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ORIGINAL PAPER

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Purification and characterization of a new hyperthermostable, allosamidin-insensitive and denaturation-resistant chitinase from the hyperthermophilic archaeon *Thermococcus chitonophagus*

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Abstract A new chitinase (1,4-β-D-*N*-acetyl-glucosaminidase, EC 3.2.1.14) was detected and purified to homogeneity in its native form from the chitinolytic enzyme system of the extremely thermophilic archaeon *Thermococcus chitonophagus*. This is the first non-recombinant chitinase purified and characterized from archaea and also constitutes the first case of a membrane-associated chitinase isolated from archaea. The enzyme is a monomer with an apparent molecular weight of 70 kDa [therefore named chitinase 70 (Chi70)] and pI of 5.9; it is hydrophobic and appears to be associated with the outer side of the cell membrane. Chi70 is optimally active at 70°C and pH 7.0 and exhibits remarkable thermostability, maintaining 50% activity even after 1 h at 120°C, and therefore the enzyme is the most thermostable chitinase so far isolated. The enzyme was not inhibited by allosamidin, the natural inhibitor of chitinolytic activity, and was also resistant to denaturation by urea and SDS. On the other hand, guanidine hydrochloride significantly reduced enzymatic activity, indicating that, apart from the hydrophobic interactions, ion pairs located on the surface of the protein could be playing an important role in maintaining the protein's fold and enzyme activity. Chi70 showed broad substrate specificity for several chitinous substrates and derivatives. The lowest K_m and highest K_{cat} values were found for pNP$(NAG)_2$ as substrate and were determined to be 0.14 mM and 23 min^{-1}, respectively. The hydrolysis pattern was similar for oligomers and polymers, with *N*,*N*′-diacetylchitobiose [$(NAG)_2$] being the final, major hydrolysis product. Chi70 was classified as an endochitinase due to its ability to release chitobiose from colloidal chitin. Additionally, the enzyme presented considerable cellulolytic activity. Analysis of the NH_2-terminal amino acid sequence showed no detectable homology with other known sequences, suggesting that Chi70 is a new protein.

Keywords Archaea · Chitinases · Hyperthermophiles · *Thermococcus chitonophagus*

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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, in a similar way to 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 1977). Chitin and its partially deacetylated derivative, chitosan, as well as other derivatives, exhibit interesting properties and constitute a valuable raw material for biomedical, agricultural, and cosmetic applications (Muzzarelli 1997; Shigemasa and Minami 1996).

In the aquatic biosphere, chitin corresponds to an annual production of billions of tons, resulting in a continuous deposition of chitin on the ocean floor. However, marine sediments contain only traces of chitin and the turnover of the polysaccharide is attributed primarily to marine bacteria that degrade and catabolize chitinous particles, allowing carbon and nitrogen to return to the marine ecosystem (Keyhani and Roseman 1999). Complete chitin hydrolysis proceeds via two probable major pathways, the most common being a three-enzyme system: an endochitinase (EC 3.2.1.14) reduces the polymer to oligomers, which are subsequently degraded to monomers by exochitinase-chitobiase (β-*N*-acetyl

hexosaminidase, EC 3.2.1.52), and finally converted to glucosamine by chitin deacetylase. Practical applications of chitinases in biotechnology and bioprocessing include its use as a protective agent against plant-pathogenic fungi (biological control) and in the production of oligosaccharides as biologically active substances (Kramer and Muthukrishnan 1997; Gooday 1996, 1999; Schickler et al. 1993).

The discovery of microorganisms that grow optimally at temperatures near and above 100°C has important ramifications in microbial physiology and metabolism, evolution, and biotechnology (Antranikian et al. 1995; Adams and Kelly 1995; Sunna et al. 1997; Bauer et al. 1998). Extremophilic microorganisms are potential sources of highly stable enzymes, they are considered as an important biotechnological resource, and their specific properties are expected to be involved in new biotechnological applications. A thorough investigation of thermostable enzymes is also of fundamental interest for the elucidation of the structural basis for their stabilization, specificity, and catalytic properties (Shoichet et al. 1995; Ladenstein and Antranikian 1998; Zeikus et al. 1998; Stetter 1999). Through such an understanding, it may be possible to engineer proteins designed for the conditions required in various industrial processes (Niehaus et al. 1999).

A large number of chitin-hydrolyzing enzymes have been isolated from eukaryotes and bacteria and their corresponding genes have been cloned and characterized. Only a few thermostable chitinolytic enzymes from bacteria are known (Takayanagi et al. 1991; Tsujibo et al. 1993; Bharat and Hoondal 1998; Gomes et al. 2001). However, studies on archaeal or hyperthermophilic chitinases are very few, with the exception of the chitinase gene isolated and cloned from the hyperthermophilic archaeon *Pyrococcus kodakaraensis* KOD1 and expressed in *Escherichia coli* (Tanaka et al. 1999, 2001).

T. chitonophagus is a novel, hyperthermophilic, anaerobic archaeon, isolated from a deep-sea hydrothermal vent site off the Mexican west coast at a depth of 2,600 m and is the first archaeon discovered to degrade chitin for nutritional purposes (Huber et al. 1995).

In this work we present data on the induction, purification and characterization of an extremely thermostable, membrane-associated chitinase from the archaeon *T. chitonophagus*. This is the first report on the purification and characterization of a native (nonrecombinant) chitinolytic enzyme from archaea.

Experimental procedures

Materials

T. chitonophagus (DSM 10152) was supplied from the German Collection of Microorganisms and Cell Cultures. Chitin and various substrates were purchased from Sigma. CM–chitin–RBV was from Loewe Biochemica (Germany). Allosamidin was a gift from Prof. K.D. Spindler, University of Ulm, Germany. The column chromatography media were purchased from Pharmacia (Sweden) and TosoHaas (Japan). Proteinase inhibitors and sequencing grade proteinases were from Boehringer (Germany), calibration-marker proteins from Phamacia and polyacrylamide electrophoresis reagents from Serva (Germany). All TLC materials were purchased from Merck. All other chemicals were from Sigma or Merck in the highest analytical grade.

Preparation of colloidal chitin

Colloidal chitin was prepared according to Shimahara and Takiguchi (1988) and adjusted to a final concentration of 10% (w/v), sterilized by autoclaving and stored at 4°C.

Growth conditions

The cells were grown anaerobically (under pure, 5.0-quality nitrogen) in a water bath at 85°C and pH 7.5, on elementary sulfur-supplemented complex medium containing the following components per liter: 20 g NaCl, 4 g Na_2SO4, 0.7 g KCl, 0.2 g NH_4Cl, 0.2 g $NaHCO_3$, 0.1 g KBr, 0.03 g H_3BO_3, 10.8 g $MgCl_2.6\ H_2O$, 1.5 g $CaCl_2.2\ H_2O$, 0.03 g $SrCl_2.2\ H_2O$, and 0.1 mg $NaWO_4$. The above minimal medium also included 0.01% (w/v) KH_2PO_4, 0.05% (v/v) vitamin mixture (Balch et al. 1979), and 0.0002% (w/v) rezasurin, and was rendered anaerobic by the addition of 0.02% (w/v) Na_2S. The nitrogen and carbon source for the above medium was 0.5% (w/v) either colloidal chitin or flakes and powdered chitin, and for rich medium it was 0.5% (w/v) peptone and 0.1% (w/v) yeast extract. Batch cultivation of *T. chitonophagus* was performed in sealed 50-ml serum flasks at 100 or 200 rpm agitation, as described by Hungate (1950) and modified by Miller and Wolin (1974).

