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Isolation, cloning, and overexpression of a chitinase gene fragment from the hyperthermophilic archaeon *Thermococcus chitonophagus*: semi-denaturing purification of the recombinant peptide and investigation of its relation with other chitinases☆

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Abstract

A 189-bp sequence was isolated from the hyperthermophilic archaeon *Thermococcus chitonophagus* and was found to present strong homology with a large number of chitinase genes from a variety of organisms and particularly with the chitinaseA gene from *Pyrococcus kodakaraensis* (Pk-chiA). This fragment was subcloned to an expression vector and overexpressed in *Escherichia coli*. The *E. coli* BLR21(DE3)pLysS transformant, harbouring the gene on the pET-31b plasmid vector, was found to overproduce the target protein at high levels. The 63 aminoacid-long peptide was efficiently purified to homogeneity, with a one-step, semi-denaturing affinity chromatography, on a metal chelation resin and was used for the production of a specific, polyclonal antibody from rabbits. The produced antibody was demonstrated to display strong and specific affinity for the chitinase A from *Serratia marcescens* (Sm-chiA), as well as, the membrane-bound chitinase70 from *Thermococcus chitonophagus* (Tc-Chi70). The strong sequence homology, in combination with the demonstrated specific immunochemical affinity, indicates that the isolated peptide is part of a chitinolytic enzyme of *T. chitonophagus*. In particular, it could belong to the membrane-bound chi70, or to a distinct chitinase, coded by a different gene, or even by the same gene, following post-transcriptional or post-translational modifications.

Keywords: Archaea; Hyperthermophiles; Chitinases; *Thermococcus chitonophagus*; Cloning; Purification

Chitin, a polysaccharide also designated "animal cellulose," constitutes the major component of insect and crustacean exoskeletons, while the wide distribution of these organisms in the marine ecosystems explains the great natural abundance of chitin. It has been estimated that only in the aquatic biosphere, more than 10^{11} metric tons of chitin are produced every year, resulting in a continuous "chitin rain" on the ocean floor [1]. However, despite the continuous deposition of enormous quantities of this insoluble source of carbon and nitrogen, only traces of chitin are found in the marine sediments, due to the rapid turnover of the polysaccharide by some abundant marine bacteria. These organisms and their chitinolytic enzymes are responsible for the effective conversion of the insoluble, polymeric chitin into useful and soluble sources of carbon and nitrogen [2,3].

In addition, the bacterial chitinases constitute an interesting means of biological control for commercially important plants, as an environmentally safer alternative, compared to harmful chemical pesticides [4–7]. On the other hand, the thermostable chitinolytic enzymes are able to hydrolyze or modify chitinous substrates at elevated temperatures and exhibit important advantages, against their mesophilic counterparts, e.g., the thermal and chemical stability, as well as, the reduction of viscosity, the increased solubility and the significant decrease of the contamination risk [8–10].

☆ The GenBank Accession number for the chitinase fragment from *T. chitonophagus* is AY438583.

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While a large number of mesophilic chitin-hydrolyzing enzymes have been isolated and cloned from eukaryotes and bacteria, the studies on thermophilic [11–14] and furthermore on archaeal or hyperthermophilic chitinases have been limited, with the exception of the chitinase gene (Pk-chiA), isolated and cloned from the hyperthermophilic archaeon *Pyrococcus kodakaraensis* KOD1 [15,16], and the hyperthermostable chitinase (Tc-Chi70), purified and characterized from the hyperthermophilic archaeon *Thermococcus chitonophagus* [17].

Thermococcus 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 the first archaeon discovered to degrade chitin for nutritional purposes [18].

In this work, we present data on the partial isolation and cloning of a potentially new chitinase gene from the above archaeon. This gene fragment was overexpressed in *Escherichia coli* and the respective peptide was purified to homogeneity. It was found to be related to various chitinolytic enzymes from mesophilic (eubacteria) and hypethermophilic (archaea) organisms, even though lacking chitinolytic activity.

