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Cloning, expression, and characterization of a chitinase gene from the Antarctic psychrotolerant bacterium *Vibrio* sp. strain Fi:7

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Abstract A marine psychrotolerant bacterium from the Antarctic Ocean showing high chitinolytic activity on chitin agar at 5°C was isolated. The sequencing of the 16S rRNA indicates taxonomic affiliation of the isolate Fi:7 to the genus *Vibrio*. By chitinase activity screening of a genomic DNA library of *Vibrio* sp. strain Fi:7 in *Escherichia coli*, three chitinolytic clones could be isolated. Sequencing revealed, for two of these clones, the same open reading frame of 2,189 nt corresponding to a protein of 79.4 kDa. The deduced amino acid sequence of the open reading frame showed homology of 82% to the chitinase ChiA from *Vibrio harveyi*. The chitinase of isolate Fi:7 contains a signal peptide of 26 amino acids. Sequence alignment with known chitinases showed that the enzyme has a chitin-binding domain and a catalytic domain typical of other bacterial chitinases. The chitinase ChiA of isolate Fi:7 was overexpressed in *E. coli* BL21(DE3) and purified by anion-exchange and hydrophobic interaction chromatography. Maximal enzymatic activity was observed at a temperature of 35°C and pH 8. Activity of the chitinase at 5°C was 40% of that observed at 35°C. Among the main cations contained in seawater, i.e., Na^+, K^+, Ca^{2+}, and Mg^{2+}, the enzymatic activity of ChiA could be enhanced twofold by the addition of Ca^{2+}.

Key words Chitinase · Cold adapted · Marine bacterium · Psychrotolerant · *Vibrio* sp.

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Introduction

Chitin represents one of the most abundant organic compounds in the marine environment. Chitin is essentially composed of alternating β-1,4-linked *N*-acetylglucosamine residues. The chitin in the marine biosphere originated primarily from crustaceans. For the entire aquatic biosphere, it is estimated that more than 10^{11} metric tons of chitin are produced annually (Keyhani and Roseman 1999).

The main site of chitin decomposition is still questionable. Microbial chitin degradation in the surface layer of the bottom sediments of the oceans seems to be of special importance. Although the deep-sea marine environment is characterized by high hydrostatic pressures and low temperatures, there seems to be a highly active microbial community that is able to decompose chitin. Helmke and Weyland (1986) investigated the formation and the activity of chitinases by psychrophilic and psychrotolerant marine Antarctic bacteria under simulated deep-sea conditions. All chitinases formed by the deep-sea bacteria under these conditions were highly barotolerant and were active up to 1,000 bar. The results suggested that the indigenous deep-sea bacteria are capable of decomposing chitin settled to or produced in the depths of the Antarctic Ocean. Furthermore, it is supposed that a considerable part of chitin degradation takes place in the digestive tracts of krill consumers such as fish, either by their own chitinases or by associated microorganisms in the gut of these animals.

The major part of the marine biosphere is characterized by permanent low temperatures, –2°–10°C. The Antarctic Ocean especially represents a stable cold habitat. Consequently, the chitinases responsible for the degradation of the krill chitin should have high catalytic activities under these low-temperature conditions. It has been demonstrated that crude chitinases of some psychrophilic marine bacteria show a similarly high catalytic activity at 3°C as that observed at 18°C (Helmke and Weyland 1986).

There are numerous reports about the cloning of chitinase genes from different mesophilic bacteria (for review, see Felse and Panda 1999). However, so far there are no

reports on the cloning of cold-adapted chitinases from psychrophilic or psychrotolerant bacteria. We report here the characterization of a cold-adapted chitinase from a marine bacterium isolated from the gut of an Antarctic fish. This psychrotolerant bacterium showed high extracellular chitinolytic activity at 5°C. The gene of one chitinase of this strain was cloned and sequenced. The chitinase was purified to near homogeneity and characterized.

