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Evi Andronopoulou · Constantinos E. Vorgias

Multiple components and induction mechanism of the chitinolytic system of the hyperthermophilic archaeon *Thermococcus chitonophagus*

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Abstract *Thermococcus chitonophagus* produces several, cellular and extracellular chitinolytic enzymes following induction with various types of chitin and chitin oligomers, as well as cellulose. Factors affecting the anaerobic culture of this archaeon, such as optimal temperature, agitation speed and type of chitin, were investigated. A series of chitinases, co-isolated with the major, cell membrane-associated endochitinase (Chi70), and a periplasmic chitobiase (Chi90) were subsequently isolated. In addition, a distinct chitinolytic activity was detected in the culture supernatant and partially purified. This enzyme exhibited an apparent molecular mass of 50 kDa (Chi50) and was optimally active at 80°C and pH 6.0. Chi50 was classified as an exochitinase based on its ability to release chitobiose as the exclusive hydrolysis product of colloidal chitin. A multi-component enzymatic apparatus, consisting of an extracellular exochitinase (Chi50), a periplasmic chitobiase (Chi90) and at least one cell-membrane-anchored endochitinase (Chi70), seems to be sufficient for effective synergistic in vivo degradation of chitin. Induction with chitin stimulates the coordinated expression of a combination of chitinolytic enzymes exhibiting different specificities for polymeric chitin and its degradation products. Among all investigated potential inducers and nutrient substrates, colloidal chitin was the strongest inducer of chitinase synthesis, whereas the highest growth rate was obtained following the addition of yeast extract and/or peptone to the minimal, mineralic culture medium in the absence of chitin. In rich medium, chitin monomer acted as a repressor of total chitinolytic activity, indicating the presence of a negative feedback regulatory mechanism. Despite the undisputable fact that the multi-component chitinolytic system of this archaeon is strongly induced by chitin, it is clear that, even in the absence of any chitinous substrates, there is low-level, basal, constitutive production of chitinolytic enzymes, which can be attributed to the presence of traces of chito-oligosaccharides and other structurally related molecules (in the undefined, rich, non-inducing medium) that act as potential inducers of chitinolytic activity. The low, basal and constitutive levels of chitinase gene expression may be sufficient to initiate chitin degradation and to release soluble oligomers, which, in turn, induce chitinase synthesis.

Keywords Archaea · Hyperthermophiles · Chitinases · *Thermococcus chitonophagus*

Abbreviations SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis · FPLC: Fast protein liquid chromatography · TLC: Thin layer chromatography · DAPI: 4,6-Diamidino-2-phenylindole · 4-MU: 4-Methyl-umbelliferyl · pNP: *p*-nitrophenyl · NAG: *N*-acetyl-D-glucosamine · $(NAG)_2$: *N*,*N*′-diacetylchitobiose · $(NAG)_3$: *N*,*N*′-diacetylchitotriose · $(NAG)_4$: *N*,*N*′-diacetylchitotetraose · $(NAG)_5$: *N*,*N*′-diacetylchitopentaose · $(NAG)_6$: *N*,*N*′-diacetylchitohexaose · $(NAG)_7$: *N*,*N*′-diacetylchitoheptaose

Introduction

Chitin, designated “animal cellulose” in the early literature, is the major component of the exoskeleton of insects and crustacea, which accounts for its widespread distribution and natural abundance. For example, copepods, a single subclass of marine zooplankton, produce billions of tons of the polysaccharide annually, resulting in a continuous rain of chitin onto the ocean floor (Muzzarelli 1977; Gooday 1996). The ecological significance of this phenomenon was already recognised early in the previous century (Johnstone 1908; Zobel and Rittenberg 1937).

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In the aquatic biosphere alone, more than 10^{11} metric tons of chitin are produced annually (Lutz et al. 1994). If this enormous quantity of insoluble carbon and nitrogen was not converted to biologically useful material, the oceans would be depleted of these elements in a few decades. Despite continuous deposition of this highly insoluble polymer, marine sediments contain, in fact, only traces of chitin and its turnover is attributed primarily to marine bacteria able to live under extreme conditions and which are responsible for the bulk of chitin recycling (Muzzarelli 1977; Gooday 1996; Keyhani and Roseman 1999).

Public concern over the harmful effects of chemical pesticides on the environment and human health has stimulated a search for safer, environmentally friendly alternatives. Control of plant pests by the application of biological agents holds great promise as an alternative to the use of chemicals (Shigemasa and Minami 1996). In this light, bacterial chitinases are important biological control agents for countering fungal infections of plants, some of which are of major economic interest (Boller 1986; Chet et al. 1986; Herrera-Estrella and Chet 1999). Due to the importance of chitinolytic enzymes in the growth and development of insects, nematodes and fungi, chitinases have been the subject of particular attention regarding their development as biopesticides, chemical-defence proteins in transgenic plants, and as microbial biocontrol agents (Schickler et al. 1993; Gooday 1996; Kramer and Muthukrishnan 1997; Gooday 1999).