To investigate the optimum growth and/or inducing conditions, 5 mg/ml of each of the following nutrients was added in the minimal medium: tryptone, peptone, yeast extract, meat extract, casein, cellulose, chitosan, trehalose, maltose, and glucose, as well as 50 μM of the potential inducers *N*-acetyl-D-glucosamine (NAG), *N*,*N*′-diacetylchitobiose [$(NAG)_2$] and *N*,*N*′-diacetylchitotriose [$(NAG)_3$]. Cell density was measured at 600 nm and by cell counting after staining with 4,6-diamidino-2-phenylindole (DAPI) and direct counting under an epifluorescent microscope according to Parsons et al. (1984) and Turley (1993). Total chitinolytic activity in the cell extract was measured according to the standard assay conditions. To examine the sensitivity to antibiotics, 100 μg/ml of each of the following antibiotics was added to rich medium: ampicillin, carbenicillin, kanamycin, neomycin, streptomycin, chloramphenicol, and tetracyclin.

Polyacrylamide gel electrophoresis and zymograms

Polyacrylamide gel electrophoresis was performed as described by Laemmli (1970), routinely carried out on 1.5-mm-thick, 12% (w/v) polyacrylamide gels containing 0.1% (w/v) SDS, at a constant current of 30 mA for 2 h at room temperature. Nondenaturing polyacrylamide gels were prepared by the Davis (1964) method. The gels were stained either with 0.25% (w/v) Coomassie Blue R-250 (Neuhoff et al. 1988) or silver nitrate (Shevchenko et al. 1996). Molecular weight marker proteins to calibrate the gel were: phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and *a*-lactalbumin (14.4 kDa).

The chitinolytic activity was also detected using the "in situ" zymogram technique in SDS-PAGE containing 0.7 mg/ml CM–chitin–RBV. After electrophoresis, the gels were washed twice in ddH_2O for 15 min, SDS was partially removed with two 30-min washes in casein renaturation buffer according to McGrew and Green (1990) and finally equilibrated in 100 mM Na-phosphate buffer, pH 7.0. Clear zones indicating chitinolytic activity appeared after incubation at 70°C, overnight.

Standard assay of Chi70 activity

Chi70 activity was routinely determined by measuring the release of *p*-nitrophenol from $pNP(NAG)_2$ which absorbs at 405 nm, according to Roberts and Selitrenikoff (1988). Chitinase assay was carried out in 50 mM Na-phosphate, pH 7.0 with 0.2 mM $pNP(NAG)_2$ at 70°C for 4 h, in a total reaction volume of 50 µl, and the reaction was stopped by adding an equal volume of 1 M glycine–NaOH, pH 10.5. One unit of chitinase activity was defined as the amount of enzyme which produces 1 µmol of *p*-nitrophenol per minute under the standard assay conditions.

Protein determination

Protein concentration was determined using the Coomassie dye binding assay (Pierce) according to Bradford (1976), using bovine serum albumin (Pierce) as standard.

Purification of Chi70

Protein extraction and ammonium sulfate fractionation

All further procedures were carried out at 4°C, unless otherwise is specified. In a routine preparation, 800 ml of anaerobic culture of *T. chitonophagus* grown in inducing medium, i.e., in the presence of 0.5% (w/v) colloidal chitin, was collected at the stationary growth phase and centrifuged at 8,000 *g* for 20 min. The cell pellet (2 g wet weight) was either stored at –80°C or processed immediately as follows. Briefly, the cell pellet was resuspended in 50 mM Na-phosphate buffer, pH 7.0, 1 mM EDTA, 1% (v/v) Tween-20, 0.1 mM phenylmethanesulfonyl fluoride (PMSF), and a cocktail of protease inhibitors (Boehringer, Germany). The suspension was vigorously mixed for 30 min, sonicated for 30 min, heated to 100°C for 15 min, and centrifuged at 12,000 *g* for 30 min. The clear supernatant was further fractionated with solid ammonium sulfate. Chi70 activity was precipitated between 40% and 80% saturation in ammonium sulfate and recovered by centrifugation at 12,000 *g* for 30 min. The pellet was dissolved in 4 ml of 50 mM Na-phosphate, 1.5 M ammonium sulfate, at pH 7.0.

Hydrophobic interaction chromatography

The Chi70-enriched protein fraction (1.12 mg protein) was applied on a butyl-TSK-NPR, HR 5/5 column (TosoHaas) pre-equilibrated in 50 mM Na-phosphate, 1.5 M ammonium sulfate, at pH 7.0. Elution of the Chi70 was performed using a combination of a linear descending gradient between 1.5 and 0 M ammonium sulfate (Fig. 1, line A–B), and a linear ascending gradient of Tween-20 between 0 and 1% (v/v) (Fig. 1, line C–D). Chi70 was eluted at 0 M ammonium sulfate and 1% (v/v) Tween-20. After this step most of the other proteins were removed. The column was further washed with a third ascending linear gradient between 0 and 40% (v/v) isopropanol (Fig. 1, line E–F) to remove the remaining strongly bound proteins.

Anion exchange chromatography

Chi70 pooled fractions (0.25 mg protein) were adjusted to 20 mM ethanolamine-NaOH pH 9.0 and directly applied on a Mono Q, HR 5/5 (Pharmacia) column, previously equilibrated in the same buffer. Bound proteins were eluted using a combined linear ascending gradient between 0 and 1 M NaCl and 0% and 1% (v/v) Tween-20 (Fig. 2, line A–B). Pure Chi70 was eluted between 0.3 and 0.4 M NaCl and 0.3%–0.4% (v/v) Tween-20. The purified enzyme was stored at 4°C without any activity loss over several weeks. All chromatography columns were monitored using the standard enzyme assay and zymogram, as described above.

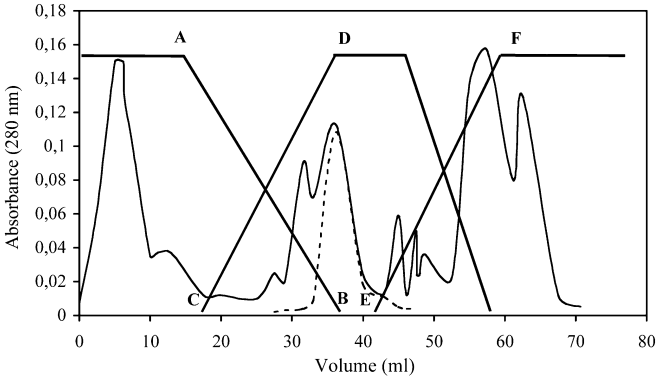

Fig. 1 Hydrophobic interaction chromatography on TSK-butyl-NPR, HR 5/5 column, applying three separate gradients, *A–B*, *C–D* and *E–F*, as described in the Experimental procedures section. The *thick lines* indicate the above gradients and the *dotted line* represents the Chi70 activity measured according to the standard assay. The *continuous line* represents the total protein measured at 280 nm