Experimental procedures

Materials

Thermococcus chitonophagus (DSM 10152) was supplied from the German Collection of Microorganisms and Cell Cultures. The *E. coli* strains XL1BlueMRF′ and BLR21(DE3)pLysS were supplied from Stratagene and Novagen, respectively. The λZapII cloning kit and Gigapack III Gold packaging extract were purchased from Stratagene (La Jolla, CA) and the pCR2.1 vector was from Invitrogen. The T4 DNA ligase and restriction enzymes were supplied from New England Biolabs. Chitin and various substrates were available from Sigma. CM-chitin-RBV was from Loewe Biochemica, Germany. The pET-31b vector and the Ni-His-Bind chromatography resin were purchased from Novagen. Marker proteins were from Pharmacia (Sweden) and polyacrylamide electrophoresis reagents were from Serva (Germany). The secondary antibody and the NBT-BCIP detection reagent were supplied from Pierce. All other chemicals and reagents were from Sigma or Merck in the highest analytical grade.

Anaerobic growth of T. chitonophagus

The cells were grown anaerobically, under nitrogen atmosphere, in a water bath, at 85 °C and pH 7.5, for 30 h, on elementary sulfur-supplemented complex medium containing the following components per liter: 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 $MgCl_2 \cdot 6\,H_2O$, 1.5 g $CaCl_2 \cdot 2\,H_2O$, 0.03 g $SrCl_2 \cdot 2\,H_2O$, 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 [19], 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) peptone and 0.1% (w/v) yeast extract. Batch cultivation of *T. chitonophagus* was performed in rubber-stoppered and aluminum-sealed, 50 ml-serum flasks at 200 rpm agitation, according to the basic anaerobic culture technique described by Hungate [20] and modified by Miller and Wolin [21].

Construction and screening of genomic libraries. Cloning of the chitinase gene fragment

Genomic DNA was routinely isolated from fully grown (30 h), anaerobic batch (50 ml) cultures of *T. chitonophagus*, using the Qiagen midi preparation kit, then partially digested with *Eco*RI (0.7 U/μg DNA), and electrophoresed on a 0.6% w/v agarose gel. The restriction fragments, of size between 4 and 6 kb, were subsequently collected by electroelution. These *Eco*RI-fragments were ligated into the dephosphorylated *Eco*RI site of the λZapII vector, by using T4 DNA ligase, according to the manufacturer's protocol. Recombinant λ-phage DNA was packaged using Gigapack III gold packaging extract and the titer and fraction of phage containing inserts were determined by plaque assay with blue–white color selection. The library was amplified, following the procedure described by the manufacturer and mass-excised, using the ExAssist helper phage (Stratagene), to produce phagemids in pBluescriptSK.

The phage and phagemid libraries were screened for genes encoding proteins that hydrolyze 4MU-$(NAG)_2$,[1] a fluorogenic analogue of chitin, using the overlay technique [22,23]. The plates, containing fully grown colonies or plaques, were overlaid with 0.4 mg/ml of 4MU-$(NAG)_2$ in 1% low melt agarose, 100 mM Tris–HCl, pH 7.0, and incubated at 50–60 °C for approximately 1 week. Fluorescent spots (putative positive clones) were detected with a portable UV illluminator, at 365 nm.

The plasmid transformants were, also, screened on plates, containing 2% w/v agar, 0.25% w/v yeast extract, and 1 mg/ml CM-chitin-RBV. Colonies forming clear

[1] *Abbreviations used:* CM-chitin-RBV, carboxymethyl-chitin-rubidium brilliant violet; CNBr, cyanogen bromide; Gu-HCl, Guanidine-hydrochloride; LB, Luria–Bertani broth; OD, optical density; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; NAG, *N*-acetyl-D-glucosamine; 4-MU, 4-methyl-umbelliferyl; 4-MU-$(NAG)_2$, 4-methyl-umbelliferyl-β-1,4-*N*,*N*′-diacetyl-chitobiose; pNP, *p*-nitrophenyl; pNP-$(NAG)_2$, *p*-nitrophenyl-β-1,4-*N*,*N*′-diacetyl-chitobiose.

holes indicated putative clones, containing hybrid plasmids with genomic inserts, coding for chitinase activity.

The PCR-screening of the selected phagemid libraries was carried out with the following specific primers, derived from the sequence alignment of the Sm-chiA and the Pk-chiA chitinase genes:

5′-TCAGTCGGCGGATGGACTCTCAGC-3′

5′-GCTGAGAGTCCATCCGCCGACTGA-3′

All subcloning procedures to the pCR2.1 vector, for sequencing purposes, as well as, to the pET-31b vector, for expression and purification, were carried out as described in Sambrook et al. [24], or according to the manufacturer's instructions.

