. marina*. Among these proteins, a chitinase with a molecular weight of 60 kDa was detected. Antiserum against the purified *Mm*Chi60 was raised in rabbit. In Western blotting analysis, the anti-*Mm*Chi60 polyclonal rabbit anti-serum reacted with the protein of 60 kDa mentioned above (Fig. 7). The N-terminal amino acid sequence of the 60 kDa protein was determined to be GTITSQDDNVV. These residues are identical to residues 23–33 of the deduced amino acid sequence of the *Mm*Chi60 (Fig. 1). These results indicate that *Mmchi60* gene is expressed and the protein is released into the medium.

Differential scanning microcalorimetry

DSC thermal denaturation experiment of recombinant *Mm*Chi60 (Fig. 8, black curve) showed a single transition peak with apparent T_m of 56.4 ± 0.5°C, at a scan rate of 1.5°C/min. The area under the heat transition peak determined the calorimetric denaturation enthalpy ($\Delta H^{\#}$) of *Mm*Chi60, which is 370 ± 12 kcal/mol. The second denaturation curve of *Mm*Chi60, after cooling (Fig. 8, grey curve), revealed a reversibility of nearly 76%.

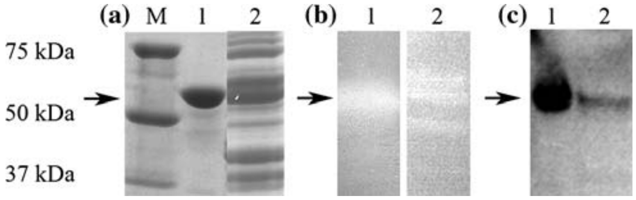

Fig. 7 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis, zymogram, and Western blot analyses of proteins in the culture supernatant of *M. marina*. **a** Gel stained with Coomassie brilliant blue R-250. **b** Chitinase activity detected on a gel containing 0.7 mg/ml carboxymethyl-chitin-Remazol Brilliant Violet (CM-chitin-RBV). **c** *Mm*Chi60 proteins detected by Western blot analysis with a polyclonal rabbit anti-serum raised against the purified *Mm*Chi60. *Lane M* molecular weight protein markers from Bio-Rad. *Lane 1* *Mm*Chi60 purified from *E. coli*. *Lane 2* culture supernatant of *M. marina* grown with colloidal chitin. *Arrows* indicate *Mm*Chi60

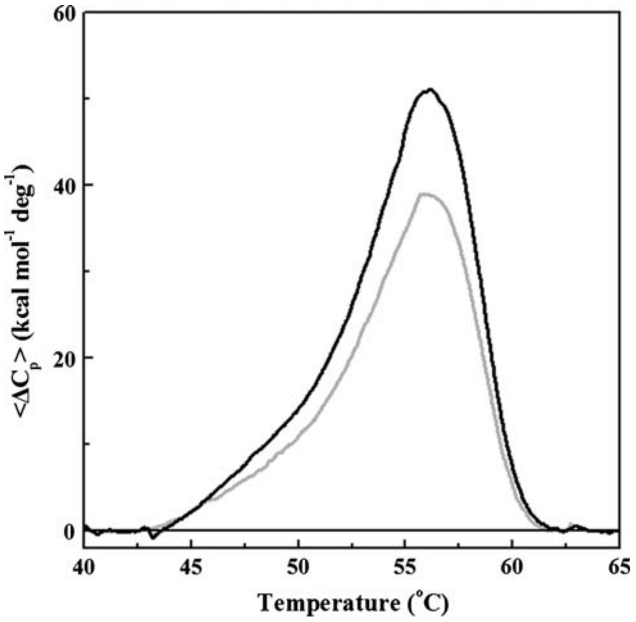

Fig. 8 Thermal unfolding of *Mm*Chi60 before (*black curve*) and after (*grey curve*) heating–cooling of the protein at 65°C. Protein concentration was 2 mg/ml, heating rate 1.5°C/min, and the buffer used was 50 mM Na phosphate (pH 8.0)