The ability of microorganisms or enzymes to hydrolyse or modify carbohydrates at elevated temperatures presents many strategic opportunities. For example, many natural polymers have low solubility at ambient temperatures and are therefore recalcitrant to enzymatic cleavage. Also, the highly viscous nature of natural polymer solutions is offset, to a great extent, as temperatures are raised, while enzymatic processing at high temperatures can also reduce the risk of contamination (Antranikian et al. 1995; Ladenstein and Antranikian 1998; Zeikus et al. 1998; Niehaus et al. 1999).

A large number of chitin-hydrolysing enzymes has been isolated from eukaryotes and bacteria, and their corresponding genes have been cloned and characterised (Collinge et al. 1993; Gaill et al. 1992; Haran et al. 1995; Muzzarelli 1997; Patil et al. 2000; Suzuki et al. 2001; Zhu et al. 2001). However, studies on thermophilic (Takayanagi et al. 1991; Tsujibo et al. 1993; Bharat and Hoondal 1998; Gomes et al. 2001), archaeal or hyperthermophilic chitinases have been limited, with the exception of the chitinase gene isolated and cloned from the hyperthermophilic archaeon *Pyrococcus kodakaraensis* KOD1 (Tanaka et al. 1999, 2001) and the hyperthermostable chitinase purified and characterised from the archaeon *Thermococcus chitonophagus* (Andronopoulou and Vorgias 2003). The latter is a novel hyperthermophilic anaerobic archaeon isolated from a deep-sea hydrothermal vent off the Mexican West coast at a depth of 2,600 m. It is the only archaeon known to degrade chitin for nutritional purposes (Huber et al. 1995).

In this work, we present data on the multiple components of the chitinolytic system of *T. chitonophagus* and on the inducing and repressing mechanisms regulating the production of chitinolytic enzymes by this archaeon. We also describe the isolation and partial characterisation of a new extracellular exochitinase from *T. chitonophagus*. This is the first report of a regulated, multi-component chitinoclastic enzyme system from Archaea and, more generally, from hyperthermophilic organisms.

Experimental procedures

Materials

T. chitonophagus (DSM 10152) was supplied from the German Collection of Microorganisms and Cell Cultures. Chitin and various substrates were purchased form Sigma. CM-chitin-RBV was from Loewe Biochemica, Germany. The column chromatography media were purchased from Pharmacia (Sweden) and Tosohaas (Japan). Proteinase inhibitors were from Boehringer (Germany), calibration-marker proteins from Pharmacia (Sweden) and polyacrylamide electrophoresis reagents from Serva (Germany). All TLC materials were purchased from Merck. All other chemicals were from Sigma or Merck and of the highest analytical grade.

Preparation of colloidal chitin

Colloidal chitin was prepared according to the method of Shimahara and Takiguchi (1988). Twenty grams of chitin powder were mixed with 200 ml of 12N hydrochloric acid and stirred for 2 h at 4°C. The suspension was poured into 4 l of ddH_2O and centrifuged at 10,000*g*, at 4°C, for 15 min. The resulting precipitate was washed with several litres of ddH_2O, until the pH reached 5.0 and then neutralised by addition of 5N KOH. The suspension was centrifuged as above and washed with 2 l of ddH_2O for desalting. The resulting precipitate was suspended in 200 ml ddH_2O to yield a final concentration of 10% (w/v) colloidal chitin, sterilized by autoclaving and stored at 4°C.

Growth conditions

The cells were grown anoxically, under nitrogen atmosphere, in a water bath, at 85°C and at pH 7.5, on elementary sulfur-supplemented complex medium containing the following components (per litre): 20 g NaCl, 4 g Na_2SO_4, 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 $MgCl_2{\cdot}6H_2O$, 1.5 g $CaCl_2{\cdot}2H_2O$, 0.03 g $SrCl_2{\cdot}2H_2O$ and 0.1 mg $NaWO_4$. The above minimal (mineralic) medium also included 0.01% (w/v) KH_2PO_4, 0.05% (v/v) vitamins mixture (Balch et al. 1979) and 0.0002% (w/v) rezasurin and was rendered anoxic by the addition of 0.02% (w/v) Na_2S. The nitrogen and carbon source for the above medium was 0.5% (w/v) colloidal chitin or untreated chitin (in flakes or powder) for the inducing conditions, and 0.5% (w/v) peptone and 0.1% (w/v) yeast extract for the non-inducing (rich) medium. Batch cultivation of *T. chitonophagus* was done in rubber-stoppered and aluminum-sealed, 50-ml serum flasks at 100 or 200 rpm agitation, according to the basic anoxic culture technique described by Hungate (1950) and modified by Miller and Wolin (1974).

To investigate optimum growth and/or inducing conditions, 5 mg of each of the following nutrients/ml minimal (mineralic) medium was added: tryptone, peptone, yeast extract, meat extract, casein, cellulose, chitosan, trehalose, maltose and glucose, as well as 50 μM of the potential inducers NAG, $(NAG)_2$ and $(NAG)_3$. Cell density was measured at 600 nm following removal of chitin and sulfur

particles and was verified by enumeration of the cells (see below), because traces of chitin in the growth medium prevented accurate determination of cellular growth phase based on estimation of biomass. Total chitinolytic activity in the clarified, soluble enzyme extract, prepared from each culture sample (Andronopoulou and Vorgias 2003), was measured at 405 nm with $pNP(NAG)_2$ as substrate and using the standard assay (see below).