Material and methods

Strains and cultivation conditions

The bacterial strain Fi:7 has been isolated from the gut of an Antarctic fish caught in the Atlantic section of the Antarctic Ocean at 60°12′ S and 50°20′ W (Helmke and Weyland 1986). This psychrotolerant strain is able to grow on ZoBell medium from 1° to 28°C. The optimal growth temperature of strain Fi:7 was 18°C. The pure culture was maintained on Marine Agar 2216 (Difco, Detroit, MI, USA) at 2°C. Tests for utilization of organic compounds as the sole carbon and nitrogen source were carried out in liquid basal medium seawater (BMS) (Helmke and Weyland 1984). The enzymatic activities of amylases, chitinases, ureases, and nitrate reductases were determined by the method of Weyland et al. (1970). Alginase activity was measured according to Ahrens (1968). All physiological tests were performed at an incubation temperature of 5°C.

The marine strain Fi:7 was cultivated at 8°C in a chitin medium consisting of 1.0 g Bacto peptone, 1.0 g Bacto-yeast extract, 0.01 g $FePO_4 \cdot 4H_2O$, 100 g wet weight (ww) precipitated chitin (prepared according to Hock 1941), and 750 ml artificial seawater (ASW) with a salinity of 27‰ (according to Burkholder 1963) containing 24 g/l NaCl, 5.3 g/l $MgCl_2 \cdot 6H_2O$, 7 g/l $MgSO_4$, 0.7 g/l KCl, 0.01 g/l $FeSO_3$, and 250 ml distilled water. Before autoclaving, the pH was adjusted to 7.5.

Escherichia coli DH5α was used for the preparation of the gene library. For overexpression of the chitinase, *E. coli* BL21(DE3) was used. *E. coli* cells were routinely cultivated under vigorous agitation at 37°C in Luria-Bertani (LB) medium if not otherwise indicated.

Nucleic acid manipulation

Chromosomal DNA from strain Fi:7 was prepared according to Sambrook et al. (1989). Plasmid DNA was purified by the alkaline lysis procedure (Sambrook et al. 1989). Chromosomal DNA was partially digested with *Sau*3AI; 4-kb fragments were isolated from 0.8% (w/v) agarose gel with the Agarose Gel DNA Extraction Kit from Roche Diagnostics (Penzberg, Germany). These fragments were ligated to *Bam*HI-digested pUC18. The ligated plasmids were transformed in competent *E. coli* DH5α cells (Hanahan 1983) and selected on LB agar plates containing 100 mg/l ampicillin. Clones with chitinolytic activity were determined on LB agar plates containing 1% (w/v) ethylene glycol chitin after overnight cultivation at 30°C and subsequent incubation at room temperature (RT) for 1 day.

The sequence of positive clones was determined by automated fluorescence sequencing with an ABI PRISM dye terminator cycle sequencing reaction mix (Perkin-Elmer, Rodgau-Jügesheim, Germany) in a 377 Perkin-Elmer DNA sequencer. The nucleotide sequences of the chitinase gene *chiA* reported in this article have been submitted to the GenBank/EMBL Data Bank with the accession number AY007314.

For overexpression of the chitinase, the coding sequence of the gene was cloned into the T7 expression vector pET-12a (Novagen, Madison, WI, USA). Ligated plasmids were transformed into *E. coli* BL21(DE3). Construction of plasmid pETchiA was performed by cloning of a 2,111-bp *Bam*HI polymerase chain reaction (PCR) fragment (primers chiAf 5′-GGG GGA TCC GGT TCA GAT ATG ACG AAT CCG and chiAr 5′-GGG GGA TCC TCT AGC TAT ATT AAG CTT ACA) covering the coding region without the potential signal sequence of *chiA* into pET-12a. The coding sequence of *chiA* was fused as a *Bam*HI fragment within the *Bam*HI restriction site in frame behind the *ompT* signal sequence of plasmid pET-12a, resulting in plasmid pETchiA. The correct orientation of the *chiA* insert was controlled by restriction analyses.

Escherichia coli BL21 cells carrying pETchiA were grown overnight on 5 ml LB medium, and 3 ml of the culture medium was transferred to 100 ml LB medium in a 500-ml shake flask. The culture was incubated at 30°C and 200 rpm until an OD (600 nm) of 0.4–0.5. Subsequently, the overexpression of the chitinase of strain Fi:7 on plasmid pETchiA was initiated by addition of 1 mM isopropyl thiogalactoside (IPTG) (final concentration). After 8 h cultivation at 20°C, the culture was centrifuged at 10,000 *g* for 10 min at 4°C to pellet the cells. The protein fraction in the supernatant was concentrated by ultrafiltration and stored at 4°C.