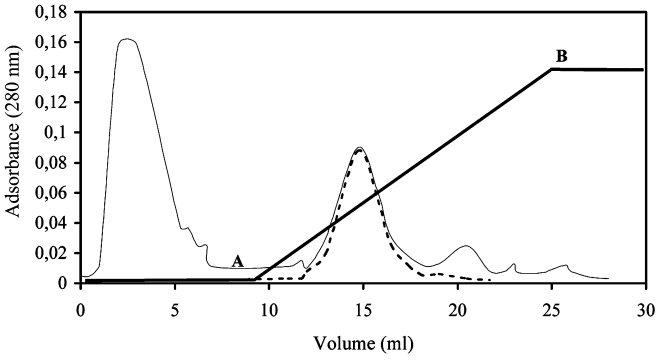

Fig. 2 Ion exchange chromatography on Mono Q, HR 5/5 column. Chi70 is eluted with a linear ascending gradient (indicated with the *thick line*) from *A* to *B*, as described in the Experimental procedures section. The *dotted line* represents the Chi70 activity measured according the standard assay, and the *continuous line* represents the total protein measured at 280 nm

Isoelectric focusing

The isoelectric point of Chi70 under native conditions was determined according to the manufacturer's instructions, on 5% (w/v) nondenaturing polyacrylamide gels containing an immobilized pH gradient between 3 and 10 (invitrogen). The proteins used for pI standards were: trypsinogen (pI = 9.3), lentin lectin (pI = 8.45), lentin lectin-acidic (pI = 8.15), horse myoglobulin (pI = 7.35), human carbonic anhydrase (pI = 6.55), bovine carbonic anhydrase (pI = 5.85), and *a*-lactoglobulin (pI = 5.2).

Determination of molecular mass by size-exclusion chromatography

The molecular weight of Chi70 was also determined by size exclusion chromatography on a Superose 6, preparative grade, HR 16–50 column (Pharmacia). The column was run in 50 mM Na-phosphate, pH 7.0, 50 mM NaCl, and 0.5% (v/v) Tween-20 at a flow rate of 0.3 ml/min. and calibrated with dextran sulfate (2,000 kDa) and pyridoxamine P and the same protein used as molecular weight markers for SDS-PAGE. Due to a low protein concentration of Chi70, the sample was applied simultaneously

with the standard proteins and its elution was monitored by activity in the standard enzyme assay.

Chitin and cellulose binding assay

The chitin binding assay was carried out in 50 mM Na-phosphate buffer, pH 7.0 and 3 mg pure colloidal chitin or cellulose using 0.8 μg of purified Chi70. After incubation for 1 h at room temperature, with occasional stirring, the supernatant containing unbound protein was separated from colloidal chitin or cellulose by centrifugation at 10,000 *g* for 10 min. The pellet was washed twice in the same buffer. The supernatants and pellets were analyzed for Chi70 by SDS-PAGE and zymograms.

Enzyme kinetics

The optimal temperature for Chi70 activity was determined in the standard assay using 0.8 μg of the purified Chi70 at temperatures ranging from 40° to 120°C, The optimum pH was measured in the range 3–12 in various buffer in the standard assay. The buffers used for various pH ranges were: 50 mM Na-citrate for pH range 3.0–5.0, 50 mM Na-acetate for pH 6.0, 50 mM Na-phosphate for pH 7.0 and 8.0, 50 mM bis-Tris propane for pH 9.0 and 10.0, and 50 mM Caps for pH 11.0 and 12.0.

To determine the effect of denaturing reagents, detergents, organic solvents, and other reagents such as inhibitors and ions (see Tables) on the enzymatic activity of Chi70, the enzyme was preincubated with various concentrations of each reagent for 20 min at room temperature; 0.2 mM $pNP(NAG)_2$ was subsequently added and the residual activity was determined under the standard assay conditions.

The substrate specificity of chitinase was determined using synthetic and natural substrates (as listed in the Tables) at concentrations up to 1.0 mM under the standard assay conditions. The natural substrates colloidal chitin, colloidal cellulose, and glycol chitosan were used in the range of 1–10 mg/ml final concentration, and the reaction was carried out at 70°C for up to 48 h. The amount of reducing sugar was quantified colorimetrically with ferric ferrocyanide reagent according to a modification of the Schales' procedure (Imoto and Yagishita 1971). Oligosaccharides derived from chitin are β,1–4-linked oligomers of NAG and are designated: $(NAG)_2$ for *N*,*N*′-diacetylchitobiose; $(NAG)_3$, $(NAG)_4$, $(NAG)_5$, $(NAG)_6$, $(NAG)_7$ for the corresponding chito-triose, -tetraose, -pentaose, -hexaose, and -heptaose derivatives, respectively. The chitooligosaccharides NAG, $(NAG)_2$, $(NAG)_3$, $(NAG)_4$, $(NAG)_5$, $(NAG)_6$ and $(NAG)_7$ were used at 1 mg/ml final assay concentration and the enzyme incubation ranged from 15 min to 8 h. The digestion of the natural, polymeric substrates was monitored by measuring the liberated reducing sugars. The kinetic constants K_m and V_{max} were determined from the respective Lineweaver–Burk plots.

For thermostability studies 0.8 μg of purified enzyme in 50 mM Na-phosphate, pH 7.0 was incubated at temperatures between 60° and 120°C for a time period of up to 48 h. At various time intervals, samples were withdrawn, cooled on ice and the residual chitinolytic activity was measured according to the standard assay. Similarly, the pH stability was determined by incubating the enzyme in the above indicated buffers, at room temperature, for a time period of up to 24 h and the residual activity was determined under the standard assay conditions, following adjustment of the pH with 1 M Na-phosphate, pH 7.0.

Analysis of the hydrolysis products of various chitinous polymers

Hydrolysis products arising from the action of the Chi70 on various oligomeric and polymeric substrates were analyzed by TLC on silica gel-60, aluminum sheets (Merck), according to Tanaka et al. (1999). Aliquots (5 μl) of each reaction mixture were analyzed twice on TLC plates with *n*-butanol:methanol:25% ammonia solution:water (5:4:2:1 v:v:v:v) and the products were detected by spraying the plate with anilin–diphenylamine reagent (4 mM of aniline, 4 g of diphenylamine, 200 ml of acetone, and 30 ml of 85% phosphoric acid) and baking it at 180°C for 5 min. The end products of the above reactions were also analyzed by HPLC on a reverse phase, aminopropyl–silica column (μ-Bondapak, 125A, Waters). The sugars were isocratically eluted with 75% acetonitrile in ddH_2O at 3 ml/min and room temperature. The separated products were detected by absorbance at 210 nm and compared to standard chitooligosaccharides. For HPLC analysis, the Chi70 assay was performed in 50 mM Hepes, pH 7.0, at 70°C, for a time period up to 48 h. Aliquots were withdrawn at specific time intervals and placed on ice until analyzed.

NH_2-terminal sequence analysis

The NH_2-terminal amino acid sequence of the purified Chi70 was determined by stepwise Edman degradation in an automated protein sequencer (Applied Biosystems), using an electroblotted protein sample on PVDF (Amersham) membrane. Electrotransfer from a 10% SDS-polyacrylamide gel onto the membrane was performed in 10 mM Caps, pH 10.0, 10% methanol, at a constant current of 100 mA, at 4°C, for 2 h.

Results

Growth of *T. chitonophagus* with chitin and Chi70 induction

To improve Chi70 production from *T. chitonophagus*, the archaeon was grown anaerobically as described in the Experimental procedures section. The optimum batch-culture conditions for chitinase production were obtained by cultivation in the presence of 0.5% (w/v) colloidal chitin as the sole carbon and nitrogen source at 200 rpm, 85°C, for 60 h. Under these conditions we have detected and isolated two distinct chitinases produced by *T. chitonophagus*: one extracellular enzyme secreted in the medium (unpublished observations) and a second enzyme associated with the cell membrane, Chi70. Zymogram analysis of clarified, soluble cell extract clearly indicated the presence of multiple clear bands of chitinolytic activity, suggesting the presence of more than one chitinolytic enzyme (Fig. 3, lane 6). *T. chitonophagus* was found to be totally insensitive to the antibiotics ampicillin, carbenicillin, kanamycin, neomycin, streptomycin, chloramphenicol, and tetracyclin.