Overexpression of the chitinase gene fragment

Escherichia coli BLR(DE3)pLysS cells, carrying the cloned peptide gene, were grown overnight with aeration, at 37 °C, in LB medium, supplemented with 50 μg/ml carbenicillin and 34 μg/ml chloramphenicol. Next day, the cells were diluted 50-fold and grown as above to $OD_{600\,nm}$ of ~0.4. Expression was induced with 1 mM IPTG and the cells were grown to $OD_{600\,nm}$ of ~1.8. The cells were harvested by centrifugation at 5000*g*, 4 °C, for 15 min and washed once with 50 mM Tris–HCl, pH 7.9. The cell pellet was either stored at −70 °C or used immediately.

Peptide purification

All further procedures were carried out at 0–4 °C, except otherwise specified. For routine protein preparation, 2-L cultures were used. The bacterial paste was resuspended in 200 ml of 40 mM Tris–HCl, pH 7.9, 0.5 M NaCl, and 5 mM imidazole and the cells were disrupted by sonication, in an ice-bath, to elimination of viscosity. The total cell lysate was centrifuged at 12,000*g*, 4 °C, for 20 min and the supernatant was discarded. The pellet was resuspended in 200 ml of 40 mM Tris–HCl, pH 7.9, 0.5 M NaCl, 5 mM imidazole, and 6 M Gu-HCl, to solubilize the dense inclusion bodies. The resolubilized pellet was applied on a Ni-His-Bind column (25 ml of 50% slurry), previously equilibrated in 40 mM Tris–HCl, pH 7.9, 0.5 M NaCl, 5 mM imidazole, and 6 M Gu-HCl. All chromatographic steps were carried out at a flow rate of 0.25 ml/min, except otherwise specified. The column was washed first with 150 ml of 40 mM Tris–HCl, pH 7.9, 0.5 M NaCl, 5 mM imidazole, and 6 M Gu-HCl, followed by a second wash with 200 ml of 40 mM Tris–HCl, pH 7.9, 0.5 M NaCl, 16 mM imidazole, and 6 M Gu-HCl. The fused peptide was eluted from the column in 150 ml of 40 mM Tris–HCl, pH 7.9, 0.5 M NaCl, and 0.3 M imidazole, in the absence of denaturant (Gu-HCl or urea) and at a higher flow rate (0.5 ml/min). Fractions containing the fused peptide were readily visible due to precipitation of the insoluble product in the eluate's tube and samples from foggy fractions (containing the precipitate) were analyzed by Tris–Tricine–SDS–PAGE, following solubilization with 8 M urea. The fractions that contained the purified fused peptide were collected, pooled, and centrifuged at 8000*g*, 4 °C, for 20 min to precipitate the insoluble complex. The precipitate was resuspended in 60 ml of 80% formic acid, with gentle agitation in the hood, and subsequently 2 g of CNBr was carefully added, followed by a quick N_2-flushing on the surface of the solution. The flask was sealed with parafilm, wrapped in aluminum foil, and the solution was incubated with continuous stirring, in the hood, at room temperature, for 22–24 h. Next day, the formic acid-CNBr was allowed to evaporate in a rotary evaporator, inside the hood. The gelatinous material was resuspended in 5 ml of 20 mM KPO_4–$NaHCO_3$, 0.1 M NaCl, pH 7.4, and the mixture was stirred overnight, at room temperature. The suspension was centrifuged at 8000*g*, 4 °C, for 20 min and finally the supernatant, containing the purified, soluble peptide, was transferred to a fresh tube, concentrated to half the original volume, and stored at 4 °C, until used.

Polyacrylamide gel electrophoresis

Slab gel electrophoresis, using homogeneous polyacrylamide gels and discontinuous buffer systems, under denaturing and reducing conditions, was performed as described by Laemmli [25]. Electrophoresis was routinely carried out on 1.5 mm-thick, 15% (w/v) polyacrylamide gels, containing 0.1% (w/v) SDS, at a constant current of 30 mA, for 2 h, at room temperature. Peptide electrophoresis was performed on 1 mm-thick, 16.5% w/v, discontinuous polyacrylamide gels, using the Tris–Tricine buffer system, under denaturing and reducing conditions and at a constant current of 20 mA, for 2.5 h, at room temperature [26].