Purification of the chitinase

The protein samples were applied to an anion-exchange column (Resource Q; Pharmacia Biotech, Freiburg, Germany) in a phosphate buffer (pH 7.5) containing K_2HPO_4, 0.842 g/l; KH_2PO_4, 0.702 g/l; glycerol, 8%; and ethylenediaminetetraacetic acid (EDTA), 1 mM. After washing the column with the same buffer, the proteins were eluted by a gradient of 0–1 M NaCl in the phosphate buffer described earlier. The eluted fractions showing activity on chitin were collected and concentrated with filter membranes (Ultrafree-30) from Millipore (Bedford, MA, USA) by centrifugation (4,000 rpm, 8°C). The proteins of the collected fractions were thus concentrated to approximately 0.5 µg/µl. These purification steps were repeated using the same conditions as described previously.

Anion-exchange chromatography was followed by hydrophobic interaction chromatography. The sample was applied to a Resource PHE column from Pharmacia

Table 1. Comparison of characteristic metabolic activities of the biovars *Vibrio splendidus* and the isolate Fi:7

Characteristics	*Vibrio splendidus* I[a]	*Vibrio splendidus* II[a]	Strain Fi:7
Growth at:			
4°C	**d**	–	**+**
35°	**d**	–	–
C			
Na^+ requirement	+	+	+
Gelatinase	+	+	+
Amylase	+	+	+
Lipase	+	+	+
Chitinase	+	+	+
Reduction of NO^{3-} to NO^{2-}	+	+	+
Arginine dihydrolase	**+**	–	**+**
Utilization of:			
D-Glucose	+	+	+
L-Arabinose	–	–	–
Lactose	–	–	–
Maltose	+	+	+
D-Mannitol	+	+	+
Salicin	–	–	–
D-Sorbitol	–	–	–
Trehalose	+	+	+
D-Xylose	–	–	–

Boldface type indicates traits useful for the preliminary identification of *Vibrio* species
d, differs among strains
[a]Data given in Baumann and Schubert (1984)

Biotech in a 50 mM Tris-HCl buffer (pH 8.5) containing 1 M $(NH_4)_2SO_4$. The proteins were eluted by the same buffer but without $(NH_4)_2SO_4$ at a flow rate of 1 ml/min. The eluted fractions showing activity on chitin were collected and concentrated with filter membranes as described previously. To improve the purity of the chitinase ChiA, a second hydrophobic interaction chromatography was followed by an additional anion-exchange chromatography with the same conditions as described earlier.

Chitinase assay

Chitinase activity was measured as described by Roberts and Selitrennikoff (1988). Enzyme solution (10 µl) containing 1 µg protein was mixed with 90 µl of the chitin analog *p*-nitrophenyl-β-D-*N*,*N*′-diacetylchitibioside (Sigma, München, Germany). The samples were incubated at 20°C for 60 min, if not otherwise indicated. For termination of the reaction, 10 µl of 1 M NaOH solution was added. After centrifugation for 5 min at 10,000 *g*, the amount of the released *p*-nitrophenol (pNP) was measured spectrophotometrically at a wavelength of 400 nm (molar extinction coefficient, 17,700) (Sakai et al. 1998). Sample blanks were used to correct for nonenzymatic release of *p*-nitrophenol. One unit of enzyme activity was defined as the amount that released 1 µmol pNP/min at 20°C. Specific activity was expressed as units per milligram of protein.

Protein electrophoresis and N-terminal protein sequencing

Denaturating protein gel electrophoresis was performed with a 10% (w/v) polyacrylamide gel as described by Sambrook et al. (1989). After electrophoresis, the gel was stained with Coomassie blue. The N-terminal protein sequencing was done as described by Völker et al. (1992).