Purification and physicochemical properties of Chi70

Chi70 was routinely purified from 2 g wet weight cells of *T. chitonophagus* produced from 800-ml batch cultures in inducing medium. By using an extraction step with Tween-20 and differential fractionation with ammonium sulfate, and by applying hydrophobic interaction and anion exchange chromatography, Chi70 was purified to homogeneity. Table 1 summarizes the entire purification procedure, where the purification factor was 15-fold and the recovery 4%. The bulk of the other proteins were removed in the first chromatographic step (Fig. 1 and Fig. 3, lane 2), where Chi70 activity and protein were

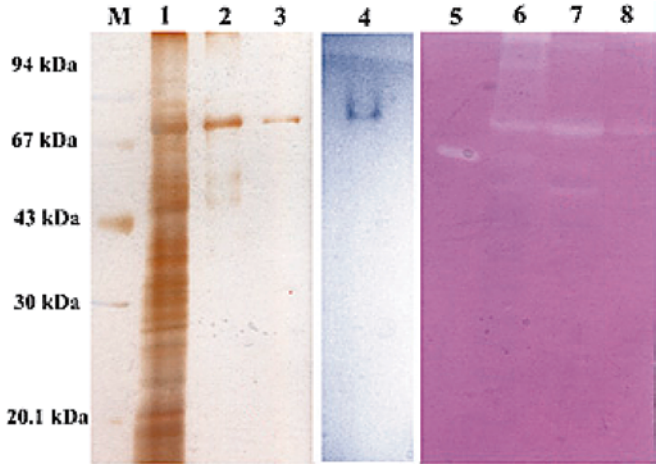

Fig. 3 SDS-PAGE analysis of peak fractions from the various Chi70 purification steps. *Lane M* contains the molecular weight markers as indicated in kDa. *Lanes 1 and 6* contain the clarified soluble enzyme extract, *lanes 2 and 7* contain the peak Chi70 fraction eluted from the TSK-butyl-NPR HR 5/5 column and *lanes 3 and 8* the purified protein eluted from the Mono Q column. Lane 4 contains the purified protein analyzed by native PAGE at pH 8.9. *Lane 5* is chitinase A from *Serratia marcescens* which was used as a positive control in the zymogram. Lanes M–3 were stained with silver. Lane 4 was stained with Coomassie Blue. Lanes 6–8 are zymograms

eluted at low ammonium sulfate concentration and 1% Tween-20; however, some other impurities were still present. Chi70 was eluted from the final anion exchange column, MonoQ at around 350 mM NaCl (Fig. 2). As shown in Fig. 3, lane 3, no other proteins detectable with silver staining were present and therefore the Chi70 was considered to be highly pure.

The apparent molecular weight of Chi70 under denaturation conditions on SDS-PAGE was estimated to be 70 kDa. Analysis by native PAGE clearly shows that the enzyme exists in a monomeric form. This was also confirmed by isoelectric focusing, where a single chain with a pI of 5.9 was resolved. The native molecular weight of Chi70 was also determined by size exclusion chromatography and found to be 77 kDa. The slight difference is within the resolution of the methods and could also be due to possible nonspecific interactions of the Chi70 protein and the polysaccharide nature of the matrix of the column.

The purified Chi70 was not found to be glycosylated, as examined with glycosylation assay (data not shown). Chi70 did not react with a polyclonal antibody raised against chitinase A from *Serratia marcescens*.

Membrane localization experiments were performed using selective, differential extraction at 37°C, with precondensed TritonX-114, in Tris-saline buffer (TBS), at pH 7.5, according to Bordier (1981) and Pryde and Philips (1986). The extract was analyzed by SDS-PAGE and zymogram and the protein amount was quantified by densitometry of the objective band on the Coomassie Blue-stained gel. About 73% of Chi70 was detected in the detergent-enriched phase of the extract (data not shown), indicating that the enzyme has regions that are highly hydrophobic and therefore able to associate with membranes.

Catalytic properties of Chi70

Chi70 was found to be active at a broad range of temperatures (70°–120°C, Fig. 4b) and in a narrow pH range (pH 6.5–7.5, Fig. 4a). Maximal activity was measured at 70°C and pH 7.0, using the substrate $pNP(NAG)_2$, under the standard assay conditions, as described in the Experimental procedures section.

Enzyme stability

The enzyme presented considerable thermostability, maintaining over 50% of the initial activity after 48 h at 90°–100°C. At 110° and 120°C, Chi70 had a half-life of 4 h and 1 h, respectively (Fig. 5a).

Chi70 presented broad pH stability, maintaining over 50% activity after 24 h in the pH range 3–9. No significant loss of activity occurred after incubation at pH 6, 7, and 8 for 24 h At pH 10 and 11, the enzyme appeared very unstable, losing half of its initial activity

Table 1 Purification scheme for Chi70

Purification step	Volume (ml)	Protein concentration (mg/ml)	Total protein (mg)	Specific activity (units/mg)	Total activity (units)	Protein yield (%)	Enzyme yield (%)	Purification factor (fold)
Clarified, soluble enzyme extract	8	0.36	2.90	0.20	0.58	100	100	1
Fractionation with ammonium sulfate (40%–80% saturation)	4	0.28	1.12	0.34	0.38	39	65	1.7
Hydrophobic interaction chromatography (Chi70 peak fraction)	5	0.05	0.25	0.40	0.1	9	17	2.0
Ion exchange chromatography (Chi70 peak fraction)	1.5	0.005	0.008	3	0.024	0.3	4	15

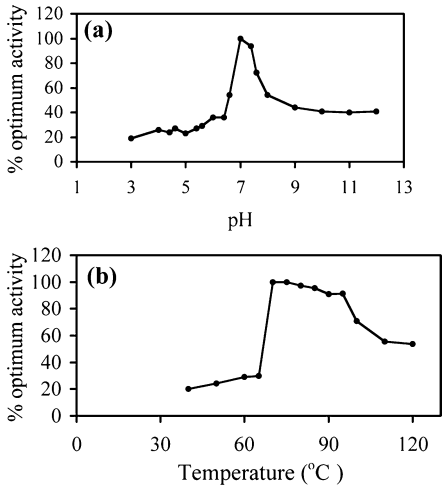

Fig. 4 The dependency of Chi70 activity on pH (**a**) and temperature (**b**). The enzyme was incubated at the indicated pH and temperatures for 4 h, and the activity was measured using the standard assay