Determination of cell number

Fixed cells taken from samples the different cultures were stained with 0.02 mg DAPI/ml and counted directly under an epifluorescent microscope (100-fold resolution), according to the procedure of Parsons et al. (1984) and Turley (1993).

Polyacrylamide gel electrophoresis and zymograms

Slab gel electrophoresis, using homogeneous polyacrylamide gels and discontinuous buffer systems, under denaturing and reducing conditions was done as described by Laemmli (1970). Electrophoresis was 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. Protein bands were made visible by staining with 0.25% (w/v) Coomassie R-250 (Neuhoff et al. 1988) or silver nitrate (Shevchenko et al. 1996). The marker proteins employed 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 α-lactalbumin (14.4 kDa).

To determine the chitinolytic activity using the in situ zymogram technique, SDS-PAGE was carried out as above using plates containing 0.7 mg CM-chitin-RBV/ml. Protein samples were prepared by boiling for 5 min in sample buffer [2% (w/v) SDS, 10% (v/v) glycerol and 1.25%(w/v) DTT in 62.5 mM Tris–HCl, pH 6.8]. Following electrophoresis, the gel was washed twice in ddH_2O for 15 min, SDS was optionally removed with two 30-min washes in casein-renaturation buffer according to the method of McGrew and Green (1990), and the gel was equilibrated in 100 mM Na–phosphate buffer, pH 7.0. Clear zones indicating chitinolytic activity appeared after overnight incubation at 70°C.

For in situ detection of chitobiase activity, a similar zymogram technique was applied, but in the absence of CM-Chitin-RBV and with polyacrylamide gels overlayed, following renaturation, with 0.4 mM 4-MU-NAG, in 1% w/v low-melt agarose and 100 mM Na–phosphate buffer, pH 7.0, as described by O'Brien and Colwell (1987). Following gelling of the agarose, the overlayed gel was incubated at 37°C overnight. The clear fluorescent band, indicating chitobiase activity, was detected at 365 nm with either a portable UV lamp or a UV transilluminator .

Standard assay of chitinase activity

Chitinase activity was routinely determined by measuring the release of *p*-nitrophenol and the subsequent increase in absorbance at 405 nm according to the procedure of Roberts and Selitrenikoff (1988). Chitinase was assayed in 50 mM Na–phosphate, pH 6.0 or 7.0, with 0.2 mM $pNP(NAG)_2$, at 70 or 80°C for 4 h in a total reaction volume of 50 µl. The reaction was stopped by cooling on ice, an equal volume of 1 M glycine–NaOH, pH 10.5, was added, and the absorbance was measured.