The model proposed of *Mm*Chi60 chitin-binding domain

The ChBD of *Mm*Chi60, which has been assigned to the C-end of the protein, exhibited considerable amino acids sequence identity with ChBDs of many bacterial chitinases (Fig. 3). *Mm*Chi60 ChBD showed 36% identity with the ChiC ChBD of *S. griseus* (Ohno et al. 1996). The model of ChBD of *Mm*Chi60 was calculated based on the crystal structure of ChiC of *S. griseus* (PDB 1wvu). The model is shown in Fig. 9 and revealed a *β*-sheets structure with two highly conserved tryptophan residues, i.e. Trp-533 and Trp-534 exposed to the solvent.

Discussion

The largest proportion of the earth's biosphere comprises organisms that thrive in cold environments, named, under the collective term, psychrophiles. The ability of psychrophiles to grow and proliferate in the cold is predicated on their capacity to synthesize cold-adapted enzymes. *M. marina* is a psychrophilic bacterium isolated from a low-temperature marine environment. The secreted chitinases detected in the culture supernatant of *M. marina* grown in the presence of colloidal chitin support the assumption that the chitinases produced by psychrophilic bacteria are responsible for the degradation of the krill chitin in marine biosphere.

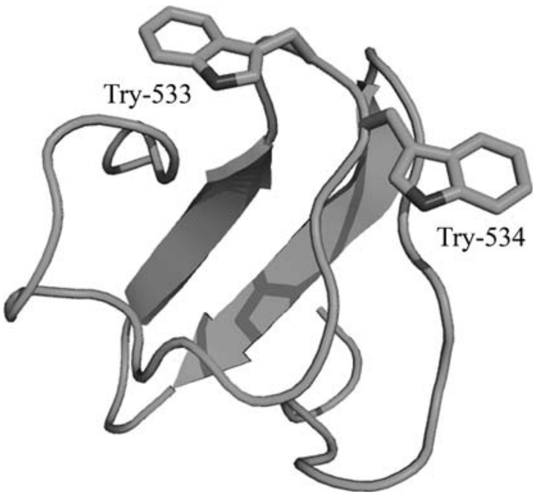

Fig. 9 The model proposed for the *Mm*Chi60 chitin-binding domain (ChBD). The model was based on the crystal structure of chitinase C of *Streptomyces griseus* (PDB 1wvu). The solvent exposed Trp-533 and Trp-534 of *Mm*Chi60 are proposed to be involved in the interaction with the chitin substrate

The analysis of the enzymes involved in the mechanism of chitin utilization in the cold marine environment at the molecular level is our goal and this paper provides the first study of this organism. We present the first chitinase that was cloned, sequenced, and characterized from *M. marina.*

The *Mmchi60* gene encodes a chitinase of 550 amino acids with modular structural organization consisting of an N-terminal catalytic region of the family 18 of glycosyl-hydrolases and a C-terminal ChBD. Comparison of the N-terminal catalytic region of *Mm*Chi60 with the respective regions of bacterial chitinases revealed the conserved sequence F_{145}DGLDIDLE_{153} of *Mm*Chi60, which is essential for chitinase activity and has been classified as the "Prosite signature" for the family 18, of glycosyl-hydrolases superfamily, in the PROSITE database (Fig. 2). Glu residue of the "Prosite signature" sequence was found to be conserved in all the catalytic regions of the indicated bacterial chitinases and was identified at positions 315 of *S. marcescens* ChiA and 153 of *M. marina* Chi60. The crystal structure of *S. marcescens* ChiA has shown that Glu-315 acts as a proton donor residue in catalysis (Papanikolau et al. 2001). Therefore, Glu-153 of *Mm*Chi60 is predicted to be the catalytic residue acting as proton donor, assuming that this enzyme uses the same catalytic mechanism.