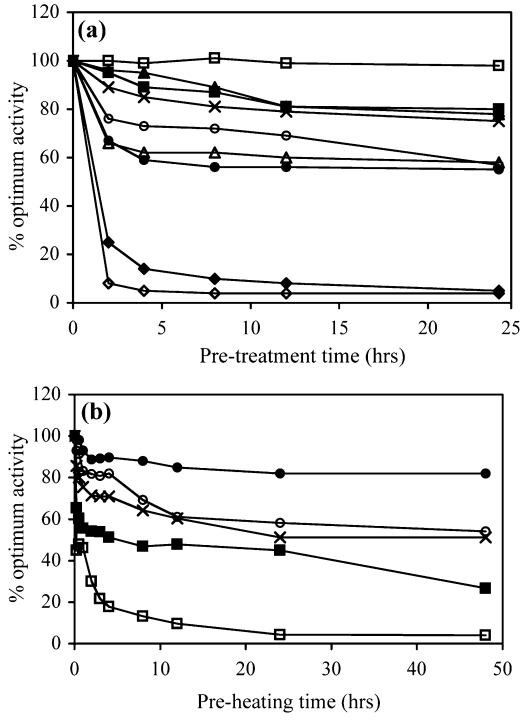

Fig. 5 **a** pH stability of Chi70. The enzyme was preincubated at various pH values, as indicated, at room temperature up to 24 h, and at various time intervals samples were withdrawn, adjusted to pH 7.0 and the residual activity was measured according to the standard assay conditions and expressed as the percentage of the initial activity. ● pH 3.0, ❍ pH 4.0, pH 5.0, ■ pH 6.0, □ pH 7.0, ∇ pH 8.0, ◆ pH 9.0, ▼ pH 10.0, ◇ pH 11.0. **b** Thermal stability of Chi70. The enzyme was preheated at the indicated temperatures, at pH 7.0, up to 48 h and at various time intervals samples were withdrawn, cooled on ice and the residual activity was measured according to the standard assay and expressed as the percentage of the initial activity. ● 80°, ❍ 90°, 100°, ■ 110°, □ 120 °C

within the first hour (Fig. 5b). Pure Chi70 can be stored without losing its activity at 4°C for a long period.

Effect of metal ions

The effect of various monovalent and bivalent cations is summarized in Table 2. No influence on the activity was observed with any of the cations indicated, with the exception of iron, which appeared to inhibit the enzyme. Correction of the absorbance values obtained for iron, using a negative control, absence of Chi70, indicated that the apparent inhibition was an artifact due to the formation of a complex between the substrate and iron ions.

EDTA was found to have no effect on the enzyme activity, even at a final concentration of 50 mM and in the presence or absence of metals, clearly showing that bivalent cations were not required for enzymatic activity.

Effect of various reagents

Most of the reagents indicated in Table 2 did not have any significant effect on the activity of Chi70. The reducing agent dithiothreitol (DTT) and the alkylating reagent iodoacetamide (IAAM) did not have any effect, indicating that thiol groups are not involved in the active site. The enzyme appeared considerably stable in organic solvents, mainly in isopropanol and less so in ethanol. On the other hand, polyethylene-glycol-400 (PEG-400) and dimethyl sulfoxide (DMSO) caused a considerable loss of the enzyme activity, which was expected, due to the hydrophobicity of the protein. High resistance was also observed against denaturation by urea. Over 50% of the initial activity was maintained in the presence of 3 M urea, while in the presence of guanidine hydrochloride at the same concentration the activity was completely absent. Chi70 was completely unaffected by the presence of various non-ionic or zwitterionic detergents and was resistant to the addition of 1% (w/v) SDS. The enzyme was also moderately resistant to high ionic strength, preserving 95% and 40% of its activity in the presence of 0.5 and 1 M NaCl, respectively.

Effect of allosamidin

Chi70 was unaffected by the addition of allosamidin to concentrations ranging from 50–200 μM in the standard assay. The enzyme was preincubated with the inhibitor at room temperature for 30 min and its activity was measured without removing the inhibitor.

Chitin and cellulose binding assays

Pure colloidal chitin and colloidal cellulose were strongly bound to the Chi70 protein molecule,

Table 2 Chemical stability of Chi70. Residual enzymatic activity, determined under the standard enzyme assay conditions, is expressed as the percentage of the initial activity following incubation at the indicated conditions

	% residual activity					
Inhibitor concentration (mM)	DTT	IAAM	EDTA	PMSF	pCMBa	pBBa
1	95.6	87.0	88.0	98.0	92.7	90.8
50	76.5	88.0	78.1	82.5	68.7	82.6
Organic solvent concentration (%v/v)	2-propanol		Ethanol	PEG-400	DMSO	
5	100.0		89.0	88.4	46.0	
40	86.8		66.0	30.0	22.0	
Denaturing reagent concentration (M)	Guanidine hydrochloride			Urea		
0.5	24.0			99.2		
3	2.1			60.0		
5	0.7			17.2		
Cation concentration (mM)	Fe^{+2}	Cu^{+2}	Mg^{+2}	Zn^{+2}	Co^{+2}	Mn^{+2}
1	69	94	88	83	82	77
5	48	78	95	82	83	73
10	21	76	96	81	79	69
Cation concentration (mM)	Ca^{+2}	Sr^{+2}	W^{+6}	Ni^{+1}	Na^{+1}	
1	80	90	97	83	98	
5	81	89	92	82	96	
10	85	87	90	81	94	

DTT = dithiothreitol, IAAM = iodoacetamide, PMSF = phenylmethanesulfonyl fluoride, pCMBa = *p*-chloromercuribenzoic acid, pBBa = *p*-bromobenzoic acid, PEG-400 = polyethylene-glycol-400, DMSO = dimethyl sulfoxide

suggesting that the enzyme may contain a chitin and cellulose binding domain (data not shown).

Substrate specificity and kinetic parameters

Kinetics experiments with synthetic substrates carrying the *p*-nitrophenol (pNP) group were performed using the standard activity assay. The pNP-releasing activity of Chi70 was highest towards $pNP(NAG)_2$. The results of the substrate specificity experiments are summarized in Table 3 and clearly show that Chi70 cleaves specific β-glycosidic bonds.

Kinetic experiments with natural substrates were performed using the reducing sugars assay and the results are summarized in Table 3. The optimum activity in this case was obtained at 12 h, due to the low solubility and viscosity of the biopolymers. The most preferred natural substrate was colloidal chitin.

On the basis of the respective Lineweaver-Burk plots for the synthetic and natural substrates, the apparent V_{max}, K_m, and K_{cat} values were calculated and are presented in Table 4. As expected, the highest V_{max} and K_{cat} values and the lowest value K_m were determined for $pNP(NAG)_2$ (among the synthetic substrates) and pure colloidal chitin (among the natural substrates).

Mode of action

The end products of the hydrolysis of colloidal chitin, CM-chitin and $(NAG)_7$ were analyzed by HPLC (Fig. 6), as described in the Experimental procedures section. The hydrolysis pattern of various natural substrates and NAG-oligomers was followed by TLC analysis (Fig. 7).