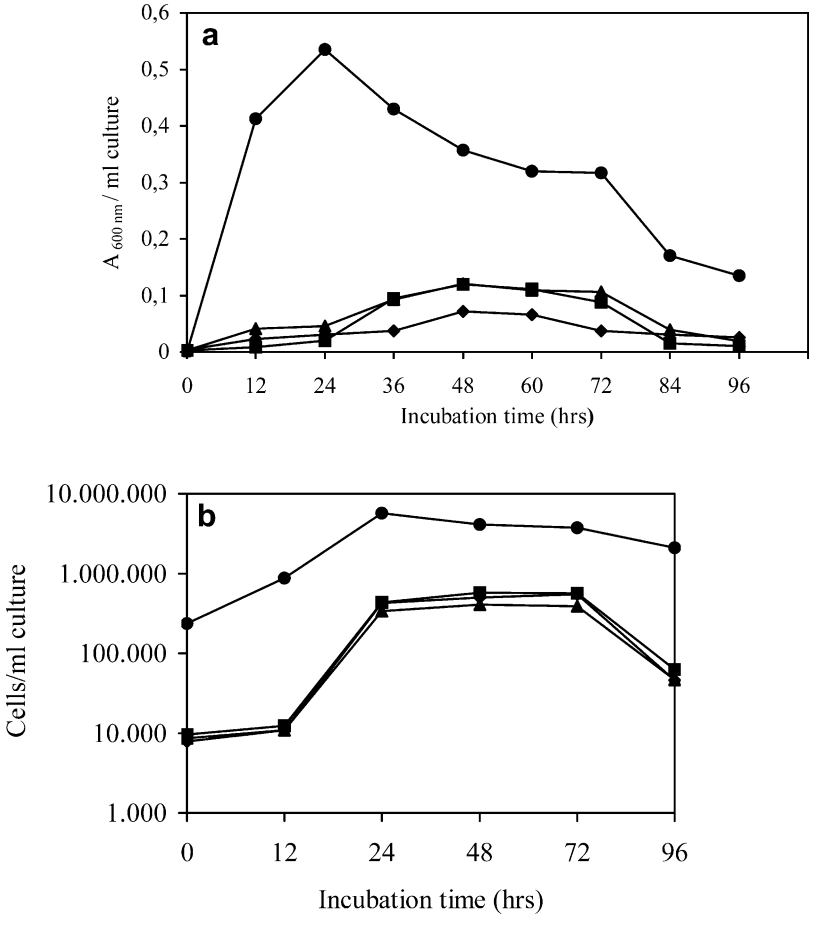

Fig. 1 **a** The cell density in the presence of various types of chitin and in rich, non-inducing medium in relation to cultivation time. The agitation speed was 200 rpm and the incubation temperature 85°C. *Filled circles* Rich, non-inducing medium, *filled diamonds* colloidal chitin, *filled squares* chitin powder, *open triangles* chitin flakes. **b** The cell titre in the presence of various types of chitin and in rich, non-inducing medium in relation to cultivation time. The agitation speed was 200 rpm and the incubation temperature 85°C. *Filled circles* rich, non-inducing medium, *filled squares* colloidal chitin, *filled diamonds* chitin powder, *open triangles* chitin flakes

Partial purification of Chi50

An anaerobic culture of *T. chitonophagus* in 800 ml of inducing medium was collected at the late stationary growth phase, filtered to remove sulfur and undigested chitin particles and centrifuged at 8,000*g* for 20 min at 4°C to harvest the cells. The supernatant was fractionated with solid ammonium sulfate. The protein fraction precipitating between 40 and 80% ammonium sulfate saturation was collected by centrifugation at 12,000*g* for 30 min at 4°C. The pellet was resuspended in 4 ml of 50 mM Na–phosphate, 1 M ammonium sulfate, pH 6.0, and supplemented with 1 mM EDTA, 0.1 mM PMSF and a cocktail of protease inhibitors. The solution was applied onto a Butyl-TSK-NPR, HR 5/5 (TosoHaas) column pre-equilibrated in 50 mM Na–phosphate, 1 M ammonium sulfate, pH 6.0. Elution of the Chi50 was carried out with a linear descending gradient between 1 and 0 M ammonium sulfate in the above buffer. Chi50 eluted at 100–0 M ammonium sulfate and the fractions were collected and pooled. The column was further washed and regenerated with a third ascending linear gradient between 0 and 40% (v/v) isopropanol in ddH_2O to remove various strongly bound proteins. The pooled fractions, containing the chitinase activity, were adjusted to pH 9.0 with 1 M ethanolamine–NaOH and directly applied onto a Mono Q, HR 5/5 (Pharmacia) column previously equilibrated in 20 mM ethanolamine–NaOH, pH 9.0. Bound proteins were separated using a linear ascending gradient between 0 and 1 M NaCl in the above buffer. Chi50, of approximately 80% purity, was eluted with 250–300 mM NaCl.

All chromatography columns were controlled using the FPLC system (Pharmacia) operating at 4°C and at a flow rate of 1 ml/min. Various protein-containing fractions were examined for chitinase activity using the standard enzyme assay and zymogram as described above.

Analysis of the hydrolysis products of colloidal chitin

Hydrolysis products, arising from the action of Chi50 on colloidal chitin, were analysed by TLC on Silica gel-60, aluminum sheets (Merck) according to the method of Tanaka et al. (1999). Aliquots (5 µl) of each reaction mixture were chromatographed 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 aniline–diphenylamine reagent (4 mM aniline, 4 g diphenylamine, 200 ml acetone and 30 ml 85% phosphoric acid) and baking it at 180°C for 5 min.

Results

T. chitonophagus was cultivated in batch cultures, under nitrogen, in two major types of complex medium, both based on the same minimal, mineralic medium and supplemented with elementary sulfur: (1) minimal, inducing medium, containing chitin or similar chitinous substrates as the exclusive carbon, nitrogen and energy source, and (2) rich, non-inducing medium, in which chitin was replaced by a variety of nutrient, non-chitinous substrates (i.e. peptone, yeast extract, tryptone, meat extract, casein). *T. chitonophagus* wagrew to high cell densities on complex proteinaceous substrates, such as yeast extract, meat extract, peptone and tryptone, whereas on chitin substrates the growth rate was considerably lower: the cell density and titre were five and ten times lower, respectively (Fig. 1a,b). No growth was observed on glucose, casein, chitosan and the β-linked glucosidic substrates maltose and trehalose. Growth was not influenced within a temperature range of 75–90°C. An agitation speed of 200 rpm was found to be optimal for growth in 50-ml serum flasks (data not shown).

