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## Molecular characterization and expression of a granzyme of an ectothermic vertebrate with chymase-like activity expressed in the cytotoxic cells of Nile tilapia (*Oreochromis niloticus*)

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**Abstract** We have identified the gene coding for a novel serine protease with close similarities to mammalian granzymes from nonspecific cytotoxic cells of a teleost fish *Oreochromis niloticus*. The genomic organization of tilapia granzyme-1 (TLGR-1) has the signature five-exon–four-intron structure shared by all granzymes and similar hematopoietic Ser proteases. Molecular modeling studies suggested a granzyme-like structure for this protein with four disulfide linkages and two additional Cys residues. The expression of this gene is found to be restricted to cytotoxic cell populations with a low level of constitutive expression when compared to similar granzymes in other teleost species. High levels of transcriptional activation of TLGR-1 with different stimuli suggested that this gene is highly induced during immune reactions. Triplet residues around the active site Ser of TLGR, which determines the primary substrate specificity of granzymes, differ significantly from that of other granzymes. Recombinant TLGR-1 was expressed in the mature and proenzyme forms using pPICZ-alpha vector in the *Pichia pastoris* expression system. Recombinant TLGR-1 was used to determine the primary substrate specificity of this protease using various synthetic thiobenzyl ester substrates. In vitro enzyme kinetics assays suggested a preference for residues with bulky side chains at the P<sub>1</sub> site, indicating a chymase-like activity for this protease. These results indicate the presence of novel granzymes in cytotoxic cells from ectothermic vertebrates.

**Keywords** Cytotoxicity · Natural killer cells · Granzyme · Teleosts · Nonspecific cytotoxic cells

### Introduction

Granzymes belong to a family of serine proteases, which constitute the major components of granules of professional killer cells such as cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells. Along with perforin, a pore-forming protein that is also found in these granules, granzymes play a major role in inducing target cell death (Lieberman 2003; Russell and Ley 2002; Trapani 2001). All the mammalian granzymes described so far are highly conserved and are located in three clusters on three chromosomes (Grossman et al. 2003). Granzymes A and B are the most extensively studied granzymes. The different pathways used by granzymes A and B to initiate cytotoxicity have been described (Lieberman and Fan 2003; Trapani and Sutton 2003). However, novel mechanisms of cytotoxicity are being discovered for these granzymes, expanding our knowledge about complex pathways involved in cell death and proliferation (Beresford et al. 2001; Fan et al. 2002, 2003a,b; Han et al. 2004; Sebbagh et al. 2005). The function of many of the other granzymes is not well understood, thus they are collectively called as orphan granzymes (Grossman et al. 2003).

Nonredundant pathways of cell-mediated cytotoxicity have been proposed for granzymes, suggesting a ‘fail-safe’ mechanism provided by multiple granzymes (Grossman et al. 2003; Lieberman 2003). The role of granzymes in inducing target cell death has been investigated extensively in vitro (Trapani and Smyth 2002). However, advances in in vivo studies demonstrating the significance of individual granzymes in cytotoxicity lag behind. Targeted gene disruption studies of granzymes have resulted in focal immune deficiencies (Ebnet et al. 1995; Heusel et al. 1994; Trapani and Smyth 2002; Wilharm et al. 1999b). Recent attempts to understand the individual effects of members of the granzyme B gene cluster has revealed the significant role played by orphan granzymes present downstream of

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the granzyme B gene in mice (Revell et al. 2005). Such results indicate the relative significance of other granzymes that have not been fully investigated.

Granzymes are classified based on their primary substrate specificities, and four enzymatic activities have been described: tryptase, Asp-ase, Met-ase, and chymase. The most extensively studied mammalian granzymes fall under three of the above specificities. Granzymes A and K have tryptase activity, granzyme B has Asp-ase activity, while granzyme M has Met-ase activity (Kam et al. 2000). Many of the orphan granzymes have chymase activity.

The presence of multiple granzymes can be interpreted as a result of molecular strategies developed by the immune system to compete with pathogens. These pathogens have coexisted and coevolved with immune systems of organisms for millions of years (Trapani and Smyth 2002). Genetic comparisons and chromosomal clustering of granzymes suggest gene duplication events as a means of generating newer granzymes (Grossman et al. 2003). Almost all the assumptions on granzyme evolution are based on the gene sequence information from human and rodents due to the lack of sequence information in other organisms. The availability of nonmammalian granzyme sequences would add new insights into the evolution of these proteases with very narrow substrate specificity, and it would also aid in the discovery of newer killing pathways (Hink-Schauer et al. 2003; Praveen et al. 2004).

Nonspecific cytotoxic cells (NCC) are the first identified cytotoxic cell population in teleosts. First described in the channel catfish (Graves et al. 1985), these cells have been well characterized in a number of lower vertebrates (Jaso-Friedmann et al. 2002; Jaso-Friedmann and Evans 1999; McKinney and Schmale 1994; Suzumura et al. 1994). Although first described as agranular (Graves et al. 1985), NCC in trout, tilapia, and catfish do have small granules, and contact with target cells leads to granule exocytosis with necrotic and apoptotic death pathways (Faisal et al. 1989; Greenlee et al. 1991; Jaso-Friedmann et al. 1990). The requirement for calcium for the induction of cytotoxicity by NCC was an early indication that these cells use the granule exocytosis killing pathway (Carlson et al. 1985). We have previously reported the existence of granzyme-like serine proteases in the NCC of channel catfish (*Ictalurus punctatus*) and tilapia (*Oreochromis niloticus*) (Praveen et al. 2004). Here, evidence is presented for the full-length sequence of the cDNA and gene for a granzyme-like protease from tilapia, which was designated as tilapia granzyme-1 (TLGR-1), its expression pattern, and the substrate specificity of the recombinant protein. These results demonstrate the molecular characterization of a granzyme from a cytotoxic cell of nonmammalian vertebrates.

## Materials and methods

### Cloning of full-length TLGR-1

The identification and partial sequence of a granzyme-like serine protease from a tilapia NCC cDNA library have been

previously reported (Praveen et al. 2004). Full-length sequence of that tilapia granzyme (TLGR-1) was obtained by rapid amplification of cDNA ends (RACE). Fresh RNA was purified from tilapia NCC from peripheral blood for the RACE as described before (Praveen et al. 2004). Briefly, mRNA (500 ng) was reverse-transcribed with granzyme-specific primers using Generacer Superscript II RT module (Invitrogen, CA). First strand cDNA was subjected to homopolymeric tailing with cytosine using terminal transferase (Roche). Later, dC-tailed cDNA was amplified using abridged anchor primers (Invitrogen), and gene-specific nested primers followed by a reamplification using a nested abridged universal amplification primer and another nested gene-specific primer (Praveen et al. 2004). The amplicons were purified and TA-cloned for sequencing. For 3' RACE, RNA was reverse-transcribed using an anchor primer, EPB-18T (Evans et al. 1998), to generate first strand cDNA. Using gene-specific nested primers and EPB, the 3' end of the mRNA was amplified and TA-cloned for sequencing. The sequences were edited and assembled to obtain the complete sequence of TLGR-1. Forward and reverse primers were designed at the two ends to amplify the full-length cDNA, which was TA-cloned and sequenced in both directions using standard protocols.

### TLGR-1 gene and promoter sequences

The complete genomic sequence of the TLGR-1 gene and its promoter elements were obtained by sequencing a cosmid clone containing the TLGR-1 gene. The tilapia genomic DNA library was constructed in SuperCos-1, as reported for channel catfish (Jaso-Friedmann et al. 2004). A directed polymerase chain reaction (PCR)-based iterative screening protocol was used to identify clones with TLGR-1 gene (Heaton et al. 1997). Specific PCR primers (TLGR COSF1 5'-gcaccacgatatgacaaggtga-3' and TLGR COSR1 5'-ggccttcaatttctcttacagaca-3') were designed based on the cDNA sequence. After several rounds of screening, several TLGR-1-positive clones were isolated, expanded, and frozen in glycerol stocks for further analysis. The complete TLGR-1 gene sequence was obtained by sequencing the cosmid clone in a 373 A DNA sequencer (Applied Biosystems, CA) at the Integrated Biotech Laboratories (University of Georgia, Athens). The upstream region of the TLGR-1 gene (promoter region: 1,200 bp) was directly sequenced from the cosmid. Primers were synthesized as needed for "primer walking" (Genosys, TX). The TRANSFAC database (Wingender et al. 2000) and MatInspector web-based software (Quandt et al. 1995) were used to locate transcription factor binding sites.

### Phylogenetic analysis and molecular modeling

Phylogenetic analysis and molecular modeling of TLGR-1 were done as described before (Praveen et al. 2004). Briefly, all the known granzymes and similar serine proteases were aligned, and phylogenetic analysis was

done with Mega version 2.1 (Kumar et al. 2001). Analysis using different methods resulted in similar trees, and reliability of the trees was tested using 1,000 bootstrap replications. The tree was rooted on a subtree consisting of adipsin-like proteases from various sources. Molecular modeling of TLGR-1 was done using SWISS-MODEL in the first approach mode accessible via the Internet (<http://www.expasy.org/swissmod>). The crystal structure of human pro-granzyme K (1MZA) was used as a template to build the TLGR-1 model. RasWin version 2.6 was used to visualize the coordinate data.