Results

Characterization of the cold-adapted chitinolytic marine bacterium

More than 200 psychrophilic and psychrotolerant marine bacteria of the bacterial strain collection of the Alfred Wegener Institute were screened on ZoBell medium agar containing 10% wet weight (ww) precipitated chitin. A fast-growing strain that showed the largest clearing zones on turbid chitin agar at 5°C was selected. This psychrotolerant marine bacterium with the designation Fi:7 was isolated from the gut of an Antarctic fish (Helmke and Weyland 1986). The partial 16S rRNA sequencing of isolate Fi:7 showed the highest similarity of this strain to two undescribed strains of the genus *Vibrio* (99.8% to strains ANG.D14 and ANG.D15). The nearest related validated species was *Vibrio splendidus*. The 16S rRNA sequence of strain Fi:7 showed 98% homology to the 16S rRNA of *V. splendidus*; 16S rRNA sequence homology to other *Vibrio* species was less than 93%. Despite the 2% difference in the

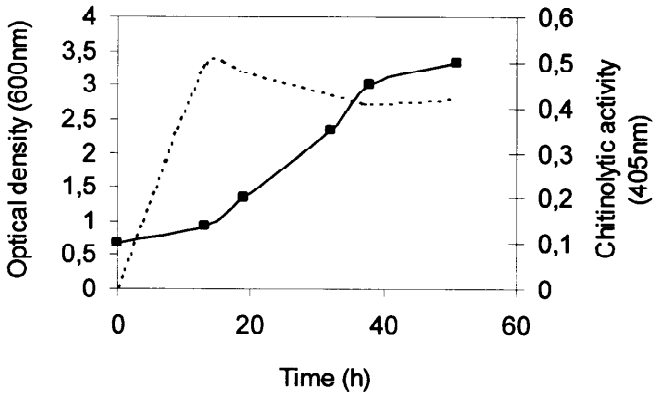

Fig. 1. Growth curve of *Vibrio* sp. strain Fi:7 (*dotted line*) cultivated at 20°C in ZoBell medium containing chitin and the corresponding extracellular chitinolytic activity (*solid squares*)

16S rRNA sequences, strain Fi:7 did not show clear differences in selected catabolic activities to two biovars of *V. splendidus* (Table 1).

The highest level of chitinolytic activity of isolate Fi:7 in chitin ZoBell medium was determined in the late stationary phase (Fig. 1). The major part of the chitinolytic activity could be found in the supernatant approximately 30 h after the onset of the stationary phase.

Cloning and sequencing of the chitinase gene of *Vibrio* sp. Fi:7

The screening of an *E. coli* DH5α genomic library of strain Fi:7 was done on LB agar plates containing 1% (w/v) ethylene glycol chitin. Three colonies from approximately 10,000 clones of the Fi:7 gene library showed halos after overnight cultivation at 30°C and subsequent incubation at 20°C for 1 day. The DNA inserts of two clones were 3.2 kb long and showed an identical restriction pattern. The third clone showed a different restriction pattern, and the insert was 2.7 kb long. The DNA sequencing revealed overlapping regions with all three clones, indicating that all clones carried at least a part of the same chitinase gene of strain Fi:7. The sequencing showed for the two identical clones an open reading frame of 2,189 nt corresponding to a protein of 79.4 kDa (Fig. 2). This protein, with a theoretical pI of 4.16, was designated as ChiA.

The deduced amino acid sequence of ChiA of isolate Fi:7 showed high homology (82%) to the chitinase ChiA from *Vibrio harveyi* (Svitil and Kirchman 1998). For the ChiA of *V. harveyi*, a C-terminal chitin-binding domain and a catalytic domain at the N-terminal part of the enzyme could be determined. Similar conserved regions could be also found for the chitinase ChiA of strain Fi:7 (Fig. 2). ChiA contains a potential signal peptide of 26 amino acids.

Purification of the chitinase ChiA

The coding sequence of *chiA* without its potential signal peptide sequence was cloned in frame to the *ompT* leader peptide sequence of the vector pET-12a. For the overexpression of ChiA in *E. coli* BL21(DE3), the expression system was induced by addition of 1 mM IPTG at 30°C. One hour after induction, the culture was further cultivated for 8 h at 20°C. Although the OmpT leader peptide usually directs the secretion of proteins into the periplasm, a considerable part of the chitinolytic activity could be found in the extracellular medium (data not shown).

For the purification of ChiA, the extracellular *E. coli* BL21 (pETchiA) protein fraction was concentrated to approximately 0.5 µg protein/µl. The chitinase was purified by anion-exchange chromatography and hydrophobic interaction chromatography (Fig. 3). Although the purified protein fraction represented only 10% of the total protein fraction before purification, its chitinolytic activity was at least twofold higher than that of the crude extract (data not shown).