Incubation of Chi70 with $(NAG)_2$ for a time period ranging from 15 min to 8 h did not yield any products

Table 3 Substrate specificity of Chi70. The values represent the maximum activity obtained after 12 h incubation for the natural substrates and 4 h for the synthetic substrates and for the optimum substrate concentration. Activity is expressed as the percentage of the enzyme activity under the standard conditions using either pNP $(NAG)_2$ or colloidal chitin

Natural substrate (10 mg/ml)	% standard activity after 12 h incubation at 70°C	Specific activity (mg product/min mg protein) after 12 h incubation at 70°C
Pure colloidal chitin	100.0	0.31
Pure colloidal cellulose	86.7	0.27
Pure chitosan (glycol)	75.0	0.23
Synthetic substrate (0.5 mM)	% standard activity after 4 h incubation at 70°C	Specific activity (units/mg) after 4 h incubation at 70°C
pNp-β-D-diacetylchitobioside	100.0	2.07
pNp-β-D-glucopyranoside	51.5	1.26
pNp-β-D-maltoside	37.1	0.96
pNp-β-D-mannopyranoside	25.4	0.63
pNp-β-D-cellobioside	13.2	0.35
pNp-β-D-lactopyranoside	10.0	0.25
pNp-β-D-galactopyranoside	9.9	0.25

Table 4 Kinetic parameters of Chi70 for natural and synthetic substrates. K_m and V_{max} have been calculated from the double reciprocal plots (Lineweaver–Burk)

Natural substrate	V_{max} (mg product/min·mg protein	K_m (mg/ml substrate)	K_{cat} (min^{-1})	K_{cat}/K_m (ml/mg min)
Pure colloidal chitin	0.37	1.3	0.30	0.23
Pure colloidal cellulose	0.37	2.0	0.20	0.10
Pure glycol chitosan	0.25	1.7	0.15	0.09
Synthetic substrate	V_{max} (units/mg)	K_m (mM)	K_{cat} (min^{-1})	K_{cat}/K_m (mM $min)^{-1}$
pNp-β-D-diacetylchitobioside	3.20	0.14	23.0	164.3
pNp-β-D-glucopyranoside	1.43	0.25	5.7	22.9
pNp-β-D-cellobioside	0.44	0.18	2.4	13.3
pNp-β-D-maltoside	1.40	0.33	4.2	12.7
pNp-β-D-mannopyranoside	1.25	0.67	1.9	2.8
pNp-β-D-lactopyranoside	0.33	0.22	1.5	6.8
pNp-β-D-galactopyranoside	0.40	0.40	1.0	2.5

(data not shown). Hydrolysis of $(NAG)_3$ produced, within the first 15 min and throughout the whole period of 8 h, $(NAG)_2$ and NAG (data not shown). The substrate $(NAG)_4$ was cleaved to $(NAG)_2$, while traces of NAG were also detected. $(NAG)_5$ was completely hydrolyzed to $(NAG)_3$ and $(NAG)_2$ and, upon longer incubation, to $(NAG)_2$ and NAG (data not shown). Incubation of Chi70 with $(NAG)_6$ yielded, within the first 15 min, multiple products: $(NAG)_2$, $(NAG)_3$, $(NAG)_4$, in approximately equal amounts, as well as uncleaved $(NAG)_6$ (Fig. 7, lane 1). Following 30 min incubation, uncleaved substrate could no longer be detected (Fig. 7, lanes 5–8). Digestion of $(NAG)_7$ with the enzyme produced within the first 10 min $(NAG)_2$, $(NAG)_3$, $(NAG)_5$ and nonhydrolyzed substrate, after 30 min $(NAG)_2$ and $(NAG)_3$, and after 1–8 h $(NAG)_2$ was the major product, while traces of NAG were also detected (data not shown).

Concerning the natural substrates, longer incubation times were required for complete hydrolysis. The main degradation product obtained from the action of Chi70 on colloidal chitin was $(NAG)_2$ which was detectable after 24 and 48 h digestion. NAG could also be detected in very small amounts (Fig. 6a). On the other hand, the water-soluble polymer CM-chitin was degraded to $(NAG)_2$ after only 1 h of incubation, whereas prolonged incubation (48 h) also yielded $(NAG)_3$ (Fig. 6b). Cellulose also constituted a substrate for Chi70 and was

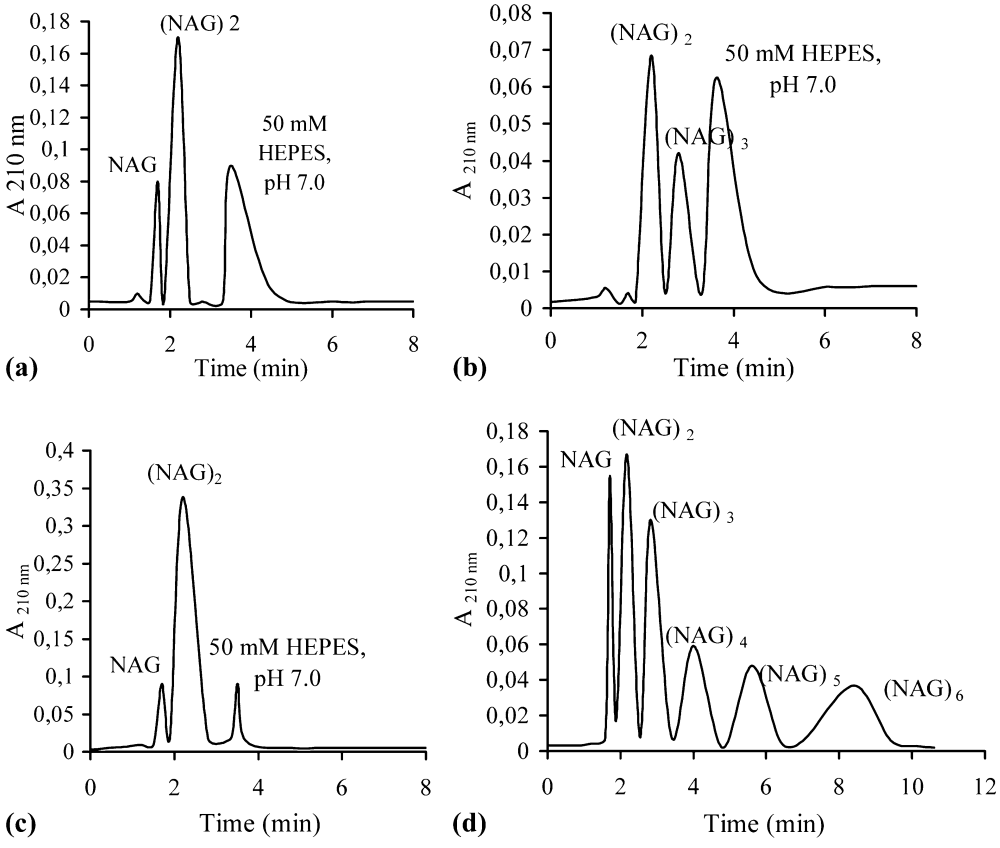

Fig. 6 HPLC-analysis on a μ-Bondapak, amino–propyl–silica column (Waters) of the end products of hydrolysis of 20 mg/ml colloidal chitin (**a**), 0.7 mg/ml CM-chitin (**b**), and 0.7 mg/ml $(NAG)_7$ (**c**). The substrates were incubated with Chi70 at 70°C, for 48 h. The analysis of the standard *N*-acetyl-D-glucosamine (NAG) oligomers is presented on plot **d**

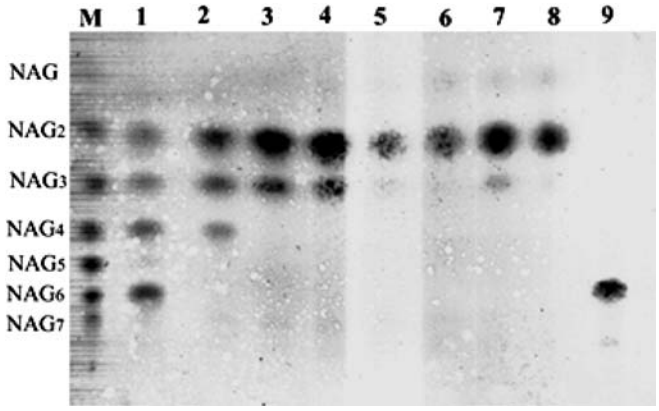

Fig. 7 TLC analysis of the hydrolysis products of $(NAG)_6$. Chi70 was incubated with the substrate for 15 min (*lane 1*), 30 min (*lane 2*), 45 min (*lane 3*), 1 h (*lane 4*), 2 h (*lane 5*), 4 h (*lane 6*), 6 h (*lane 7*), 8 h (*lane 8*) at 70°C. *Lane 9* contains the negative control (absence of enzyme). *Lane M* contains the NAG standards as indicated

degraded to cellobiose and glucose after incubation for 12 h, indicating the presence of an additional cellulase activity, probably of the endo-type (data not shown). The last finding implies that Chi70 is an enzyme with dual active sites, one containing the chitinolytic activity and a second responsible for the cellulolytic activity.