The C-terminal ChDB 3-D model of *Mm*Chi60 was constructed by homology modelling using the structural data available for the ChiC of *S. griseus*, due to significant sequence similarity (PDB 1wvu) (Fig. 9). It has been reported that the ChBD of *S. griseus* is an all-beta protein with the Trp-59 and Trp-60 residues faced to the solvent, that have been assigned to be important for the interaction of the enzyme with colloidal chitin (Itoh et al. 2006). On the basis of the amino acid sequence alignment of the ChBDs of bacterial chitinases (Fig. 3), two tryptophans were found to be highly conserved at positions 533 and 534 of *Mm*Chi60, and 59 and 60 of *S. griseus* ChiC. Moreover, the model proposed for the *Mm*Chi60 ChBD exhibited a beta protein with Trp-533 and Trp-534 exposed to the solvent. As a result, we suggest that Trp-533 and Trp-534 of *Mm*Chi60 may be involved in the interaction with the chitin substrate.

The highly pure recombinant *Mm*Chi60 was used to carry out a comprehensive biochemical analysis. Generally, the enzyme showed very high activity only on chitin substrates at low temperatures and therefore it was necessary to reduce both synthetic substrate concentration and time of reaction in order to measure initial enzymatic velocity. *Mm*Chi60 activity was completely abolished by the specific inhibitor of chitinases, which is allosamidin (Spindler and Spindler-Barth 1999). This strongly supports that *Mm*Chi60 is a chitinolytic enzyme. Moreover, *Mm*Chi60 is proposed to be an endochitinase for the following reasons: (1) incubation with $(NAG)_2$ did not yield any products, indicating that *Mm*Chi60 is not a 1,4-*β*-*N*-acetyl-glucosaminidase, (2) when $(NAG)_4$ and $(NAG)_6$ were used, *Mm*Chi60 did not produce only $(NAG)_2$, but also NAG and $(NAG)_3$, indicating that it is not a chitobiosidase, and (3) concerning colloidal chitin, multiple oligomers were detectable as degradation products.

The insensitivity of *Mm*Chi60 to urea indicates a decrease of hydrophobic interactions in the protein. The insensitivity of the protein to DTT shows that thiol groups do not participate in the overall stability and activity of the enzyme. These observations have also been reported for cold-adapted enzymes (Asgeirsson et al. 2003; Suzuki et al. 2005).

*Mm*Chi60 showed a relatively high level of catalysis at low temperatures (Table 4). The high values of K_m constant are due to the fact that enzymes produced by marine bacteria work at saturating concentrations of chitin, since chitin is very abundant in marine sediments. Therefore, the strategy used to maintain sustainable activity at a permanently low temperature is to enhance k_{cat} and k_{cat}/K_m, instead of decreasing K_m (D'Amico et al. 2002).

The results obtained by the enzyme activity (Fig. 5a) demonstrated that *Mm*Chi60 exhibited low optimal temperature of 28°C, under the indicated conditions, which is comparable to those reported for cold-adapted chitinase B of *Alteromonas* sp. strain O-7 (30°C) (Orikoshi et al. 2003) and chitinase A of *Vibrio* sp. strain Fi:7 (30°C) (Bendt et al. 2001).

DSC analysis showed a low apparent T_m for *Mm*Chi60 (56.4 ± 0.5°C), which is quite close to those found for the psychrophilic chitinases *Arthrobacter* sp. strain TAD20 chitinase A (*Ar*ChiA) (54.3°C) and B (*Ar*ChiB) and (54°C) significantly lower than the mesophilic *S. marcescens* chitinase A (*Sm*ChiA) T_m (64.2°C) (Lonhienne et al. 2001). The value of the denaturation enthalpy per mol for *Mm*Chi60 was lower (370 ± 12 kcal/mol), compared to those of *Ar*ChiA (415 kcal/mol) and *Sm*ChiA (449 kcal/mol) (Table 5) (Lonhienne et al. 2001). These findings suggest that *Mm*Chi60 has properties in common with other cold-adapted enzymes.