Surprisingly, during the purification process two protein bands of 80 and 82 kDa were accumulated. Both protein bands were determined by N-terminal sequencing of the first ten amino acids. In both cases, the same N-terminal sequence of STGSGSDMTN could be found. The first five amino acids (STGSG) originate from the *ompT* signal peptide sequence of plasmid pET-12a, which remained after cleavage of the signal peptidase at the N-terminus of the protein. The following five amino acids (SDMTN) represent the first five N-terminal amino acids of the mature Fi:7 chitinase.

Enzyme characterization

The enzyme characterization of the crude extract of Fi:7 showed apparent maximal chitinolytic activity at 25°C and pH 8 (data not shown). The apparent maximal enzymatic activity of the purified chitinase ChiA was found at 35°C (Fig. 4A). The remaining chitinase activity of the purified enzyme at 5°C was 40% of that observed at 35°C. The optimal enzyme activity of the purified chitinase could be determined at pH 8 (Fig. 4B). The apparent maximal specific activity of the purified ChiA protein fraction at 35°C was 0.11 units. ChiA showed a high stability at 30° and even at 40°C, whereas its half-life at 50° and 60°C was 60 and 30 min, respectively (Fig. 5). The chitinase activity of the purified protein fraction was stimulated twofold by the addition of 0.5 or 1 mM Ca^{2+}, whereas the addition of 1 mM EDTA resulted in an approximately twofold decrease of the activity of the chitinase of Fi:7 (data not shown). The presence or absence of Na^{+}, K^{+}, or Mg^{2+} did not influence the enzymatic activity of ChiA.

Discussion

Up to the present time, cold-adapted chitinolytic marine microorganisms have not been intensively investigated although a significant part of the chitin degradation in the marine biosphere takes place under permanently low tem-