### Real-time polymerase chain reaction analysis

After exposing NCC to activation treatments, total RNA was isolated using RNeasy mini-kit (Qiagen, CA) following the manufacturer's protocol, quantified spectrophotometrically, and treated with RNase free DNase (Promega, WI). The first strand cDNA was synthesized from 3 µg of total RNA using First Strand cDNA synthesis kit (Invitrogen). A cDNA stock for constructing a relative standard curve was synthesized using the same method. Primers were designed based on available gene sequences to amplify ~150 bp amplicons for TLGR-1 and β-actin. Forward and reverse primers were selected at positions to include an intron so that genomic DNA contamination in the samples could be easily detected. A relative standard curve was constructed for target gene and housekeeping gene (β-actin) using either cDNA stock or full-length cDNA clone for TLGR-1 and β-actin. Briefly, reactions were set up with different concentrations ranging from 10 to 3000 pg per reaction for the standard curve. The reactions were carried out using Brilliant SYBR Green QPCR master mix (Stratagene, CA) according to manufacturer's instructions. The efficiencies of the primer pairs were determined, and appropriate adjustments were made to optimize reaction conditions. Diluted cDNA samples were used as a template for every 25-µl reaction. Each sample was set up in triplicates for the PCR. The following PCR protocol was used for gene amplification: 95°C, 3 min; 40 cycles: denature 95°C, 30 s; anneal 60°C, 30 s; extend 72°C, 30 s, followed by 4°C hold using Mx3000P real-time PCR (RT-PCR) system (Stratagene).

### Production of mature and pro-TLGR-1 in *Pichia pastoris*

The cDNAs coding for pro and mature forms of TLGR-1 were PCR-amplified and cloned in to pPICZ-alpha vector for expression in GS115 strain of *P. pastoris* (Invitrogen). The coding region of TLGR-1 contained a recognition site for *Xho*I. Because digestion of DNA with *Sal*I has been shown to generate cohesive ends compatible with that made with *Xho*I digestion, forward primers TLGRYSTF1 (5'-aactgtc gacaaaagaatcataaatggca-3') and TLGRYSTF2 (5'-aactgtcga caaaagaagtgaatcataaatggca-3') were designed to engineer an *Sal*I site upstream of mature and pro TLGR-1, re-

spectively. A reverse primer TLGRYSTR1 (5'-ggggttctaga tggcattgctttttattgagaatg-3') was designed to incorporate an *Xba*I site. The combination of these primers was used to PCR amplify the insert using a full-length TLGR-1 clone. The PCR products were purified, digested with a combination of restriction enzymes (*Xho*I and *Sal*I), and ligated in to the pPICZ-alpha vector digested with *Xho*I and *Xba*I. The insert was introduced into this vector downstream of alpha factor signal sequence and Kex2 protease cleavage site. This position facilitated the production of recombinant proteins with native N-terminus and a polyhistidine tag at the C-terminus, which would be secreted to the medium with minimum contamination from yeast proteins. The ligation reaction was used to initially transform competent JM109 *Escherichia coli* cells, and positive clones were selected on a low-salt Luria-Bertani plate with 25 µg/ml Zeocin (Invitrogen). The clones were verified for the presence of insert by restriction digestion and colony PCR methods. Recombinant plasmids (pPICZ-alpha-TLGR-1 and pPICZ-alpha-pro-TLGR-1) were sequenced in both directions using 5' and 3' AOX1 primers (Invitrogen) to verify the correct reading frame. Subsequently, the recombinant plasmids were linearized by digesting with *Sac*I before electroporating in to the GS115 strain of *P. pastoris* according manufacturer's instructions. Positive clones were selected on yeast peptone dextrose (YPD) agar plates containing 100 µg/ml Zeocin. Positive clones were tested for insert by PCR. Expression of recombinant proteins was assessed by a small-scale expression trial followed by Western blot analysis of the trichloroacetic acid (TCA)-precipitated supernatants. Clones with high level of expression were selected to scale-up production.

To obtain enough recombinant protein for purification, the selected clones were grown in baffled flasks for optimum aeration. Using a single colony, 25 ml of buffered glycerol-complex medium [BMGY: 1% yeast extract, 2% peptone, 100 mM potassium phosphate (pH 6.0), 1.34% yeast nitrogen base, 4×10<sup>-5</sup>% biotin, 1% glycerol] in a 250-ml baffled flask was incubated at 30°C for 18 h until the OD<sub>600</sub>=2–6. Cells were harvested by centrifugation, and the pellet was resuspended in buffered methanol-complex medium (BMMY: BMGY with glycerol replaced with 0.5% methanol) to an OD<sub>600</sub> of 1.0 in a 1-l baffled flask. The flasks were incubated at 30°C for various time points until the optimum production was verified by Western blotting of supernatants collected at different time points. The supernatants were collected and stored at -80°C until ready for purification.

For purification of the His-tagged recombinant proteins, the supernatants were concentrated by ammonium sulfate precipitation followed by dialysis in phosphate-buffered saline (PBS, pH 7.2). The recombinant proteins were purified by metal affinity chromatography using Ni-NTA agarose (Qiagen) according to manufacturer's instructions. The positive fractions were pooled, desalted, and concentrated using Centricon YM-3 centrifugal filters (Millipore, MA). Aliquots were stored at -80°C. Protein concentrations were estimated using Biorad Protein Assay Kit (Biorad, CA).

Western blot analysis

Proteins were resolved on 12.5% sodium dodecyl sulfate (SDS) gels, transferred to nitrocellulose membrane, and probed with INDIA-His probe-horse radish peroxidase (HRP) (Pierce, IL) or anti-His monoclonal antibody (Qiagen). Proteins were detected with SuperSignal West Pico Chemiluminescent Substrate (Pierce).

In vitro enzyme assays

Activity assays for proteases were performed at 37°C in 50 mM Tris-HCl, 0.15 M NaCl, 0.01% Triton X-100 (pH 7.6) containing 0.2 mM Ellman's reagent [5,5'-

dithiobis(2-nitrobenzoic acid), Sigma, MO] in 96-well microtiter plates with a reaction volume of 200 µl. Various thiobenzyl ester substrates at a concentration of 0.2 mM were used to determine the enzyme kinetics by measuring an increase in absorbance at the 405-nm wavelength over time with a Spectra Max Plus microplate reader (Molecular Devices, CA). Results for controls and samples are expressed as change in mOD/min as described before (Wilharm et al. 1999a). Stock solutions of Z-Lys-SBzl (Calbiochem, CA) were made in ethanol, while Z-Arg-SBzl, Boc-Ala-Ala-Asp-SBzl, Boc-Ala-Ala-Met-SBzl (MP Biomedicals, OH), N-Succinyl-Phe-Leu-Phe-SBzl, and N-Succinyl-Ala-Ala-Pro-Phe-pNA (Sigma) were dissolved in dimethyl sulfoxide (DMSO) and stored at -20°C and added to the reaction buffer prior to the assay.

**Fig. 1** Genomic organization of TLGR-1 gene and promoter region. Exon sequences are represented in *upper case letters*, while introns and promoter region are represented as *lower case letters*. Putative transcription factor binding sites in the promoter region are represented as *underlined bold letters* with the name of the factor above the sequence. The predicted signal sequence at the N-terminus is highlighted, while the dipeptide is represented with *double underline*. A putative glycosylation site is *boxed*, and the polyadenylation signal is represented by *underlined bold letters* in the 3' UTR. Triad residues constituting the active site are represented as *bold letters* in the sequence of amino acid chain