NH_2-terminal amino acid sequence

The first 21 amino acids from the N-terminus of Chi70 were determined as described previously. The LTYD(E/Y) GGLHKNEYKSNRAXLT peptide contained one unidentified amino acid X, and in one position the E/Y could not be resolved. The sequence was compared against the SwissProt protein database, and showed no detectable homology with other known sequences, indicating that Chi70 is a new protein.

Discussion

T. chitonophagus produces several, not yet characterized, chitinolytic enzymes when it grows in a minimum medium containing colloidal chitin as the exclusive carbon and nitrogen source (unpublished results). These enzymes are most likely either to be associated with the inner and outer part of the cell membrane or to be secreted. As has been reported for other microorganisms, chitinase gene expression is controlled by a repressor/inducer system, in which chitin or its degradation products act as inducers (Felse and Panda 1999). During the course of our study, two chitinases have been detected: a secreted chitinase-50 (Chi50) and a cell membrane-associated Chi70. Chi50 is an endochitinase, with an apparent molecular weight of 50 kDa, isolated from the culture supernatant and with exhibited temperature and pH optima at 80°C and 6.0, respectively (unpublished observations). Chi70 is a cell membrane-associated enzyme, isolated from the total extract of *T. chitonophagus*. We have focused our study on Chi70 since this enzyme appears to be produced in greater amounts. Here we describe the first native (nonrecombinant) chitinase purified to homogeneity and characterized from an archaeon. The good agreement of the molecular weights estimated by SDS-PAGE (70 kDa) and size exclusion chromatography (77 kDa) clearly shows that Chi70 exists as a single polypeptide. The protein is not glucosylated and preliminary data, partially described in the Results section, suggest that Chi70 is associated with the outer face of the cell membrane. This is also supported by the fact that Chi70 activity can be measured on intact cells.

Low yield and purification factors were obtained during the purification of the enzyme because the protein is hydrophobic and it is very likely that various gluco- or lipid moieties, bound to the molecule, had strongly influenced its ammonium sulfate fractionation and chromatographic behavior. This problem was solved after we included Tween-20 in the extraction procedure and the chromatography steps. High initial chitinolytic activity measured in the total extract could reflect the activity of several chitinolytic enzymes, resulting in overestimation of the initial chitinase activity.

Generally, known chitinases have been classified into two major groups: (1) endochitinases, which cleave glucosidic linkages randomly along the chitin chain, eventually producing oligomers of NAG, and (2) exochitinases. Exochitinases can be divided into two subgroups: (a) chitobiosidases, which catalyze the progressive release of $(NAG)_2$ (chitobiose), starting at the nonreducing end of the chitin microfibril, and (b) 1,4-β-*N*-acetyl-glucosaminidases, which cleave the oligomeric products of endochitinases and chitobiosidases at the nonreducing end, generating monomers of NAG. Chi70 displays a rather broad substrate specificity and is suggested to be an endo-type enzyme for the following reasons: (1) Chi70 does not hydrolyze pNP-NAG and $(NAG)_2$, indicating that it is not a 1,4-β-*N*-acetyl-glucosaminidase, (2) when $(NAG)_6$ is used as a substrate, Chi70 does not produce $(NAG)_4$ as a major product at the early stages of the reaction, indicating that it is not a chitobiosidase.

Another archaean chitinase with two catalytic domains was detected and cloned for the first time from the hyperthermophilic archaeon *Pyrococcus kodakaraensis* (Tanaka et al. 1999). The primary structure of this chitinase has been considered, so far, to be unique among bacterial and eukaryotic chitinolytic enzymes because it exhibits dual activity (chitinolytic and cellulolytic). The ability of Chi70 from *T. chitonophagus* to efficiently hydrolyze cellulose, apart from chitin, supports the view that this enzyme also exhibits dual activity. The considerable activity of the enzyme towards several glucose-containing substrates that are not *N*-acetylglucosamine derivatives, such as pNP-β-

D-glucopyranoside, pNP-β-D-cellobioside and pNP-β-D-maltoside, can be attributed to the second inherent cellulolytic activity of Chi70.

It has been proposed for cellulose-binding domains, and is also true for chitin-binding domains, that they mediate the nonhydrolytic disruption of the crystalline chitin or cellulose, thereby facilitating the subsequent enzymatic hydrolysis by the catalytic domains (Blackwell 1982; Watanabe et al. 2001). The binding of Chi70 to cellulose and chitin substrates suggests that the protein molecule could contain both chitin- and cellulose-binding domains.

The complete resistance of Chi70 to inhibition by allosamidin, the natural, (generally) competitive inhibitor of chitinolytic activity (Spindler and Spindler-Barth 1999), differentiates the enzyme from most other chitinases so far characterized. The fact that this trisaccharide analog of a chitin oligomer does not block the enzyme active site suggests the inability to bind to the protein molecule, rather than interference with the catalytic mechanism. Metal ions and other cations are not required for the stabilization or activation of the enzyme.

Generally, Chi70 is characterized by high resistance to several denaturing agents, such as urea and SDS, but not to guanidinium hydrochloride. The protein was moderately unaffected by high ionic strength and its activity was not affected in a highly hydrophobic environment. Apart from its considerable chemical stability, Chi70 is also characterized by remarkable heat tolerance, remaining active for at least 1 h at 120°C and 4 h at 110°C. These observations classify Chi70 as a hyperthermophilic enzyme.

According the current knowledge for the structural properties required in order to achieve hyperthermophilicity, tight packing in combination with minimum flexibility of the protein is primarily achieved by hydrophobic interactions (Sterner and Liebl 2001). The inactivation of the enzyme in the presence of guanidine hydrochloride, even at low concentrations, contrary to the observed resistance to urea, suggests the presence of an arginine-involved ion-pairs network on the surface of the protein that substantially contributes to the overall stability of the molecule. The insensitivity to DTT indicates that thiol groups do not participate in the overall stability and activity of the enzyme, as expected since hyperthermophilic proteins contain very low sulfur-containing residues (Ladenstein and Antranikian 1998; Shoichet et al. 1995; Stetter 1999).

In conclusion, its high thermostability and chemical stability make Chi70 unique among other thermostable chitinases so far isolated and characterized from thermophilic bacteria (Takayanagi et al. 1991; Tsujibo et al. 1993; Bharat and Hoondal 1998; Gomes et al. 2001) and the hyperthermophilic archaeon *Pyrococcus kodakaraensis* KOD1 (Tanaka et al. 1999, 2001). Moreover, this is the first membrane-associated chitinase so far isolated and studied, as well as being one of the few cases of membrane proteins purified and characterized from archaea. Its high thermal and chemical stability, its independence of metal ions, and its substrate specificity qualify Chi70 as an attractive candidate for possible industrial and biotechnological applications.