Chitinolytic activity was highly induced in cells grown in minimal medium in the presence of 0.5% (w/v) colloidal chitin (Table 1 and Fig. 2a). Two chitin preparations were used: commercially available, untreated, crystalline chitin, in powder or flakes, isolated from crab shells (Sigma), and colloidal chitin prepared as described above (see "Experimental procedures"). Treated (colloidal) chitin was a more efficient inducer than chitin in the form of powder or flakes (crystalline chitin was a less active inducer due to its insolubility) (Table 1 and Fig. 2a). Induction of chitinolytic activity, albeit to lower levels, was achieved by the addition of cellulose, $(NAG)_3$ and $(NAG)_2$ in the same minimal mineralic medium (Table 1 and Fig. 2b). Low levels of total chitinolytic activity were also detected in the presence of non-inducing rich medium without chitin and containing basal salts and yeast extract, indicating constitutive expression of the chitinolytic genes (Table 1). The highest specific chitinolytic activity in the presence of colloidal chitin was six times higher than the respective activity in the presence of non-inducing rich medium. The untreated types of chitin (powder or flakes) yielded only half of the highest specific activity of colloidal chitin (Table 1). Chitin monomer (NAG), when added in peptone and yeast-extract-supplemented minimal medium (which does not support cell growth on its own), clearly acted as a repressor of total chitinolytic activity (Table 1 and Fig. 2b).

The initial production of total chitinolytic activity coincided with the onset of stationary phase, while the highest levels of chitinase activity were detected with the onset of autolysis (Figs. 1a, 2b).

In *T. chitonophagus*, only a part of the total chitinolytic activity (approximately 20%) is secreted into the medium, while most of the enzymatic activity is cell-associated.

Table 1 Growth of *Thermococcus chitonophagus* under various nutritional conditions that induce chitinolytic activity under anoxic conditions. Final concentrations of substrates or inducers, added to minimal medium, are shown in parentheses. The incubation times correspond to the time required for the maximum ratio values. The minimal (mineralic) medium was the same in all cases. The basal level of chitinolytic activity was obtained in the presence of meat extract

Substrate or nutrient (final concentrations in the medium)	Chitinolytic activity/cell density (A_{405}/A_{600} per ml culture)	Incubation time (h)
Colloidal chitin (5 mg/ml)	5.968	60
Chitin powder (5 mg/ml)	2.91	72
Chitin flakes (5 mg/ml)	2.00	72
Cellulose (5 mg/ml)	0.760	96
NAG (50 µM)	0.110	24
$(NAG)_2$ (50 µM)	0.262	24
$(NAG)_3$ (50 µM)	0.351	24
Yeast extract (5 mg/ml)	0.250	48
Meat extract (5 mg/ml)	0.202	48

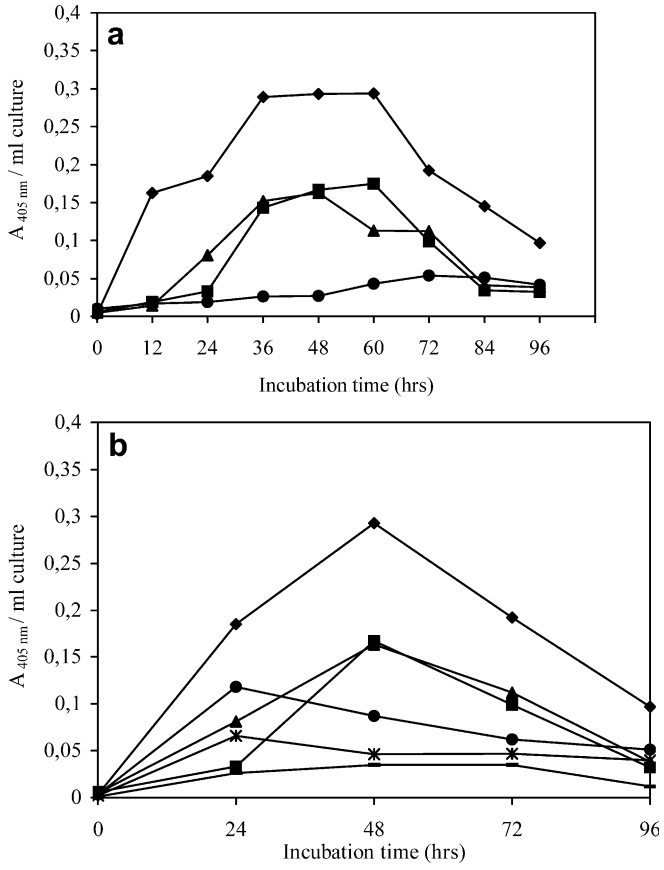

Fig. 2 **a** Total chitinolytic activity of clarified, soluble enzyme extract in the presence of either various types of chitin or in rich, non-inducing medium and the relation to cultivation time. The agitation speed was 200 rpm and the incubation temperature 85°C. *Filled circles* Rich, non-inducing medium, *filled diamonds* colloidal chitin, *filled squares* chitin powder, *open triangles* chitin flakes. **b** Total chitinolytic activity of the clarified, soluble enzyme extract in the presence of various chitinous substrates and in relation to cultivation time. The agitation speed was 200 rpm and the incubation temperature 85°C. *Filled diamonds* colloidal chitin, *filled squares* chitin powder, *open triangle* chitin flakes, *filled circle* $(ClcNAc)_3$, *cross marks* $(ClcNAc)_2$, *dotted line* GlcNAc