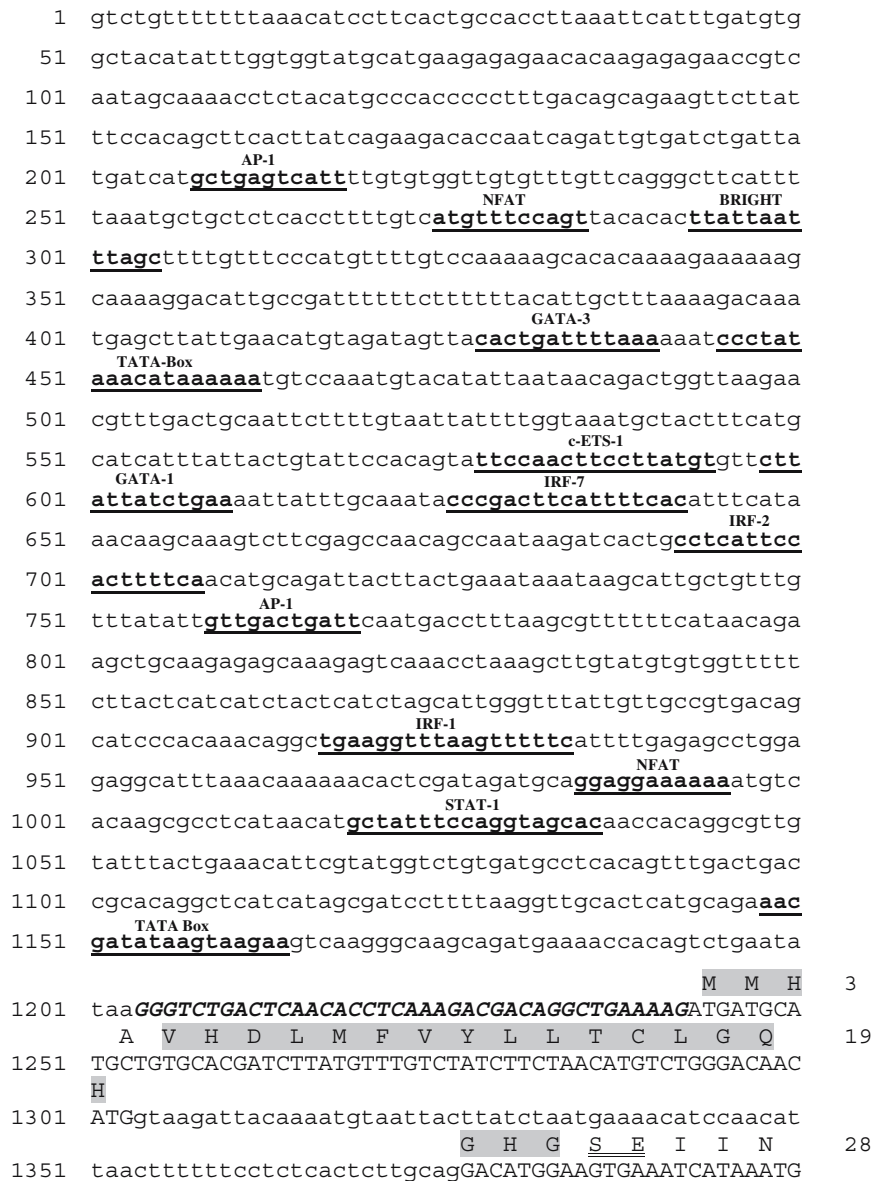




Fig. 1 (continued)

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G K N V P Q N S M Q Y M A S V Q I 45
1401 GCAAAAATGTCCACAGAACTCAATGCAGTATATGGCTTCTGTGCAGATC
D G K H V C G G F L V S E D F V L 62
1451 GATGAAAACACGTATGTGGAGATTCTTGTGTCAGTGAAGACTTTGTGCT
T A A H C Y K N 70
1501 CACGGCTGCACATTGTTACAAAAgtaagtgaacaacctgtgtactttta
1551 tttgtttttctcaaaaaaacccagctaacacctacttaataattcgctcgc
S P M E V V I G T H N L K K V 85
1601 tcagTTCTCCTATGGAGTTGTAATTGGAACCCACAATCTGAAGAAGTT
N N N K M R Y S V K T C K H P R Y 102
1651 AATAACAACAAAATGAGATACAGTGTAAAGACATGCAAGCACCCACGATA
D K V E S G N D I M L L K 115
1701 TGACAAAGTTGAGTCTGGTAATGACATCATGCTCCTCAAagtaagtaaat
1751 atctttgaatgtgattcattacattacaataactccacttcgtacttcac
1801 caaagacttgatataagatcagaaatgtacagagttggtttctatcagtc
L S R K L Q 121
1851 tttttatTTTTTTTcctTTTTTggttcttcttagCTCTCGAGGAAACTTCA
L D K K V K P I Q L A R K E I K 137
1901 ACTGGACAAGAAAGTAAACCGATACAACCTGGCAAGGAAGGAGATTAAG
A K D N V K C Q V A G W G F T E T 154
1951 CAAAAGACAATGTAAAGTGTGAGGTGGCTGGATGGGGTTTCACAGAAACC
N G K A V D V L R W V D V P L I D 171
2001 AATGGCAAAGCTGTTGATGTGCTGAGATGGGTAGATGTGCTCCTTATTGA
L N V C K R K L K G K L P K G V 187
2051 CCTTAATGTCTGTAAGAGAAAATTGAAAGGCAAACCTCCAAAAGGTGTTA
I C A G G S D T K N G F C Q 201
2101 TCTGTGCAGGAGATCTGACACAAAGAATGGATTCTGCCAGgtatgtcct
2151 ctgttcttttaagaaagtatcacttcacttgtagtattaaataaaagat
G D S G G P L 208
2201 aaataaaacaagcttcttcccccttacagGGTATTCTGGTGCCCTTG
V C [N G T A] V G V V S F N I N G N 225
2251 GTGTGCAATGGAACAGCAGTTGGTGTGTGCTTTCAACATAAATGGAAA
C K Y P N Y P N V Y T D I S K H 241
2301 TTGTAAATACCCAAATTACCCCAACGTCTATACAGATATATCAAAGCACC
L S W I K N I L N K K Q C * 254
2351 TTTCTGGATCAAAAACATCTCAATAAAAAGCAATGCTAAGTATACTACT
2401 GTAACAATGTGCAATCTGCAATATCTGGCATTATAAATACTGTATGTGAAC
2451 TGCAGGATAAATGTAAATAGCTTAAGACATCTCTCTGTATTGCAAAGA
2501 TATTCCTGCTTGCTCTTAAATTAAGTAAAAAATATTGAAG

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## Results

### Identification and analysis of full-length cDNA and gene for TLGR-1

For identification of genes involved in cytotoxic functions of NCC, a cDNA library was constructed using activated cytotoxic cells. The identification of a partial sequence of a serine protease with novel predicted substrate specificity from a tilapia NCC library has been reported (Praveen et al. 2004). A full-length cDNA for that protease was cloned from a cDNA library. Primers were designed based on this sequence to screen a tilapia genomic library, and a cosmid clone containing the gene and promoter elements was identified (Fig. 1). Surprisingly, the TLGR-1 gene had an approximate size of 1.32 kb (starting from the 5' UTR and ending at the polyadenylation signal), which is much smaller than mammalian granzyme genes. However, the comparison of the cDNA and genomic sequences of the TLGR-1 gene revealed a five-exon and four-intron organization, which is shared among many members of the

granzyme family. The teleost granzyme gene has shorter introns than the mammalian granzyme genes. All of the intron/exon boundaries of TLGR-1 gene fulfill the GT-AG rule (Mount 1982).

The proximal promoter region of TLGR-1 was analyzed for the putative transcription factor binding sites. Similar to other granzyme gene promoters, TLGR-1 has many binding sites for transcription factors specific for the immune system (Fig. 1). There are two AP-1 and NFAT binding sites along with sites for binding of interferon response factors and STATs. This would appear to suggest that there may be a significant correlation between the transcriptional upregulation of TLGR-1 and activated immune response reactions.

The TLGR-1 cDNA sequence was initially obtained by RT-PCR using specific primers at the 5' and 3' end of the gene, and the sequence was verified by comparing it with the genomic sequence. The cDNA and genomic sequences were submitted to GenBank (accession numbers AY918866 and AY918867, respectively). This cDNA has a total length of 999 bp, with an open reading frame of 765 bp that

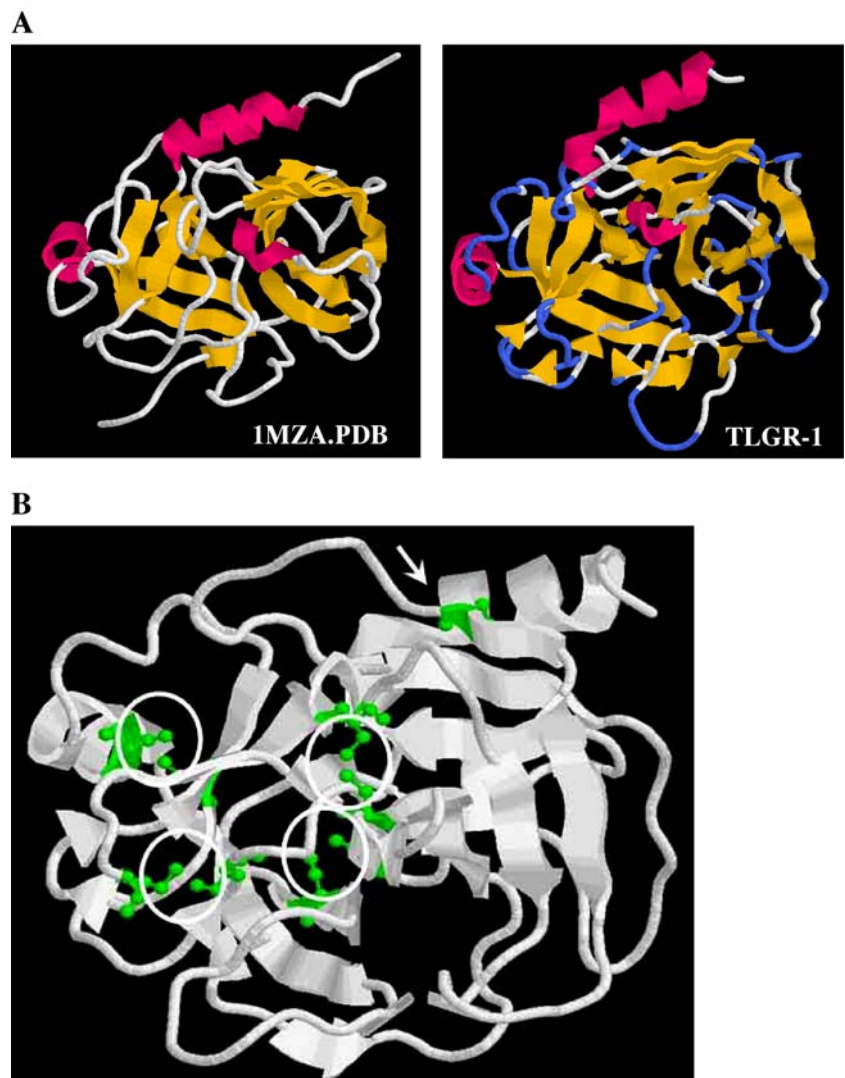
encodes a putative protein with 254 amino acids (Fig. 1). Using algorithms to predict signal sequences, a stretch of 23 residues at the N-terminal end was identified as a signal sequence, indicating that this protein is sorted to the secretory pathway. Signal sequence cleavage sites were predicted using weight matrix analysis of von Heijne (1986). TLGR-1 is predicted to have a dipeptide (Ser-Glu) as propeptide, much similar to the majority of mammalian granzymes. There is a single putative glycosylation site (NGTA) located toward the C-terminus of the protein (Fig. 1). The predicted molecular mass of the non-glycosylated mature TLGR-1 was calculated as 25,339 Da (229 amino acids) with an isoelectric point of 9.57. Other granzymes also have a highly basic charge.