Protein bands were made visible by staining with 0.25% (w/v) Coomassie R-250 [27]. The marker proteins employed to calibrate the gels 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).

Protein determination

Protein concentration was determined using the Coomassie dye binding assay (Pierce) according to Bradford [28], with bovine serum albumin (Pierce) as standard.

Production of polyclonal, monospecific antibody

Antibody to the recombinant peptide was produced in two rabbits by immunization with 100 μg of purified antigen, emulsified in Freund's complete adjuvant. Anti-

gens were administered by subcutaneous injection on each of 6 occasions, at intervals of 2 weeks. Rabbits were bled 8 days after each boost. Specificity and titer of the antibodies was tested by ELISA and Western blot.

ELISA test

The test was performed on microwell plates with pre-adsorbed purified peptide (0.5 μg/well), according to Voller et al. [29]. Anti-serum, following each bleed, was added at 1:50, 1:100, 1:500, 1:1000, 1:1500, and 1:2000 dilutions in PBS and incubated overnight, at 4 °C, with gentle agitation. The phosphatase-labelled secondary antibody was used at 1:5000 dilution and incubated for 2 h, at room temperature. Detection was performed with 10 mg/ml pNP and the absorbance was measured on a plate reader at 405 nm.

Western blot

Proteins were analyzed on 15% w/v polyacrylamide gels, under denaturing and reducing conditions and subsequently transferred onto nitrocellulose membrane, in 25 mM Tris–HCl, pH 8.3, 0.15 M glycine, and 20% v/v methanol, at a constant current of 100 mA, for 2 h, at 4 °C, according to Towbin et al. [30]. Proteins were incubated on the membrane, for 2 h, with immunized serum at 1:1000 dilution and with the phosphatase-labelled secondary antibody at 1:5000 dilution. Detection was performed with the BCIP-NBT reagent, in 0.1 M Tris–HCl, pH 9.5, 0.1 M NaCl.

Standard assay of chitinase activity

Chitinase activity was determined by measuring the release of *p*-nitrophenol and the subsequent increase in absorbance at 405 nm, according to Roberts and Selitrenikoff [31]. 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, containing 0.8 μg of purified protein. The absorbance was measured after the reaction was stopped by cooling on ice and adding equal volume of 1 M glycine–NaOH, pH 10.5.

Results and discussion

A high-efficiency λ-phage cloning vector (λZapII) was used to produce genomic libraries from the archaeon *T. chitonophagus*. It is well known that the phage vector λ constitutes the preferred cloning system for many hyperthermophilic archaea, since it presents high cloning efficiency, while the broad range of fragments of insert DNA (up to 12 kb) provides sufficient representation of the typical archaeal genome [32,33].

The phage, as well as, phagemid libraries were screened with specific and sensitive substrates, such as the fluorogenic analogue of chitin (4MU-$(NAG)_2$) and the soluble dye-labelled form of chitin (CM-chitin-RBV), to identify chitinase genes. Detection of a cloned chitinase by expression requires cloning of a native promoter with the chitinase gene, or in the case this is not applicable, the alignment of the cloned gene with the reading frame of the lacZ promoter on the vector. The phagemid (plasmid) libraries allow the expression of the cloned genes to higher levels and for prolonged time periods, in comparison with the phage libraries [34,35]. This is particularly useful in the case of the functional screening of the library, using specific and sensitive substrates.

Two of the prepared phagemid libraries were found to contain putative positive clones for chitinolytic enzymes, after two rounds of functional screening. Due to technical problems, the recovery of these clones was proved unfeasible. These two plasmid libraries were selected for further screening by PCR, employing specific primers that resulted from the alignment of the chiA genes from the mesophilic eubacterium *Serratia marcescens* and the hyperthemophilic archaeon *Pyrococcus kodakaraensis*. The resulting DNA fragment was subcloned into the pCR2.1 plasmid vector and sequenced in both directions (Fig. 1).