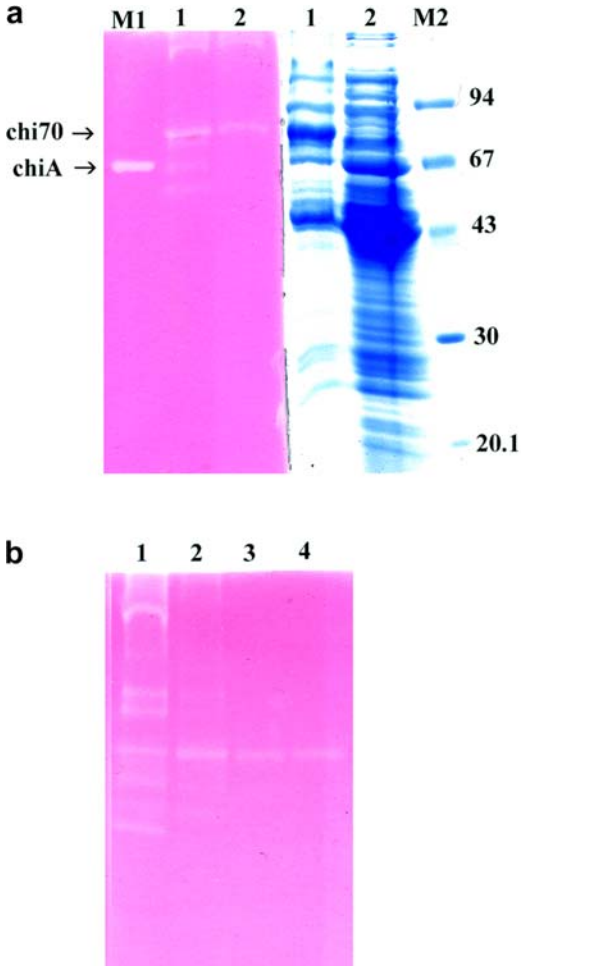

Fig. 3 **a** Analysis by 12% SDS-PAGE (followed by Coomassie staining) and zymogram of proteins in the clarified, soluble enzyme extract prepared from a colloidal chitin-induced (*lane 1*) and a non-induced (*lane 2*) culture . *Lane M1* ChitinaseA from *Serratia marcescens* (61 kDa), used as positive control for the zymogram; *lane M2* molecular mass markers in kDa. **b** Zymogram analysis by 10% SDS-PAGE, of proteins in the clarified, soluble enzyme extract from a culture induced with colloidal chitin (*lane 1*), chitin powder (*lane 2*), or chitin flakes (*lane 3*) and a non-induced culture in rich medium (*lane 4*)

Zymogram analysis of the clarified-membrane extract from induced cultures clearly indicated the presence of multiple bands of chitinolytic activity that co-isolated with the major, membrane chitinase Chi70 (Andronopoulou and Vorgias 2003), suggesting the presence of more than one chitinases or chitinase-isoforms associated with the cell membrane of *T. chitonophagus*. The same analysis, using extracts from non-induced cultures, also demonstrated the presence of multiple chitinolytic enzymes, indicating a constitutive low-level expression of the *T. chitonophagus* chitinolytic gene system, even in the absence of chitin or chitin oligomers in the culture medium (Figs. 3, 4).

The optimum batch-culture conditions for chitinase production were determined by cultivation at 85°C, for 60 h, at 200 rpm in the presence of 0.5% (w/v) colloidal chitin as the sole carbon, nitrogen and energy source . Under these conditions, several distinct chitinolytic

Table 2 Summary of the multiple components of the chitinoclastic system of *T. chitonophagus*. One unit of chitinolytic activity was defined as the amount of enzyme that produces 1 μmol *p*-nitrophenol per minute under the standard assay conditions. N/A, not available

Chitinolytic enzyme	Mode of action	Subcellular location	Specific activity (units/mg protein)
Chi70	Endochitinase	Cell membrane	3
Chi50	Exochitinase	Extracellular (secreted)	~1.2
Chi90	Chitobiase	Periplasm	N/A

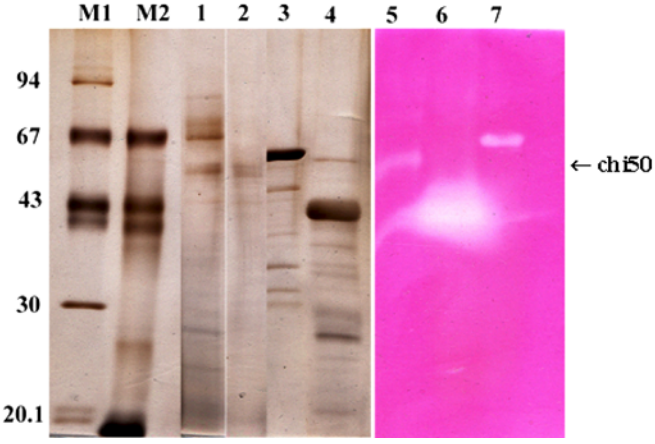

Fig. 4 Analysis of peak fractions from the various Chi50 purification steps by 12% SDS-PAGE. *Lanes M1–4* 12% SDS-PAGE followed by silver-staining, *lanes 5–7* zymogram, containing 0.7 mg/ml CM-chitin-RBV, *lanes M1 and M2* molecular mass markers, as indicated in kDa, *lane 1* peak Chi50 fraction eluted from the TSK-butyl column, *lanes 2 and 5* peak Chi50 fraction eluted from the Mono Q column, *lanes 3 and 7* chitinaseA from *Serratia marcescens* (61 kDa), *lanes 4 and 6* chitinase Chi40 (40 kDa) from *Trichoderma harzianum*. The latter two proteins were used as positive controls for the zymogram and to estimate the molecular mass of Chi50