Disulfide linkages are crucial for the proper folding of fully active granzymes. Overall structure of the tilapia granzyme had a close similarity to available crystal structures of mammalian granzymes. TLGR-1 can be predicted to have four disulfide linkages (Fig. 2), similar to what was shown for catfish granzyme (Praveen et al. 2004). Formation of disulfide bonds between Cys residues can be predicted as follows: 42 with 58, 136 with 168, 182 with 201.

Cys191 and Cys220 (chymotrypsinogen numbering) could be analogous to a fourth disulfide linkage which bridges the active site serine, as in chymotrypsin. Although the tilapia granzyme has additional Cys residues (Fig. 2), these are not present at corresponding positions to that of granzyme A, which is crucial in the dimerization (Hink-Schauer et al. 2003).

Multiple sequence comparisons of TLGR-1 with related granzyme sequences enabled the identification of signature motifs shared by members of the granzyme family. While the N-terminal IIXG motif is conserved in the TLGR-1, the PHSRPYMA motif is not entirely conserved (Fig. 3). However, the residues in corresponding position share close similarity to those in the mammalian sequences. The three key amino acid residues representing the catalytic triad (charge relay system) of serine proteases (His57, Asp102, and Ser195; chymotrypsinogen numbering system) as well as their neighboring residues are well conserved in the TLGR-1 (Fig. 3). The combination of amino acid residues, which is crucial in the formation of the substrate specificity pocket, can be used as a prediction tool for primary specificity for a protease (Wouters et al. 2003).

**Fig. 2** Predicted three-dimensional model for TLGR-1. **a** Comparison of TLGR-1 structure to human granzyme K (1MZA.pdb). **b** Predicted disulfide bonds in TLGR-1. Cys residues are represented in *green*. Four disulfide bonds which are conserved in other granzyme sequences are represented by *circles*. The extra Cys residue is marked with an *arrow* (one additional Cys residue at the C-terminus of the molecule is omitted from the model and not shown)



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TLGR-1 : IINGKNV PONS MQYMASV QIDG---KHVCGGFLVSEDFVLTAAHCYKNSP-- : 47
CFGR1 AAQ54830 : IIGGREVK-KPKP WMA SVQSN--N--SHICGGTLHQQVNLTAHCKTFLQFK : 48
Human grnzA A31372 : IIGGNEVTPH SRPYMVL SLD----RKTICAGALIAKD VNLTAHCNLNKR-- : 47
Human grnzB AAH30195 : IIGGHEAKPHSRPYMAYLMIWDQKSL-KRCGGFLIQDDEFVLTAHCWGSS--- : 49
Human grnzK P49863 : IIGGKEVSPH SRPFMASIQYG--G--HHVCGCVLIDPQVNLTAHCQYRFTKG : 49
Human grnzM AAH25701 : IIGGREVTPH SRPFMASIQRNG----SHLCGGVLVHPK VNLTAHCLAQR--M : 47
Human grnzH A32692 : IIGGHEAKPHSRPYMAFVQFLQEKSR-KRCGGILVRKDFVLTAHCQGSS--- : 49
Mus grnzC NP_034501 : IIGGNEVSPH SRPFMAFVYEF LKVG GKKMF CCGGFLVRDKEVNLTAHCKGRS--- : 50
Mus grnzD NP_034502 : IIGGHVVKPH SRPFMAFVMSVDIKGNRIYCGGFLIQDDEFVLTAHCKNSS--- : 50
Mus grnzE NP_034503 : IIGGHVVKPH SRPFMAFVMSVDIEGNRRYCGGFLVQDDEFVLTAHCNRRT--- : 50
Mus grnzF AAA37741 : IIGGHEVKPH SRPFMARVRFVKDNGKRHS CCGGFLVQDYFVLTAHCTGSS--- : 50
Mus grnzG NP_034505 : IIGGHEVKPH SRPFMAFIKSV DIEGKKKYCGGFLVQDDEFVLTAHCNRNS--- : 50

TLGR-1 : --MEVVTGTHNLK-KVNNNKMYRSVKTCKHPRYDKV-ESGNDIMLLKLSRRLQ : 96
CFGR1 AAQ54830 : P-IEVLLGAHSLT-KDKNAMRVKLCFHISPKFSAT-TRVHDIMLLKLDQNVQ : 98
Human grnzA A31372 : --SQVILGAHSTIREEPTQCFIPVKKRPIPHPAYNPK-NFSNDIMLLQLERKAK : 97
Human grnzB AAH30195 : --INVTLGAHNITKEQEQPTQCFIPVKKRPIPHPAYNPK-NFSNDIMLLQLERKAK : 99
Human grnzK P49863 : QSPTVVLGAHSLSKNEASKQTEIETKFIETFSRVTS-DPQNSNDIMLVKLQTAAK : 101
Human grnzM AAH25701 : AQLRLVVLGLHTLD---SPGLTFHIKAAIQHPRYKVPALENDLALLQLDGVK : 97
Human grnzH A32692 : --INVTLGAHNITKEQEQPTQCFIPVKKRPIPHPAYNPK-NFSNDIMLLQLERKAK : 99
Mus grnzC NP_034501 : --MTVTLGAHNITAKEETQCFIPVAKAI PHPDYNDP-DRSNDIMLLKLVNRNAK : 100
Mus grnzD NP_034502 : --MTVTLGAHNITAKEETQCFIPVAKAI PHPDYNAT-IFYSNDIMLLKLESKAK : 100
Mus grnzE NP_034503 : --MTVTLGAHNITAKEETQCFIPVAKAI PHPDYNAT-AFFSNDIMLLKLESKAK : 100
Mus grnzF AAA37741 : --MRVTLGAHNITAKEETQCFIPVAKAI PHPAYDDK-DNTSDIMLLKLESKAK : 100
Mus grnzG NP_034505 : --MTVTLGAHNITAKEETQCFIPVAKAI PHPAFNRK-HGTNDIMLLKLESKAK : 100

TLGR-1 : LDK-KVMPVQLARKEIKADNVKCOVAGWGFTE TN-GKAVDVRWVDVPLIDL : 147
CFGR1 AAQ54830 : LKKNKVDVKKI PKSGKDI PAETKCEVRRGWGTTTHVKNPKACDITQEELEVTVVDR : 151
Human grnzA A31372 : INK-YVTILHL PKKGDDVXPCTMCOVAGWGRTHN-SASWSDTLREVNITLIDR : 148
Human grnzB AAH30195 : RTR-AVQPLRLPSNKAQVVPQTCVAVGWGQTAP-LGKHSHTLQEVKMTVQED : 150
Human grnzK P49863 : LNK-HVIMLHIR-SKTSLSRSTCKCKVTGWGATDPDSL RPSDTRREVTVTVLSR : 152
Human grnzM AAH25701 : PSR-TIRPLALPSKRQVVAATRCSMAGWGLTHQ-GGRLSRVIRELDLQVLDT : 148
Human grnzH A32692 : WTT-AVRPLRLPSKAQVVPQCLCSVAVGWGYVS--MSTLATTLQEVLLTVQKD : 149
Mus grnzC NP_034501 : RTR-AVRPLNLRPRNAHVKPEDECYVAGWKVTP-DGEFPKTHHEVKLTVQKD : 151
Mus grnzD NP_034502 : RTK-AVRPLKLRPNARVVKPEDEVCSVAVGWSRSINDTKASARLREAVQLVQED : 152
Mus grnzE NP_034503 : RTK-AVRPLKLRPNARVVKPEDEVCSVAVGWP RSINDTKASARLREAVQLVQED : 152
Mus grnzF AAA37741 : RTK-AVRPLKLRPNARVVKPEDEVCSVAVGWRTSINATORSSCLREAVQLVQKD : 152
Mus grnzG NP_034505 : RTK-AVRPLKLRPNARVVKPEDEVCSVAVGWKTSINATKASARLREAVQLVQED : 152