This gene fragment showed significant homology—despite the short length of the overlapping sequence—with a large number of chitinase genes, isolated from a variety of organisms, including archaea, bacteria, fungi, plants, nematodes, and insects. Fig. 2 presents the short but highly conserved regions, shared by three sequences: the Sm-chiA, the PK-chiA, and the isolated peptide. The conserved region spans the active site of both chitinases, which is the typical family 18-chitinase active site [36] and includes the highly conserved glutamate, involved in the catalytic mechanism and probably acting as a proton donor [37]. This glutamate is located at the extremity of the best conserved region of the family 18-chitinases. It should be emphasized about the high degree of identity

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5'- GTC GGC GGA TGG ACT CTC AGC AAG TAC TTC TCA
     V   G   G   W   T   L   S   K   Y   F   S
    GTA ATA GCT GCT GAT CCA GCA AAG AGG CAA AGG
     V   I   A   A   D   P   A   K   R   Q   R
    TTT GCT GAA ACT GCA CTA GAA ATA ATC AGG AAG
     F   A   E   T   A   L   E   I   I   R   K
    TAC GAT CTC GAT GGT GTG GAT ATT GAT TGG GAG
     Y   D   L   D   G   V   D   I   D   W   E
    TAT CCA GGC GGC GGT GGA ATC GAG GGC AAC TAC
     Y   P   G   G   G   G   M   E   G   N   Y
    GTT AGT CCC GAC GAC GGT AAG AAC - 3'
     V   S   P   D   D   G   K   N
```

Fig. 1. The sequence of the isolated chitinase gene fragment.

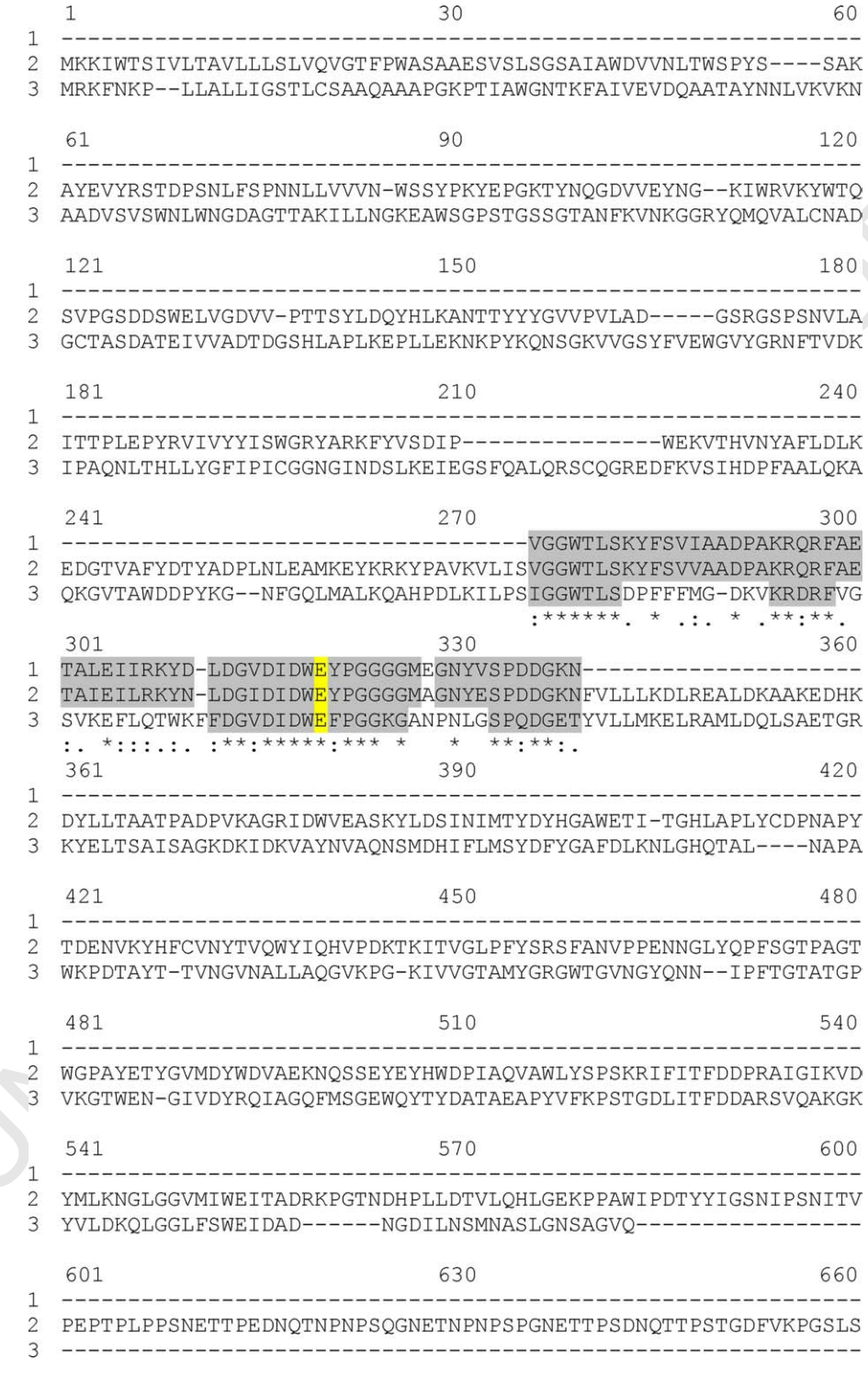

Fig. 2. Multiple sequence alignment of the isolated chitinase fragment and two selected and well-studied chitinases: (1) the 63 aa-peptide; (2) the Pk-chiA, 1215 aa; and (3) the Sm-chiA, 563 aa. The highlighted regions illustrate the well-conserved domains. The yellow color indicates the highly conserved residue of the active site (glutamate), which plays a key role in the catalytic mechanism. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

(88%) of the peptide with the Pk-chiA and the somewhat lower, but still significant, identity (47%) with the Sm-chiA. It is evident that the two chitinases (the peptide and the Pk-chiA), both isolated from hyperthermophilc archaea, classified in the same family (Thermococcales) are closely related molecules.

The 189-bp fragment was subcloned, fused with an insoluble peptide tag, in the dephosphorylated, *Alw*NI