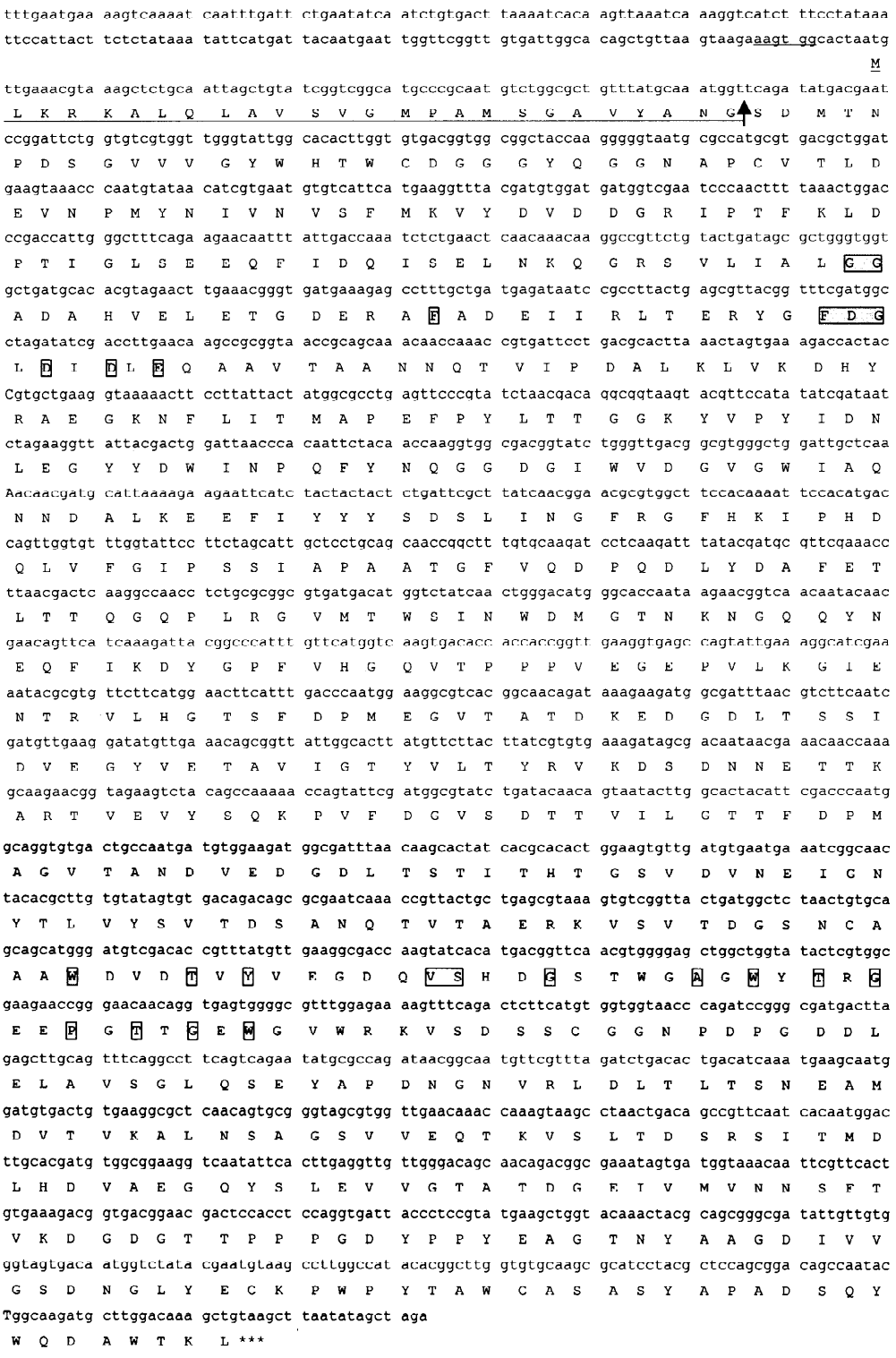

Fig. 2. Nucleotide sequence of the *chiA* gene of strain *Vibrio* Fi:7. The putative ribosomal binding site is *double underlined*. The deduced amino acid sequence of ChiA is given *below* the nucleotide sequence. The putative signal peptide is *underlined*. The potential cleavage site of the signal peptidase is indicated by an *arrow*. The conserved amino acids of the putative catalytic and chitin-binding domains are indicated by *shaded boxes* and *unshaded boxes*, respectively. The stop codon is indicated by *asterisks*

perature conditions. We have identified a psychrotolerant Antarctic marine bacterium that showed high chitinolytic activity at 5°C. Sequencing of the 16S rRNA of the Antarctic chitinase-producing isolate Fi:7 indicated an affiliation of this strain to the genus *Vibrio*. According to the 98% homology of the 16S rRNA of the isolate Fi:7 to *V. splendidus*, and despite their consistency in selected catabolic activities, this psychrotolerant strain could represent a new species of the genus *Vibrio*. The high chitinolytic activity of this bacterium, which was isolated from the gut of an Ant-

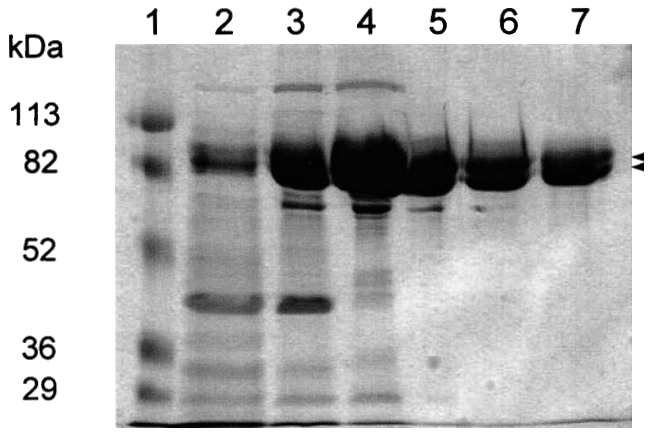

Fig. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of chitinase ChiA after overexpression with the T7 expression system in *Escherichia coli* BL21(DE3) and after purification. *Lane 1*, molecular weight protein standard; *lane 2*, extracellular protein fraction of *E. coli* BL21(DE3) pETchiA cells after overexpression of ChiA; *lane 3*, purified ChiA protein fraction after first anion-exchange chromatography; *lane 4*, purified ChiA protein fraction after second anion-exchange chromatography, *lane 5*, purified ChiA protein fraction after first hydrophobic interaction chromatography; *lane 6*, purified ChiA protein fraction after second hydrophobic interaction chromatography; *lane 7*, purified ChiA protein fraction after third anion-exchange chromatography. *Arrows* indicate two proteins (80 and 82 kDa) that were shown to have the same initial 10 N-terminal amino acids