TLGR-1 : NVCKRKLKG---KLPKGVICAGGSDTKNGFCQGDSSGGLVNG--TAVGVVS : 194
CFGR1 AAQ54830 : ELONCYNSK--PTITANMLCAGNKQRDKDACWGDSSGGLIECKK--NIVGVVS : 200
Human grnzA A31372 : KVONDRNHYNFNPNVIGMNV CAGSLRGRDSCNGDSSGSPLECG--VFRGVTS : 199
Human grnzB AAH30195 : RKCESDLRH---YDSTIELCVGDPEIKKTSFKGDSGGPLVGNK--VAQGLVS : 198
Human grnzK P49863 : KLONSQSYYNGDPFITKDMV CAGDAKGQKDSCKGDSGGPLICKG--VFHAIVS : 203
Human grnzM AAH25701 : RMCNNSRFWN--GSLSPSMVCLAADSKDQAPCKGDSGGPLVCGKGRVLA GVLS : 199
Human grnzH A32692 : CQCEQLFHS---NYSRATEICVGDPKKTQTGFKGDSGGPLVCKD--VAQGILS : 197
Mus grnzC NP_034501 : QVCESQFQS---SYNRANEICVGDPSKIKGASFEEDSGGPLVCKR--AAAGIVS : 199
Mus grnzD NP_034502 : EECCKRFR---YYTETTEICAGDLKKIKTPFKGDSGGPLVCHN--QAYGLFA : 199
Mus grnzE NP_034503 : EECCKRFR---HYTETTEICAGDLKKIKTPFKGDSGGPLVCDN--KAYGLLA : 199
Mus grnzF AAA37741 : KECKKYFY---KYFKTMQICAGDPKKIQSTYSGDSGGPLVCDN--KAYGLLT : 199
Mus grnzG NP_034505 : EECCKLWY---TYSKTTOICAGDPKKVQAPYEGESGGPLVCDN--LAYGVVS : 199

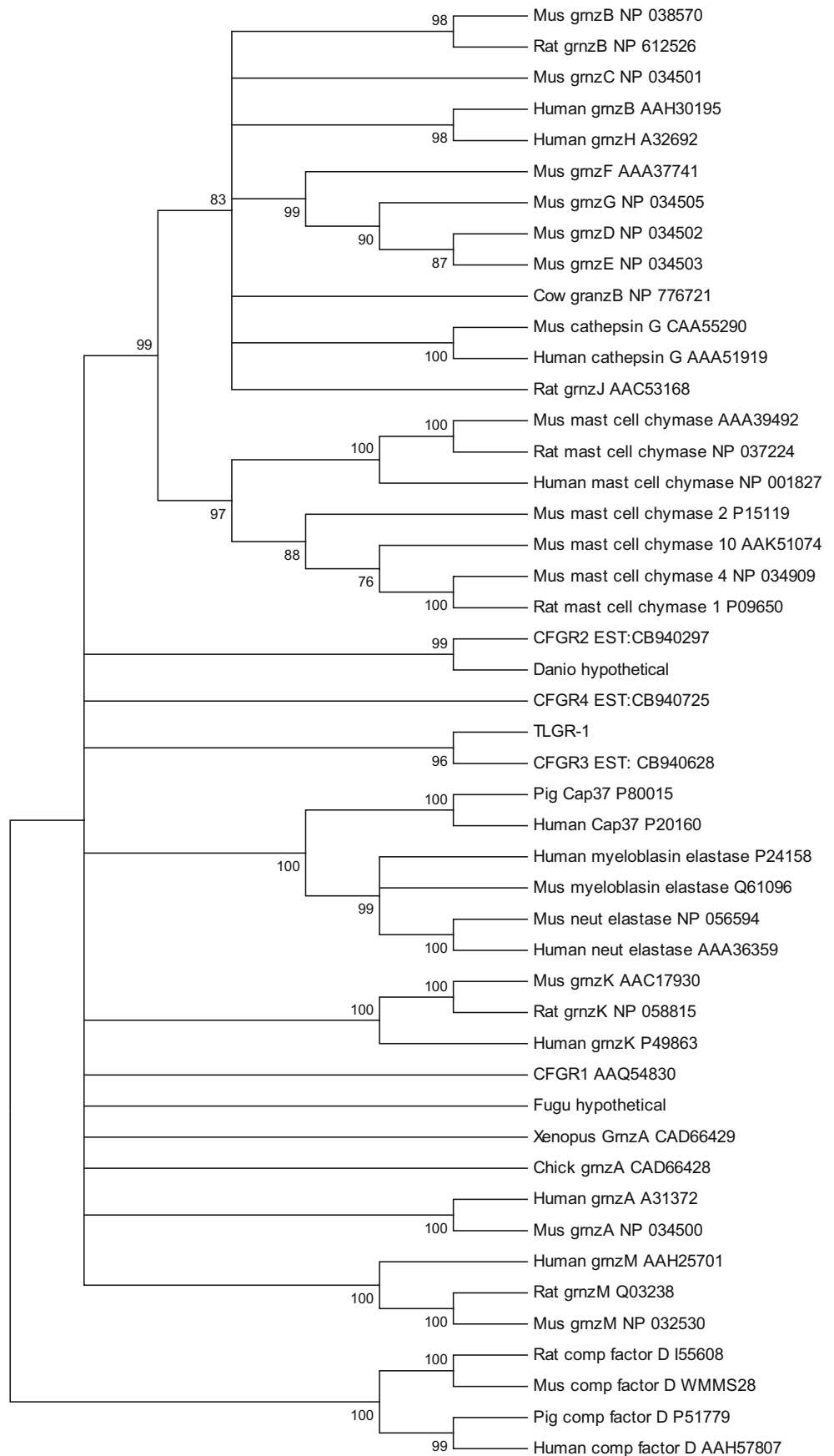
TLGR-1 : ENINGNCKYPNYPN-VYTDISKHLSWIKNII LNKKQC- : 229
CFGR1 AAQ54830 : GSGCGNPKK--PGVYTLLESKEHIDWINKI I KK--- : 231
Human grnzA A31372 : FGLNCKGDP RGPVYI LLSKKH LNWIMTIK GAV-- : 234
Human grnzB AAH30195 : YGRNNGMP---PR-ACTK VSSFVHWIKKTKMRH--- : 227
Human grnzK P49863 : GGHECGVATK--PGIYTLTKK YQTWIKSNLVPPHTN : 238
Human grnzM AAH25701 : FSSRVCTDIFKPP--VATAVAPYVSWIRKVTGRSA-- : 232
Human grnzH A32692 : YGNKKGTPPG-----VYIKVSHFLPWI KRTMKRL--- : 226
Mus grnzC NP_034501 : YGQTDGSAPQ-----VFTRVLSFVSWIKKTMKHS--- : 228
Mus grnzD NP_034502 : YAKNGTISSG-----IFTKVHFLPWI SWNMKLL--- : 228
Mus grnzE NP_034503 : YAKNRTISSG-----VFTKVHFLPWI SRNMKLL--- : 228
Mus grnzF AAA37741 : YGLNRTIGPG-----VFTKVHFLPWI SRNMKLL--- : 228
Mus grnzG NP_034505 : YGINRTITPG-----VFTKVHFLPWI STNMKLL--- : 228

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**Fig. 3** Comparison of TLGR-1 with other granzyme sequences. Multiple sequence alignments showing the conserved regions in TLGR-1 sequence, which represent the signature motifs for granzyme-like proteases. Conserved residues at the catalytic triad

are represented with a black circle above the alignment. Darker shading represents identical or residues with similar properties in 100% of the sequences, and lighter shading represents identical or residues with similar properties in 80% or more of all the sequences

**Fig. 4** Phylogenetic analysis of TLGR-1. Phylogram represents the evolutionary position of TLGR-1 compared to related serine proteases. The tree was derived by parsimony analysis, with Mega version 2.1. Sequences of mature protease were used in the analysis, and accession numbers are provided after the name. Numbers shown above the branches are bootstrap values based upon 1,000 replicates for parsimony. The tree was rooted on a subtree containing complement factor D to determine the clustering of TLGR-1 with similar granzyme





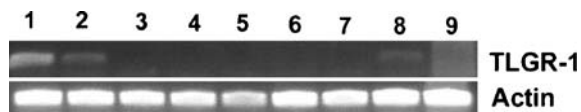
Based on the comparison of the residues in the TLGR-1, alignment with other granzyme sequences, and manual editing of the alignment based on crystallography structure profile (Hink-Schauer et al. 2002), a novel substrate specificity pocket triplet (GNN) can be predicted for this teleost granzyme. None of the other granzymes described to date share a specificity pocket triplet with TLGR-1. TLGR-1 has sequence similarity with almost all of the mammalian granzymes, with highest sequence similarity to granzyme B and G sequences.