restriction site of the expression vector pET-31b. It was overexpressed at high levels (approximately 10% of the total bacterial protein) in the *E. coli* strain BLR(DE3)pLysS. The purification of the fused peptide, from inclusion bodies, was performed with His-bind chromatography, under semi-denaturing conditions, following a simplified protocol that enabled fast recovery of the final soluble peptide. Specifically, the omission of the solubilizing reagent (Gu-HCl or urea), in combination with the higher flow rate during the elution step, allowed the quantitative elution of the fusion construct from the column (without any precipitation on the column) and the simultaneous aggregation in the eluate tubes, which was visible as fogginess. Hence, the eluate fractions, containing the peptide fused with the insoluble partner, were readily distinguished. Furthermore, the time-consuming dialysis step, to remove the solubilizing reagent (Gu-HCl), was no longer required. Following a quick analysis of the eluate fractions, by SDS–PAGE in Tris–Tricine buffer system, the fractions of the highest purity were collected, to proceed directly to chemical cleavage of the insoluble tag. The release of the soluble peptide, by CNBr–formic acid cleavage of the produced fusion protein, was quantitative. The 7 kDa, 63-amino-acid coded peptide was isolated to homogeneity and to a concentration and total amount high enough (0.6 mg/ml and 1.5 mg, respectively, from a 2-L cultures) to allow immunization experiments (Fig. 3). It should be noted, at this point, that the 63 aa-peptide displays no detectable chitinolytic activity, as it was demonstrated using the substrate pNP-$(NAG)_2$ and the standard chitinase assay, at 37–60 °C (data not shown).

A series of ELISA tests, followed by Western blots, using the purified peptide and various dilutions of the serum, were employed to estimate the titer of the antibody and to select the most enriched serum (data not shown). The resulting polyclonal antibody was found to be highly specific (Figs. 4A and B) and of high titer, and was further used to investigate the probable immunochemical affinity of the 63 aminoacid-peptide to other chitinases. Strong and specific reactions was observed with the eubacterial Sm-chiA, as well as, the archaeal,

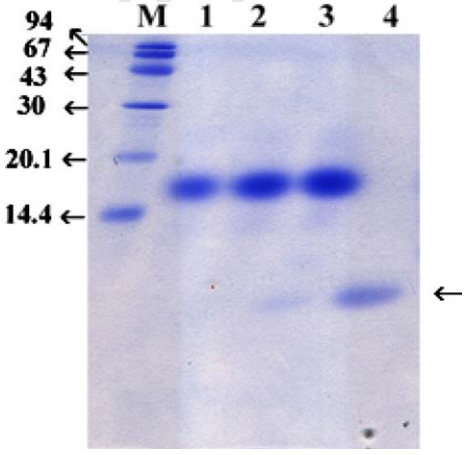

Fig. 3. Analysis, by 16.5% w/v Tricine-SDS–PAGE, of the fused protein following chemical cleavage with CNBr–formic acid and release of the insoluble fusion partner. The pellet contains the insoluble fusion tag (lanes 1, 2, and 3) and the supernatant contains the purified, soluble peptide, of calculated molecular weight of 7 kDa (indicated by the arrow on lane 4). Lane M contains the molecular weight markers, as indicated in kDa.

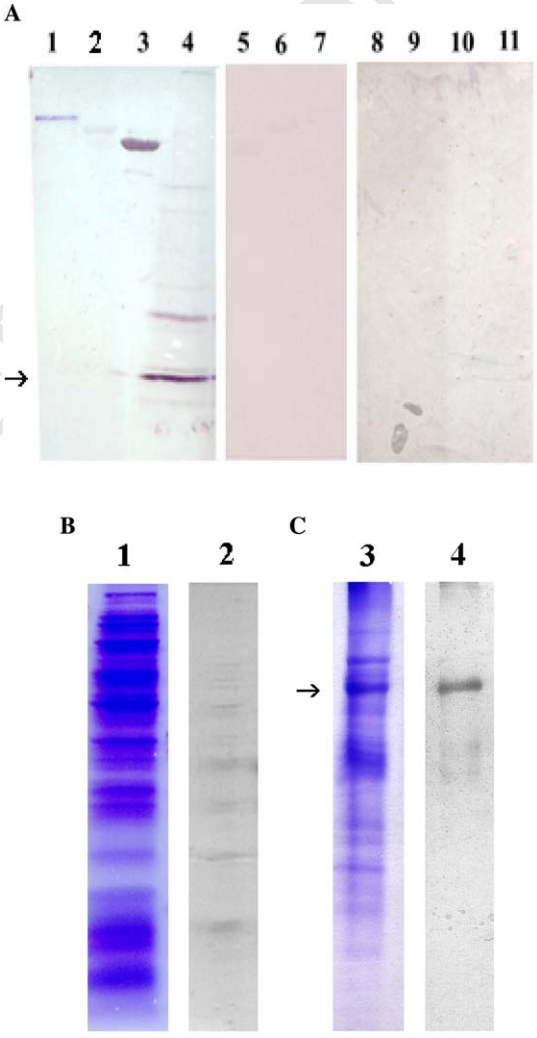