activities weredetected, including two distinct chitinases and a chitobiase, produced by *T. chitonophagus*. More specifically, a cell-membrane-associated chitinase (Chi70), an extracellular chitinase secreted into the medium (Chi50) and a periplasmic chitobiase (Chi90) most probably associated with the outer face of the cell membrane or the inner face of the cell wall were found (Table 2).

Chi70 was isolated from the cell membrane extract of *T. chitonophagus* and is the first native (non-recombinant) chitinase from an archaeon purified to homogeneity and biochemically characterised. The enzyme has been classified as an endochitinase with a low substrate specificity that hydrolyses a variety of chitinous and non-chitinous, polymeric and oligomeric, natural and synthetic substrates, including cellulose. Furthermore, it seems to be the major component of the multiple chitinolytic system of this archaeon (Andronopoulou and Vorgias 2003).

A distinct chitinolytic activity was detected in the culture supernatant and isolated following differential fractionation with ammonium sulfate and subsequent chromatographic purification, as described in "Experimental procedures". The secreted chitinase (Chi50) was partially purified and the apparent molecular mass was determined by SDS-PAGE at 50 kDa (Fig. 5). Further purification to homogeneity has not been unfeasible due to low protein concentration and enzyme activity (Table 2). It should be noted that Chi50 is the only chitinolytic activity detectable in the culture supernatant. The optimum pH and temperature values, in the presence of 0.2 mM pNP$(NAG)_2$, were found to be 6.0 and 80°C, respectively. No activity was detected in the presence of 0.2 mM pNP-NAG (data not shown). TLC analysis of the degradation products of colloidal chitin, following incubation for 15 min to 48 h with the enzyme, showed that Chi50 cleaves exclusively chitobiose $[(GlcNAc)_2]$ from $(GlcNAc)_n$. Chitobiose is the only detectable molecule (no other monosaccharides or oligosaccharides were detected) and only after extended incubation (no degradation products were found at incubation times shorter than 12 h). The latter observation confirms the inherent low activity of the enzyme. The combined results of the pNP assays and TLC analyses indicate that Chi50 possesses an exo-*N*,*N*′-diacetylchitobiohydrolase activity.

Total chitobiase activity was detected in intact cells, in the absence of any traces of medium, using pNP-NAG as substrate and was visualised on chitobiase-zymograms of whole cell-lysates (in SDS-sample buffer) as a distinct band of approximately 90 kDa (Chi90) (data not shown). Chitobiase activity was, however, undetectable (using pNP-NAG) in the cell membrane extract, cytosolic fraction, or culture supernatant, suggesting its probable association with the periplasmic space, between the cell membrane and the cell wall, or on the cell wall of the archaeon.

Discussion

Chitinolytic enzymes are divided into three principal types: (1) Endochitinases (E.C. 3.2.1.14) are defined as enzymes catalysing the random hydrolysis of 1,4-β-linkages of GlcNAc at internal sites, over the entire length of the chitin microfibrils. The products of this reaction are soluble, low-molecular-mass multimers of GlcNAc, such as chitotetraose and chitotriose, with the dimer diacetylchitobiose being predominant. (2) Exochitinases, also termed chitobiosidases, or chitin-1,4-β-chitobiosidases, catalyse the progressive release of diacetylchitobiose units in a stepwise fashion from the non-reducing end of the chitin chain, so that no monosaccharides or oligosaccharides are formed. (3) *N*-acetyl-β-1,4-D-glucosaminidases, or β-*N*-acetyl hexosaminidases (E.C. 3.2.1.52), are chitinolytic enzymes that also act in an exo-splitting mode on diacetylchitobiose and higher analogues of chitin, including chitotriose and chitotetraose, and cleave off GlcNAc monomers from the non-reducing end of the molecule; this definition inherently includes the chitobiase

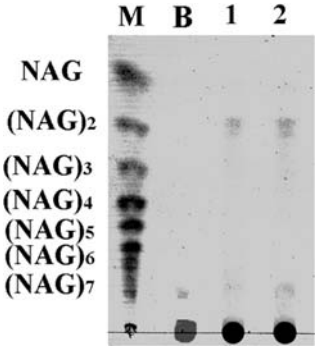

Fig. 5 TLC analysis of the hydrolysis products of colloidal chitin following cleavage by Chi50. The enzyme was incubated with the substrate for 12 h (*lane 1*) and 24 h (*lane 2*), at 80°C. *Lane B* Negative control (substrate without enzyme), *lane M* markers

activity that specifically hydrolyses diacetylchitobiose, forming GlcNAc monomers.