#### Phylogenetic analysis of TLGR-1

The TLGR-1 sequence was compared with known serine proteases, and the data were analyzed by different methods to obtain clues on the evolutionary position of this granzyme. Phylogenetic analysis using different methods yielded trees with a similar topology, and a representative tree is depicted in Fig. 4. To resolve the difference between closely related granzyme sequences, the tree was rooted on a subtree comprising of complement factor D, which has been shown to be evolutionarily distinct from granzymes while sharing many sequence features. TLGR-1 did not cluster with any of the mammalian granzymes. However, it grouped with other teleost granzymes identified by Expressed Sequence Tags (EST) database searches. Comparison of substrate specificity pocket triplet residues in the teleost sequences revealed a close similarity to TLGR-1, suggesting that they may share similar primary specificities.

#### Expression pattern of TLGR-1

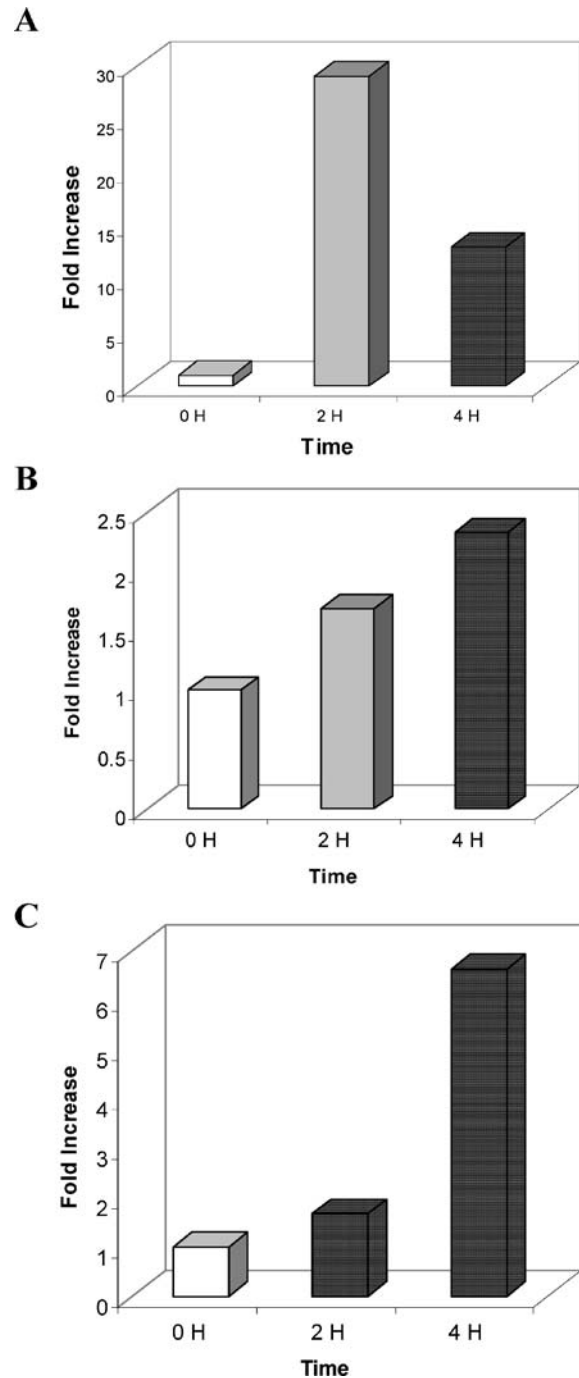
The expression of the TLGR-1 gene in various tissues was assessed by RT-PCR analysis using specific primers. Purified NCC were shown to be a major source for the expression of this gene (Fig. 5). Tissues rich in NCC [peripheral blood leukocytes (PBL) and anterior kidney] were the only places where the expression could be detected. Compared with the granzymes from catfish NCC, constitutive expression of TLGR-1 was found to be very low based on the intensity of the amplified bands.



**Fig. 5** Analysis of tissue expression of TLGR-1. RT-PCR analysis for TLGR-1 using cDNA from various tilapia tissues. Lanes: 1 purified NCC from peripheral blood, 2 total peripheral blood leukocytes, 3 total blood, 4 muscle, 5 liver, 6 gill, 7 spleen, 8 kidney, 9 TMB8. (A tilapia continuous cell line TMB-8 was provided by Dr. R. Hedrick, University of California-Davis. These cells were originally established from *Oreochromis mossambicus* cardiac tissue.) Beta actin was used as a normalizer

#### Transcriptional regulation of TLGR-1

The inducibility of the TLGR-1 message in tilapia NCC was assessed by RT-PCR. NCC were treated with various



**Fig. 6** Transcriptional regulation of TLGR-1 in NCC. RT-PCR analysis of cDNA from NCC purified from tilapia peripheral blood after subjecting to different treatments. **a** NCC were treated with recombinant tilapia TNF-alpha and incubated for 2 or 4 h and analyzed for TLGR-1 expression. **b** NCC treated with PMA and calcium ionophore (A23187) for 2 or 4 h. **c** NCC treated with lipopolysaccharides (from *E. coli*)

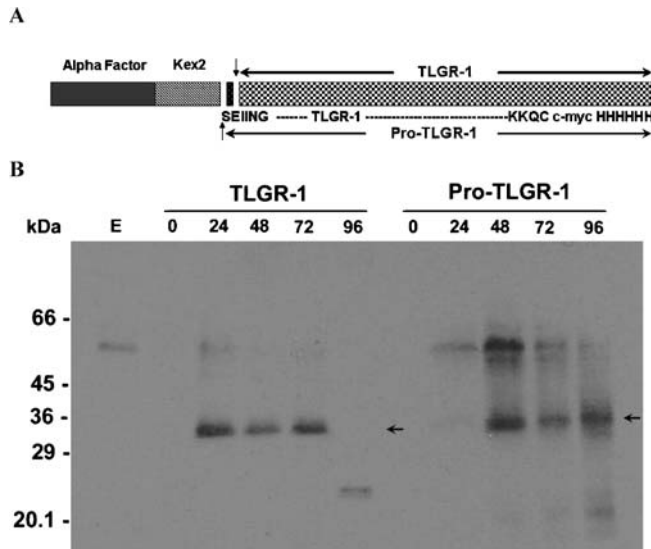
stimuli, and cDNA were prepared for analysis. Recombinant tilapia tumor necrosis factor (TNF) was found to have a profound effect on the transcription of TLGR-1 (Fig. 6a). Within 2 h of treatment with 100 ng/ml recombinant tilapia TNF, there was a 29-fold increase in TLGR-1 expression. The expression was reduced after 2 h but remained higher than base levels even after 4 h. Similarly, treatment of NCC with phorbol 12-myristate-13-acetate (PMA)/calcium ionophores resulted in more than twofold increase in TLGR-1 expression (Fig. 6b). More than sixfold increase in expression could be observed by treating the cells with lipopolysaccharide (LPS) for 4 h (Fig. 6c).

### Expression of recombinant TLGR-1

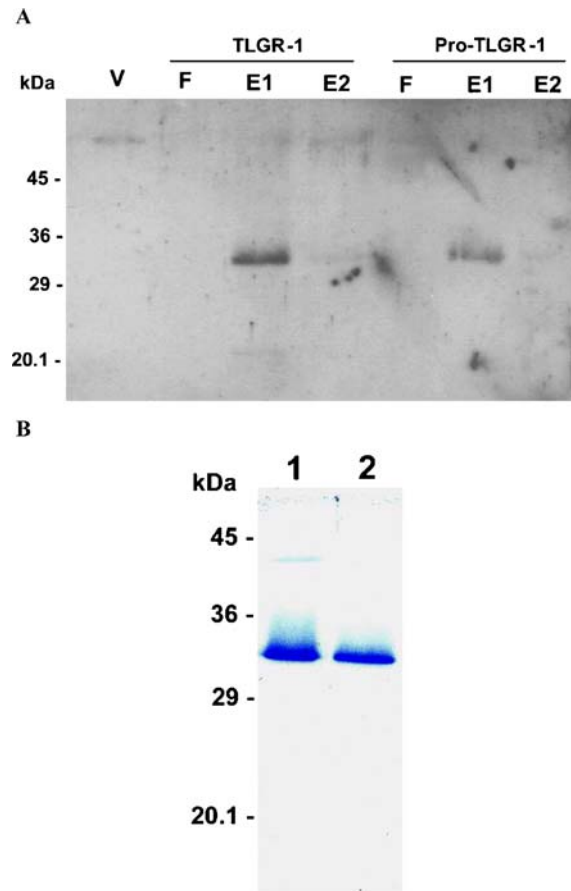
To express the pro and mature forms of TLGR-1, expression cassettes were engineered so that it could be inserted between *XhoI* and *XbaI* sites of yeast expression vector, pPICZ-alpha. Recombinant vectors were introduced in to the GS115 strain of *P. pastoris* so that the recombinant proteins were expressed as a fusion protein with an N-terminal alpha signal sequence and C-terminal granzyme. By inserting a Kex2 recognition sequence in

between the signal sequence and granzyme, active (cleaved) TLGR-1 could be secreted in to the culture supernatant. Incorporation of small C-terminal tags without affecting the protease activity has been demonstrated for many proteases before (Beresford et al. 1997; Huang et al. 1997, 1998; Xia et al. 1998). Both pro and mature recombinant TLGR-1 had a *c-myc* tag followed by a 6x-His-tag at the C-terminus (Fig. 7a). Expression of pro-TLGR-1 was achieved by retaining the activation dipeptide at the N-terminus. The dipeptide is known to yield inactive enzyme due to improper protein folding.