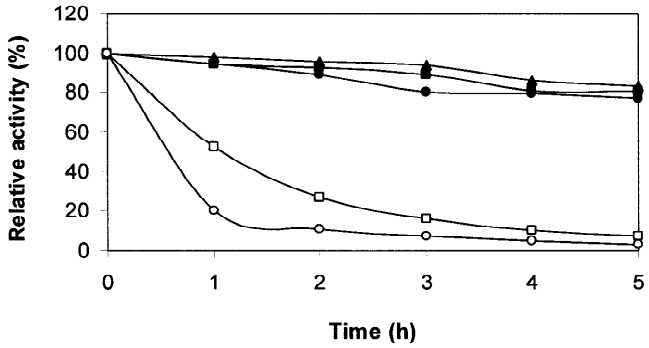

Fig. 5. Thermostability of chitinase ChiA at 20°C (*solid triangles*), 30°C (*solid squares*), 40°C (*solid circles*), 50°C (*open squares*), and 60°C (*open circles*) was determined every hour by measuring the remaining enzymatic activity in 50 mM Tris-HCl buffer (pH 8.0) containing 2 mM pNP-(GlcNAc) for 60 min at 20°C

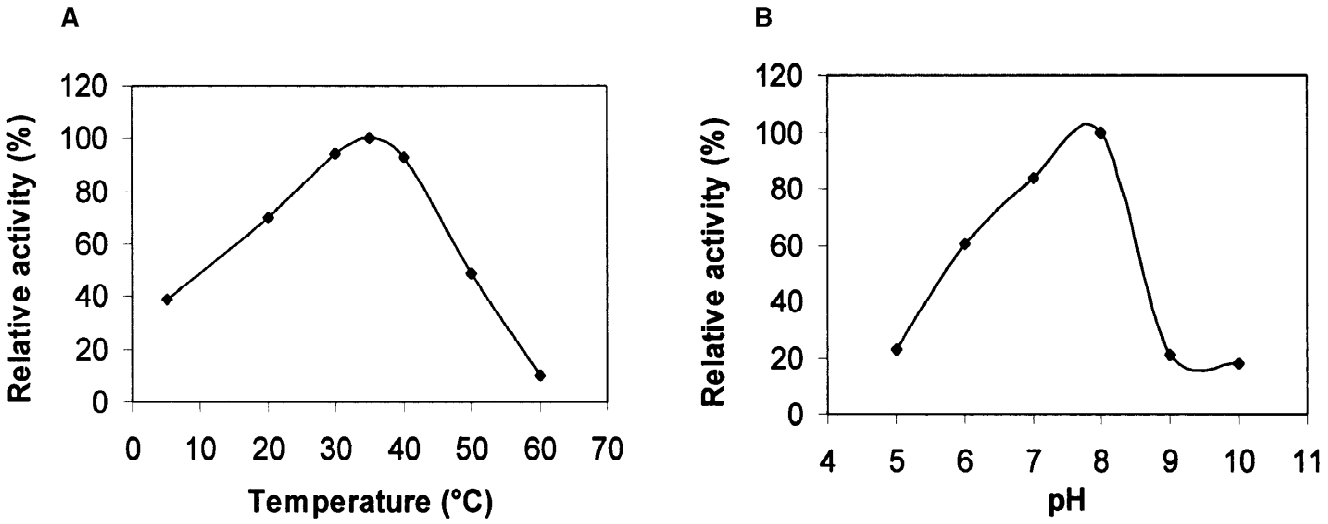

Fig. 4. Effect of temperature (**A**) and pH (**B**) on activity of the purified chitinase ChiA of *Vibrio* sp. strain Fi:7. ChiA protein solution was incubated in 50 mM Tris-HCl buffer containing 2 mM *p*-nitrophenol (pNP)-(GlcNAc)

arctic fish, supports the assumption that a considerable part of chitin degradation also takes place in the digestive tracts of krill consumers.