It is well established that increased flexibility is the most important factor for the catalytic efficiency of cold-adapted enzymes at low temperatures (D'Amico et al. 2002; Gianese et al. 2002). Several structural factors are potentially responsible for the low thermal stability, which is related to increased flexibility of the cold-adapted enzymes. A structural role for arginine in multiple hydrogen bonds to backbone carbonyl and side chain oxygens has been proposed (Mrabet et al. 1992). Proline residues are thought to modulate the entropy of protein unfolding by affecting backbone flexibility (Matthews et al. 1987). On the other hand, the lack of side chain in glycine residue allows chain rotations and dihedral angles not available to other residues (Van den Burg et al. 1998). Analysis of structural features indicates that each cold-adapted enzyme uses different small selections of structural adjustments for gaining increased molecular flexibility that in turn give rise to increased catalytic efficiency and reduced stability (Gerday et al. 1997; Smalas et al. 2000). Thus, we compared the arginine, proline, and glycine contents of *Mm*Chi60 (optimum temperature 28°C) and *P. aeruginosa* ChiC (*Pa*ChiC) (optimum temperature 50°C) (Folders et al. 2001) (Table 6). The ratio of

Table 5 Calculation of values of T_m and denaturation enthalpy per mol for *Mm*Chi60, *Arthrobacter* sp. strain TAD20 chitinase A (*Ar*ChiA) and B (*Ar*ChiB), and *S. marcescens* chitinase A (*Sm*ChiA) (adapted from Lonhienne et al. 2001)

Protein	T_m	Denaturation enthalpy/mol
*Mm*Chi60	56.4 ± 0.5°C	370 ± 12 kcal/mol
*Ar*ChiA	54.3°C	415 kcal/mol
*Ar*ChiB	54°C	270 kcal/mol
*Sm*ChiA	64.2°C	449 kcal/mol

Table 6 Calculation of values of Arg, Pro, and Gly contents of *Mm*Chi60 and *P. aeruginosa* chitinase C (*Pa*ChiC) (adapted from Folders et al. 2001)

Protein	Full length enzyme			Chitin-binding domain (ChBD)		
	Pro (%)	Arg (%)	Gly (%)	Pro (%)	Arg (%)	Gly (%)
*Mm*Chi60	4.0	2.5	8.5	2.3	2.3	13.6
*Pa*ChiC	4.6	6.0	9.9	8.7	8.7	6.5

arginine residues to the total number of amino acid residues of *Mm*Chi60 was lower than in *Pa*ChiC. However, *Mm*Chi60 had lower glycine and nearly the same proline content compared to *Pa*ChiC. Therefore, unlike the glycine and proline contents, the arginine content seems to contribute to the flexibility of the *Mm*Chi60 overall protein. Focusing only on the ChBD of *Mm*Chi60, there was a clear relationship between proline, arginine, and glycine contents of *Mm*Chi60 and *Pa*ChiC and their thermostabilities. These results suggest that the increased flexibility related to the increased heat lability of *Mm*Chi60 could be explained by its arginine content. Furthermore, this finding provides the idea that individual protein domains within the same protein facilitate different stabilization strategies.

DSC thermal denaturation–renaturation experiments showed that the protein unfolds reversibly at 65°C, by nearly 76% (Fig. 8), strongly supporting the correct folding of the recombinant enzyme. The reversibility of the thermal denaturation of *Mm*Chi60 makes this cold-adapted enzyme a valuable tool for enzyme engineering and protein design experiments in order to tailor stable enzymes with high enzymatic activity at low temperatures. Additionally, this explains the overestimated values of thermal stability of the enzyme (Fig. 6a).

To clarify the strategy that *Mm*Chi60 uses to adapt its function to low temperatures, a comprehensive thermodynamic analysis on the relationship between stability, activity, and flexibility for *Mm*Chi60 is in progress in our laboratory.

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