Fig. 4. Detection of immunochemical affinity of various chitinases by Western blotting, from 15% w/v SDS–PA gels, using the antiserum against the peptide. (A) Lanes 1, 6, and 8 contain 0.1 μg of the purified Tc-Chi70. Lanes 2 and 9 contain 1 μg of each of the proteins that were used as negative control (lysozyme and BSA). Lanes 3, 5, and 10 contain 0.5 μg of the Sm-chiA. Lanes 4, 7, and 11 contain 0.5 μg of the purified peptide (indicated by the arrow). Samples in lanes 1–4 have been incubated with a 1:1000 dilution of the anti-serum raised against the peptide, while samples in lanes 5–7 have been incubated with the pre-immune serum, and in lanes 8–11 with the secondary antibody alone (background control). (B) Lanes 1 and 2 contain the total *E. coli* cell lysate, following Coomassie blue R-250 staining (lane 1) or incubation with a 1:1000 dilution of the anti-serum raised against the peptide (lane 2). (C) Lanes 1 and 2 contain the clarified soluble extract from *T. chitonophagus*, following Coomassie blue R-250 staining (lane 1) or incubation with a 1:1000 dilution of the anti-serum raised against the peptide (lane 2). The arrow indicates the Tc-Chi70 protein band.

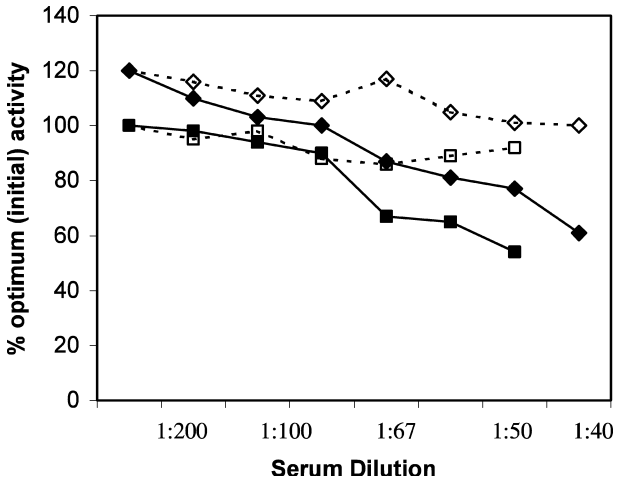

Fig. 5. Detection of the residual chitinolytic activity of the purified Tc-Chi70 protein and the clarified soluble extract from *T. chitonophagus*, following immunoreaction with the anti-serum raised against the peptide, or the alkaline phosphatase-labelled, anti-rabbit IgG (used as a negative control antibody) and immunoprecipitation of the resulting complex with Protein A–agarose beads. The pre-adsorbed purified enzyme (0.8 μg Tc-Chi70) or clarified extract (1.5 μg Tc-Chi70) was incubated at pH 7.0 and 70 °C, in the presence of 0.2 mM $pNP(NAG)_2$, for 4 h and the residual activities were measured, in the supernatant of the immunoreaction mixtures, according to the standard assay conditions and expressed as the percentage of the initial (optimum) activity of the non-adsorbed enzyme chi70 or clarified extract. (■) Purified Tc-Chi70 protein; (◆) clarified, soluble extract of *T. chitonophagus*; (□) negative control for the purified Tc-Chi70; and (◇) negative control for the clarified, soluble extract of *T. chitonophagus*.

cell membrane-associated Tc-Chi70 (Figs. 4A and C). The lower intensity of the band (Fig. 4A, lane 1), corresponding to the Tc-Chi70 protein, is exclusively due to the low concentration (5 μg/ml) of the highly pure protein preparation of this chitinase [17]. A strong signal is produced in the presence of the clarified soluble extract from *T. chitonophagus* (Fig. 4C), which contains a 10-times higher concentration of Tc-Chi70 protein, i.e., 50 μg/ml, as well as additional chitinases, at lower levels, contributing to the overall chitinolytic activity of the extract [17]. The additional chitinases of the extract seem to react, yet with lower affinity, with the antibody, indicating the presence of a common epitope, shared by the peptide, the Tc-Chi70 protein and at least two other cellular chitinases from the archaeon (Fig. 4C).