Several expression clones were identified by plating the transformed cells on YPD plates with Zeocin. Small-scale expression trials were done to assess the level of expression of selected clones upon induction with methanol (data not shown). Clones with highest expression levels were selected for further studies. Single yeast colony was grown and induced to secrete the recombinant proteins in 300 ml culture volume in 1,000-ml baffled flasks, and

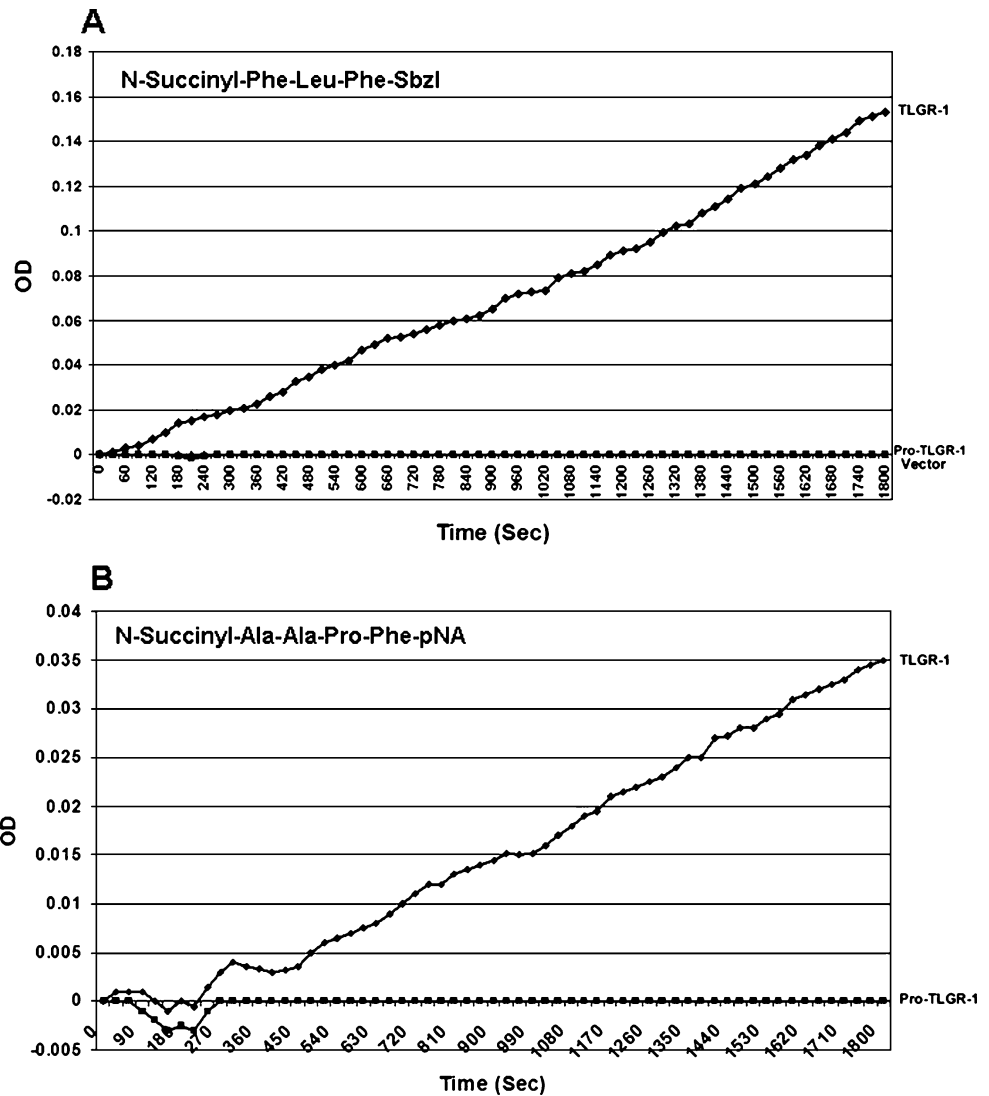


**Fig. 7** Production of recombinant TLGR-1 in *P. pastoris*. **a** Representation of expression cassettes engineered to generate pro and mature TLGR-1 using pPICZ-alpha expression vector. Pro and mature forms of TLGR-1 were expressed as fusion proteins starting with the yeast alpha factor at the N-terminus and polyhistidine and *c-myc* tagged granzyme at the C-terminus. Presence of a Kex2 site in between the alpha factor and the granzyme allowed the expression of granzyme with a native N-terminus, which can be secreted in to the culture medium with minimum contaminating yeast proteins. **b** Optimization of recombinant protein expression. The culture supernatants were collected at the time points indicated and subjected to TCA precipitation followed by Western blotting. His-tagged proteins were detected with INDIA-His probe conjugated to HRP followed by chemiluminescence. Yeast transfected with empty vector alone (E) also were checked for the expression of proteins, and only the 72 h time point is shown. The predicted bands representing TLGR and pro-TLGR are highlighted with arrowheads



**Fig. 8** Purification of pro and mature TLGR-1 recombinant proteins. Supernatants were collected after methanol induction for 72 h, and total proteins were concentrated by ammonium sulfate precipitation. His-tagged proteins were purified by nickel affinity chromatography. **a** Purification success of recombinant proteins verified by Western blotting and detection with INDIA-His probe. Lanes: V vector alone; F flow through from the column; E1, E2, and E3 three consecutive elutions of the column using buffer containing 250 mM imidazole. **b** Purity of the protein preparations verified by Coomassie staining of the gel. Lanes: 1 Pro-TLGR, 2 TLGR

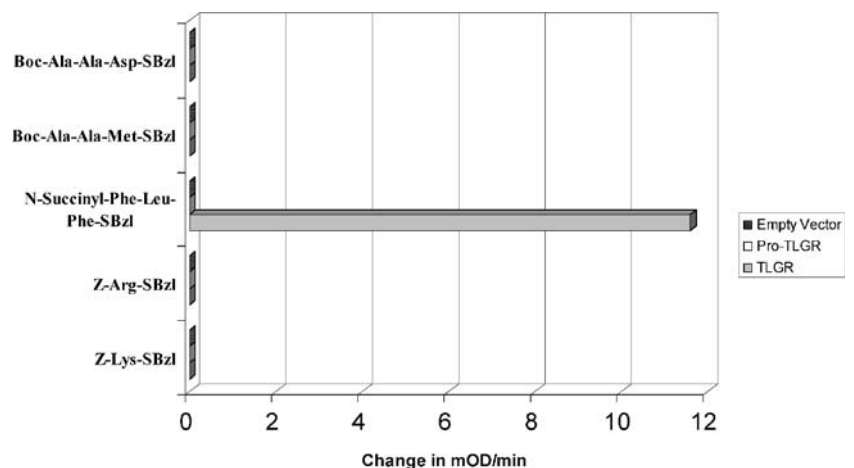
**Fig. 9** Enzymatic activity of recombinant TLGR-1. **a** Plot depicting the change in optical density against time from an in vitro enzyme kinetic assay using *N*-succinyl-Phe-Leu-Phe-SBzl as a substrate. **b** Hydrolysis of *N*-succinyl-Ala-Ala-Pro-Phe-pNA by mature and pro-TLGR-1. Both the substrates were used at a final concentration of 0.3 mM, and enzyme concentration was approximately 1 nM for both mature and pro-TLGR-1



culture supernatants were collected at various time points for TCA precipitation. The proteins were resolved on a 12.5% SDS–polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose. Expression levels of recombinant proteins were assessed by staining with His

probe. A protein of approximately 33 kDa was found to be upregulated in both mature and pro TLGR-1 culture supernatants (Fig. 7b). For mature TLGR-1, a high level of expression of recombinant proteins was observed from 24 h lasting until 72 h. From that point, there was

**Fig. 10** Substrate specificity of TLGR-1. The enzymatic activity of mature and pro-TLGR-1 was measured using the indicated thioester substrates in a final concentration of 0.3 mM. Enzyme concentration was approximately 1 nM for both mature and pro-TLGR-1. Change in optical density was measured for 30 min, and rate of hydrolysis is depicted as change in mOD/min



significant degradation of the proteins. Similarly, expression levels were high for pro-TLGR-1 from 48 h and remained high for 96 h (Fig. 7b).