T. chitonophagus produces several chitinolytic enzymes, cellular and extracellular, when grown in medium containing colloidal chitin as the exclusive nitrogen and carbon source. At least three glucosidases convert chitin oligosaccharides into products that can be translocated across the *T. chitonophagus* cytoplasmic membrane. The role of the secreted exochitinase Chi50 is to remove chitobiose units from the chitin microfibril, whereas the cell-membrane-anchored endochitinase Chi70 digests the chitin chain and the shorter GlcNAc multimers (produced by Chi50) into tetramers, trimers and predominantly dimers (Andronopoulou and Vorgias 2003). The third chitinolytic enzyme, the chitobiase Chi90 (undetectable either in the cytosolic and cell-membrane fractions or in the extracellular fraction), is most probably located in the periplasm or the cell wall facing the outside of the cell where its role is most probably to convert the chitobiose molecules—produced by Chi50 and Chi70—into GlcNAc monomers. GlcNAc monomer, chitobiose and small oligomeric molecules are subsequently able to enter the periplasm and the cell membrane, thereby constituting a nutrient source for the cell. On the basis of the present study, it seems that a multi-component enzymatic apparatus, consisting of an extracellular exochitinase (Chi50), a periplasmic chitobiase (Chi90) and at least one cell-membrane-anchored endochitinase (Chi70), are sufficient for effective in vivo degradation of chitin. Therefore, the above results confirm that a multiple system of chitinases in a single chitinolytic bacterium is required for efficient synergistic degradation of chitin.

As was reported in fungi and other microorganisms, chitinase gene expression seems to be controlled by a repressor/inducer system in which chitin or other products of degradation act as inducers (Ulhoa and Peberdy 1991; Felse and Panda 1999; Keyhani and Roseman 1999). In *T. chitonophagus*, both colloidal chitin and the low-molecular-weight breakdown products of chitin were found to be necessary for the induction of chitinolytic enzyme production. Induction with high-molecular-weight colloidal chitin remained, however, an absolute requirement for high expression of chitinolytic activity, even in the presence of substrate-level inducers. It is significant that induction with chitin stimulates the coordinated expression of a combination of chitinolytic enzymes exhibiting different specificities for the polymeric chitin and its degradation products.

High-level chitinase activity was found only in cultures supplied with chitin, but not with cellulose, chitosan, or chitobiose. GlcNAc (as well as glucose) repressed chitinase synthesis, thus preventing, wasteful synthesis of the enzyme in the presence of abundant quantities of the ultimate product of chitin hydrolysis as well as indicating regulation by a negative feedback mechanism. Similar induction-repression mechanisms have been identified in *Trichoderma harzianum*, in which both glucose and GlcNAc repressed the synthesis of induced chitinase (Ulhoa and Peberdy 1991), and in *Metarhizium anisopliae*, in which chitinase synthesis was repressed by the addition of alanine, a readily utilized nutrient source (St Leger et al. 1993).

The induction levels of the chitinases were found to be dependent upon whether cells were incubated with purified colloidal chitin or a crude preparation of crystalline chitin. This implies that *T. chitonophagus* can detect differing configurations of chitin and perhaps cross-linking of the polysaccharide to other polymers in the growth medium. The colloidal chitin used in our experiments was prepared by partial hydrolysis with 12N HCl and sterilised by autoclaving. Under these conditions, various chito-oligosaccharides, known inducers for the synthesis of chitinase, are released. In previous studies, colloidal chitin prepared by this technique was shown to induce a wide spectrum of chitinolytic enzymes, including *N*-acetyl-glucosaminidase, endochitinase and chitobiosidase, in the bacterial species *Aeromonas caviae* (Inbar and Chet 1991), *Enterobacter agglomerans* (Chernin et al. 1995, 1997a) and *Bacillus cereus* (Pleban et al. 1997), and in the fungal species *T. harzianum* (Haran et al. 1995) and *Hirshutella* sp. (Chernin et al. 1997b).

Low-level constitutive expression of chitinolytic genes was detected even in the absence of any chitinous inducers. A probable explanation is that the presence of chito-oligosaccharides, or structurally similar molecules, in the yeast extract and peptone-containing non-inducing medium act as potential inducers of chitinolytic activity. Partial degradation of the chitinous material in the surrounding environment, by low levels of chitinase, secreted constitutively, will yield a range of oligomers; these may stimulate transcription of chitinase genes (by interacting with receptors in the cell membrane) and subsequently induce chitinase synthesis. It is known that trace quantities of constitutively expressed chitinolytic enzymes, continuously released even under starved conditions, may be sufficient to initiate chitin degradation and release soluble oligomers, which subsequently act as inducers of chitinase synthesis (Felse and Panda 1999). This observation can be correlated with the fact that the cell-membrane-associated endochitinase Chi70 displays low substrate specificity and is able to hydrolyse a broad range of substrates (Andronopoulou and Vorgias 2003).

The extensive multi-component chitinolytic system of *T. chitonophagus* provides an interesting model for further studies of chitinase induction and repression, phenomena that are important in plant defence, entomo-parasitism and myco-parasitism as well as in the production of bacterial and fungal chitinases that enable biodegradation of chitin in the environment.

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