The alignment of the amino acid sequence of the chitinase ChiA of strain Fi:7 with known protein sequences showed a remarkably high homology of 82% to the chitinase ChiA of *V. harveyi*. In this respect, it is noteworthy that the amino acid sequence of ChiA from *V. harveyi* is not very similar overall to other known chitinases (Svitil and Kirchman 1998), except for two regions, the catalytic and the chitin-binding domains. These two conserved domains could be also identified in the chitinase ChiA of isolate Fi:7. Although the third chitinolytic clone of the *E. coli* Fi:7 genomic library carried a truncated chitinase gene without this putative chitin-binding domain, it showed significant chitin degradation. Svitil and Kirchman (1998) demon-

strated that a truncated chitinase without the chitin-binding domain did not bind to chitin, but it could still hydrolyze chitin. The chitinase ChiA of *V. harveyi* was classified as an endochitinase, which randomly cleaves the center of chitin molecules and produces soluble oligosaccharides (Svitil et al. 1997). Similar to the *V. harveyi* chitinase, the chitinase of strain Fi:7 was highly active on ethylene glycol chitin. Based on these data and the high homology of the Fi:7 chitinase to the chitinase ChiA of *V. harveyi*, it is most probable that the chitinase ChiA investigated in this study is also an endochitinase.

The purification process of the chitinase led to an accumulation of two different protein fragments of approximately 80 and 82 kDa. N-terminal sequencing revealed that both fragments have the same N-terminus. This result demonstrated that the ompT signal peptide of plasmid pETchiA was correctly processed after overexpression of the Fi:7 chitinase. The size of the smallest fragment, 80 kDa, is very similar to the theoretical molecular weight of 79.4 kDa deduced from the ChiA amino acid sequence. The reason for the accumulation of the two protein bands of the chitinase ChiA during the purification process remains obscure. One potential explanation could be that the chitinase ChiA of strain Fi:7 forms two isoforms. Such isoforms have been also reported for two chitinases purified from *Streptomyces* sp. (Okazaki et al. 1995). Both enzymes had an identical N-terminal amino acid sequence and the same enzymatic properties but different isoelectric points.

Several chitinases from mesophilic bacteria, above all from soil bacteria but also from marine bacteria, have been cloned and characterized (Felse and Panda 1999). However, to our knowledge there have not been any reports on cloning of cold-adapted chitinases from psychrophilic or psychrotolerant bacteria. Recently, a chitinase of a psychrotolerant fungi (*Verticillium lecanii*) from continental Antarctica has been purified and characterized (Fenice et al. 1998). This enzyme was active over a broad range of temperatures (5°–60°C). At 5°C, the relative activity of this chitinase was still 50% of that recorded at its optimal temperature of 40°C. The temperature range of this fungal chitinase is very similar to that of the bacterial chitinase of strain Fi:7 described in this study. At 5°C, the chitinase ChiA still showed 40% of the maximal activity determined at 35°C. However, in contrast to the fungal chitinase from *V. lecanii*, the bacterial chitinase of strain Fi:7 was rapidly inactivated at 60°C.

The thermoflexibility of cold-adapted enzymes is supposed to be a prerequisite for the high catalytic activity of these proteins at low temperature (Gerday et al. 2000). However, this adaptation of psychrophilic proteins to low-temperature conditions also determines an instability of these proteins at higher temperatures. During overexpression of the chitinase of strain Fi:7 in *E. coli*, the highest enzyme activity could be determined at 30°C (data not shown). At 37°C, 40% lower chitinase activity of the recombinant *E. coli* cells could be observed. Feller et al. (1998) revealed that the cold-adapted amylase of the Antarctic bacterium *Pseudoalteromonas haloplanktis* could not be functionally expressed in *E. coli* at 37°C; significant amylase activity could be only found at temperatures below 25°C. Feller et al. (1998) argued that cold-adapted enzymes need lower temperatures than mesophilic enzymes for their proper folding.

The enzyme of strain Fi:7 investigated in this study is the first chitinase cloned from a cold-adapted bacterium. The temperature optima of chitinases from mesophilic bacteria usually fall into the range 50°–60°C (Tsujibo et al. 1998). The maximal enzyme activity of the chitinase of *Vibrio* sp. strain Fi:7 is clearly shifted to the low-temperature range, and thus it can be classified as a cold-adapted chitinase.

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