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References

Adams MWW, Kelly RM (1995) Enzymes from microorganisms from extreme environments. Chem Eng News 73:32–42

Antranikian G, Rudiger A, Canganella F, Klingeberg M (1995) Biodegradation of polymers at temperatures up to 130°C. Pure Appl Chem 32:661–669

Balch WE, Fox CE, Magrun LJ, Woese CR, Wolfe RS (1979) Methanogens: reevaluation of a unique biological group. Microbiol Rev 43:260–296

Bauer MW, Driskill LE, Kelly RM (1998) Glycosyl hydrolases from hyperthermophilic microorganisms. Curr Opin Biotechnol 9:141–145

Bharat B, Hoondal GS (1998) Isolation, purification and properties of a thermostable chitinase from the alkalophilic *Bacillus* sp. BG-11. Biotechnol Lett 20:157–159

Blackwell J (1982) The macromolecular organization of cellulose and chitin. In: Brown RM Jr (ed) Cellulose and other natural polymer systems. Plenum, New York, pp 403–428

Bordier C (1981) Phase separation of integral membrane proteins in Triton X-114 solution. J Biol Chem 256:1604–1607

Bradford M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Davis B (1964) Disc electrophoresis: method and application to human serum proteins. Ann N Y Acad Sci 121:404–427

Felse PA, Panda T (1999) Regulation and cloning of microbial chitinase genes. Appl Microbiol Biotechnol 51:141–151

Gomes RC, Semedo LT, Soares RM, Linhares LF, Ulhoa CJ, Alviano CS, Coelho RR (2001) Purification of a thermostable chitinase from *Streptomyces* RC1071 isolated from a cerrado soil and its antagonism against phytopathogenic fungi. J Appl Microbiol 90:653–661

Gooday GW (1996) In: Muzzarelli RAA (ed) Chitin enzymology. European Chitin Society, Ancona, Italy, pp 125–133

Gooday GW (1999) Aggressive and defensive roles for chitinases. Extremophiles 87:157–169

Huber R, Stohr J, Hohenhaus S, Rachel R, Burggraf S, Jannasch HW, Stetter KO (1995) *Thermococcus chitonophagus* sp. nov. :a novel chitin-degrading, hyperthermophilic archaeum from a deep-sea hydrothermal vent environment. Arch Microbiol 164:255–264

Hungate RE (1950) The anaerobic mesophilic cellulolytic bacteria. Bacteriol Rev 14:1–49

Imoto T, Yagishita K (1971) A simple activity measurement of lysozyme. Agric Biol Chem 35:1154–1156

Keyhani NO, Roseman S (1999) Physiological aspects of chitin catabolism in marine bacteria. Biochim Biophys Acta 1473:108–122

Kramer KJ, Muthukrishnan S (1997) Insect chitinases: molecular biology and potential use as biopesticides. Insect Biochem Mol Biol 27:887–900

Ladenstein R, Antranikian G (1998) Proteins from hyperthermophiles: stability and enzymatic catalysis close to the boiling point of water. Adv Biochem Eng Biotechnol 61:37–85

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature 227:680–685

McGrew BR, Green M (1990) Enhanced removal of detergent and recovery of enzymatic activity following SDS-PAGE: use of casein in gel wash buffer. Anal Biochem 189:68–74

Miller TL, Wolin MJ (1974) A serum bottle modification of the Hungate technique for cultivating obligate anaerobes. Appl Microbiol 27:985–987

Muzzarelli RAA (1977) In: Chitin. Pergamon, Oxford, pp 155–181

Muzzarelli RAA (1997) Human enzymatic activities related to the therapeutic administration of chitin derivatives. Cell Mol Life Sci 53:131–140

Neuhoff V, Arold N, Taube D, Ehrhardt W (1988) Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. Electrophoresis 9:255–262

Niehaus F, Bertoldo C, Kahler M, Antranikian G (1999) Extremophiles as a source of novel enzymes for industrial applications. Appl Microbiol Biotechnol 51:711–729

Parsons PT, Haita Y, Lalli CM (1984) Direct counting of bacteria by fluorescence microscopy. In: A manual of chemical and biological methods for sea water analysis. Pergamon, Oxford, pp 123–126

Pryde DK, Philips JH (1986) Fractionation of membrane proteins by temperature-induced phase separation in Triton X-114. Biochem J 233:525–533

Roberts WK, Selitrenikoff CP (1988) Plant and bacterial chitinases differ in antifungal activity. J Gen Microbiol 134:169–176

Schickler H, Haran S, Oppenheim A, Chet I (1993) Cloned chitinases and their role in biological control of plant pathogenic fungi. In: Muzzarelli RAA (ed) European Chitin Society, Ancona, Italy, pp 375–380

Shevchenko A, Wilm M, Vorm O, Mann M (1996) Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. Anal Chem 68:850–858

Shigemasa Y, Minami S (1996) Applications of chitin and chitosan for biomaterials. Biotechnol Genet Eng Rev 1:383–420

Shimahara K, Takiguchi Y (1988) Preparation of crustacean chitin. Methods Enzymol 161:417–423

Shoichet BK, Baase WA, Kuroki R, Matthews BW (1995) A relationship between protein stability and protein function. Proc Natl Acad Sci USA 92:452–456

Spindler KD, Spindler-Barth M (1999) Inhibitors of chitinases. Extremophiles 87:201–209

Sterner R, Liebl W (2001) Thermophilic adaptations of proteins. Crit Rev Biochem Mol Biol 36:39–106

Stetter CO (1999) Extremophiles and their adaptations to hot environments. FEBS Lett 452:22–25

Sunna A, Moracci M, Antranikian G (1997) Glycosyl hydrolases from hyperthermophilic microorganisms. Extremophiles 1:2–13

Takayanagi T, Ajisaka K, Takiguchi Y, Shimahara K (1991) Isolation and characterisation of thermostable chitinases from *Bacillus licheniformis* X-74. Biochim Biophys Acta 1078:404–410

Tanaka T, Fujiwara S, Nishikori S, Fukui T, Takagi M, Imanaka T (1999) A unique chitinase with dual active sites and triple substrate binding sites from the hyperthermophilic archaeon *Pyrococcus kodakaerensis* KOD1. Appl Environ Microbiol 65:5338–5344

Tanaka T, Fukui T, Imanaka T (2001) Different cleavage specificities of the dual catalytic domains in the chitinase from the hyperthermophilic archaeon *Thermococcus kodakaerensis* KOD1. J Biol Chem 276:35629–35635

Tsujibo H, Minoura K, Katsuhiro M, Endo H, Moriwaki M, Inamori Y (1993) Purification and properties of a thermostable chitinase from *Streptomyces thermoviolaceus* OPC-520. Appl Environ Microbiol 59:620–622

Turley CM (1993) Direct estimates of bacterial numbers in sea water samples without incurring cell loss due to sample storage. In: Kemp PF, Sherr BF, Sherr EB, Cole JJ (eds) Handbook of methods in aquatic microbial ecology. Lewis, Boca Raton, Fla., pp 143–154

Watanabe K, Ishibashi A, Ariga Y, Hashimoto M, Nikaidou N, Sugiyama J, Matsumoto T, Nonaka T (2001) FEBS Lett 494:74–78

Zeikus JG, Vieille C, Savehenko A (1998) Thermozymes: biotechnology and structure–function relationships. Extremophiles 2:179–183