The strong and specific affinities of the antibody towards the Tc-Chi70 was verified by measuring the remaining chitinolytic activity of the enzyme, in the final purified preparation, as well as in the clarified soluble extract from *T. chitonophagus*, following immunoprecipitation with the complex anti-serum–protein A–agarose (Fig. 5). A 1:50 dilution of the anti-serum was found to be sufficient for a 50% decrease of the enzymatic activity of the purified Tc-Chi70. For the clarified soluble extract, containing much higher amounts of the Tc-Chi70 protein, as well as other chitinases, a 1:40 dilution of the anti-serum is required for a 40% inhibition of the total chitinolytic activity. Despite the synergistic action of the remaining cellular chitinases of the clarified, soluble extract of the archaeon, the total chitinolytic activity is considerably reduced, in the presence of the anti-serum, indicating that the Tc-Chi70 is an essential component of the chitinoclastic system of *T. chitonophagus*.

Therefore, the produced antibody includes epitopes that recognize the few, short conserved regions, common to both the eubacterial Sm-chiA and the archaeal Tc-Chi70. It can be expected that this antibody would, also, present strong affinity towards the Pk-chiA. The above results of the specific immunochemical affinity, in combination with the sequence homology results, strongly indicate that this peptide constitutes part of a chitinase enzyme from the archaeon *T. chitonophagus*. It seems to be directly related to the cell membrane-associated Tc-Chi70, but could also be related to isoforms of this enzyme, or other chitinolytic enzymes from the same archaeon. It should be noted that this archaeon has been found to produce several cellular and extracellular chitinolytic enzymes (Andronopoulou and Vorgias, unpublished data). These could be coded by an equal number of genes, or could be products of the same gene, following post-transcriptional or post-translational modifications.

The new peptide sequence information, as well as the generated antibody can be used, in future experiments, towards the full-length gene isolation of the membrane chitinase (Tc-Chi70), or other cellular chitinases from *T. chitonophagus*.

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