#### High level expression and purification of recombinant TLGR-1

To assess the success of metal affinity chromatography, Ni-NTA agarose beads were mixed with a small volume (20 ml) of the culture supernatants, and His-tagged proteins were purified eluting the bound proteins in a buffer containing 250 mM imidazole. Almost all of the recombinant proteins were eluted in the first elutions, detected by Western blotting and protease activity assays (data not shown). Later, large volumes (1–2 l) of the culture supernatants were subjected to ammonium sulfate precipitation to concentrate the proteins. This was followed by nickel affinity chromatography to purify His-tagged proteins (Fig. 8).

#### In vitro esterolytic activity of recombinant TLGR-1

Due to the novel specificity pocket triplet residues in TLGR-1, it was expected not to hydrolyze thiobenzyl esters specially designed for granzymes with tryptase, Met-ase, or Asp-ase activity. The hydrolysis of synthetic chymase substrates was used to analyze the activity of TLGR-1. Effective hydrolysis of *N*-Succinyl-Phe-Leu-Phe-SBzl (Fig. 9a) and *N*-Succinyl-Ala-Ala-Pro-Phe-pNA (Fig. 9b) by TLGR-1 suggested its strong preference for bulky residues at the P<sub>1</sub> site. Later, a comparison of the hydrolysis of various thiobenzyl ester substrates revealed in fact that TPCR-1 had chymase activity TLGR-1 (Fig. 10). The pro-TLGR-1 failed to cleave any of the substrates, indicating that the presence of pro-peptide at the N-terminus prevented proper folding of the protease, which is crucial for its activity.

## Discussion

We have previously reported the expression of granzyme-like serine proteases in NCC purified from the peripheral blood of tilapia (Praveen et al. 2004). The full-length cDNA coding for TLGR-1 was identified and sequenced in two directions to obtain the putative amino acid sequence of the protein and to identify sequence motifs shared with other granzymes. Later, a genomic library was screened using specific primers to obtain a cosmid clone containing full-length TLGR-1 gene. We sequenced genomic DNA comprising the full gene and the promoter region (Fig. 1). Compared to other granzyme sequences, the TLGR-1 gene had a smaller size. However, the intron–exon structure of the gene had closest similarity to other mammalian granzymes (Kelly et al. 1996; Trapani 2001). Additionally, the number and position of introns of TLGR-1 suggest that this could be a member of the sixth class of serine proteases

(Irwin et al. 1988), like NK cell-specific granzyme M (Kelly et al. 1996; Pilat et al. 1994). The first one of the substrate specificity triplet residues (Gly 173; –6 with reference to active site Ser) is encoded within the fourth exon (Fig. 1), as in the case of other chymotrypsin-like proteases (Bell et al. 1984).

Studies on proximal promoter elements of mammalian granzymes had revealed the conserved sequences enabling cell-specific expression of these proteases. Granzyme B promoter has binding sites for T cell-specific transcription factors such as Ikaros and core-binding factor as well as for more ubiquitous transcription factors like AP-1 (Haddad et al. 1993; Kamachi et al. 1990; Wang and Speck 1992). These sequences are found to be sufficient to drive reporter gene expression in T cell lines where these factors are constitutively active (Fregeau and Bleackley 1991; Hanson et al. 1991). The promoter region of granzyme M gene has been shown to have binding sites for factors like AP-2, AP-3, and glucagons-G3A, influencing its bias for expression in NK cells (Kelly et al. 1996; Smyth et al. 1995a). The presence of multiple transcription factors in the promoter region of the TLGR-1 gene in NCC is an indirect indication that this gene is highly regulated during the immune response.

Highly conserved Cys residues at similar positions in all granzyme sequences give rise to three pairs of disulfide bonds, which are crucial in proper folding and activation of granzyme during processing. Among these disulfide bonds, the ones between Cys26 and Cys42 as well as between Cys151 and Cys167 link the primary and secondary substrate-binding sites, forming S1 subsites in granzymes A, K, and M (Sattar et al. 2003). A fourth pair of Cys residues forms an additional disulfide bond, which bridges the active site Ser residue is found in granzymes M and K (Kelly et al. 1996; Przetak et al. 1995). Granzyme A is the only mammalian granzyme shown to have one additional Cys residue, which is responsible for intermolecular disulfide bonds (Bell et al. 2003; Hink-Schauer et al. 2003). TLGR-1 was shown to have two additional Cys residues (Fig. 2). At this time, their function is unknown, as their positions do not match with those corresponding to the dimer-forming Cys residue of granzyme A (Hink-Schauer et al. 2003).

Multiple sequence alignment of TLGR-1 with other granzymes revealed highly conserved regions shared by all known granzymes (Fig. 3). The N-terminal IIXG motif and catalytic triad residues that make up the charge relay system were highly conserved. Moreover, residues around the triad residues were highly conserved among all the sequences. The presence of Gly residue at position –6 upstream of the active site Ser indicated a substrate specificity that was different from that of granzymes A/K, B, and M. Other residues at positions +15 to +17 and +28 relative to the active site Ser are found to be important in determining the primary substrate specificity of Ser protease (Bode et al. 1989). TLGR-1 has Ser-Phe-Asn (+15 to +17) and Asn (+28) at these positions, which is quite different from all other known granzymes. A comparison of crystal structures of Ser proteases has led



to a theory that three residues of the S<sub>1</sub> substrate-binding pocket confer primary specificity. These specificity-conferring residues are located at positions 189, 216, and 226 (chymotrypsin numbering; Wouters et al. 2003). Aligning the TLGR-1 sequence with other granzymes and matching the secondary structure elements, the substrate specificity pocket residues for this protease were determined as Gly-Asn-Asn. These triplet amino acid residues have not been previously described in mammalian granzymes.

Multiple sequence comparisons alone are not sufficient to draw conclusions on the evolutionary positions of closely related proteins like granzymes. Phylogenetic analysis of mammalian granzymes has revealed the clustering of granzymes with similar substrate specificity, giving rise to the classification of granzymes into three major groups (Grossman et al. 2003; Wouters et al. 2003). Similar analysis of teleost granzymes along with mammalian serine protease placed TLGR-1 in a separate cluster, indicating a parallel evolution for teleost granzymes. Catfish granzymes with similar predicted substrate specificity were the other members of that cluster. The experimental verification of the enzymatic activity for the catfish granzymes would be crucial to make the correlation between substrate specificity and sequence similarity.

The pattern of tissue expression of TLGR-1 suggests that this protease is associated with cytotoxic cell populations (Fig. 5). Tissues with appreciable TLGR-1 expression were the ones with high NCC density. The use of cDNA derived from total tissues for expression studies could be misleading due to the interference from different cell populations that might be present in these tissues. The cell line (TMB-8), which has been derived from heart tissue, was used to assess the specificity of expression with more confidence. TMB-8 cells have been used as nonhematopoietic and noncytotoxic controls in previous experiments (Taylor et al. 2001). Compared to the CFGR-1, which had a high level of constitutive expression in NCC from different tissues (Praveen et al. 2004), TLGR-1 expression levels were very low in resting NCC (Fig. 5). Transcriptional upregulation of TLGR-1 with various stimuli indicated the high degree of inducibility for this tilapia granzyme (Fig. 6). Transcriptional activation of granzyme A in response to TNF has been previously reported in lymphokine-activated killer cells and CTLs (Owen-Schaub et al. 1989; Robinet et al. 1990).

Purification of catalytically active recombinant granzymes is critical in understanding their individual physiological roles, compared to combined effects of multiple granzymes in purified cytotoxic granules. The generation of mammalian granzymes in recombinant forms has been reported (Beresford et al. 1997; Kummer et al. 1996; Pham et al. 1998; Smyth et al. 1995b; Sun et al. 1999; Wilharm et al. 1999a; Xia et al. 1998). The use of the *P. pastoris* expression system with pPICZ-alpha expression vector allowed the generation of recombinant granzymes with the desired N-terminal end. By inserting the tilapia granzyme expression sequence downstream of an alpha mating signal sequence, secretion of the protein to the expression me-

dium was possible. The pro-TLGR-1 was generated by retaining two residues at the N-terminus of the mature protease (Fig. 7a). This has been shown to prevent the proper folding and activation of mammalian recombinant granzymes (Kummer et al. 1996; Sayers et al. 1996; Sun et al. 1999). The supernatants were collected at various time points, and expression levels were compared by Western blotting (Fig. 7b).

Purification of recombinant TLGR-1 from culture supernatants was carried out after concentrating the proteins by ammonium sulfate precipitation. This method had been previously used without affecting the proteolytic activities of human mast cell chymase (Lockhart et al. 2005). After the ammonium sulfate precipitation, the pellet was dissolved in PBS and concentrated to 50 ml followed by dialyzing against PBS to remove traces of ammonium sulfate. His-tagged proteins were purified by nickel affinity chromatography (Fig. 8). The Western blot analysis of TLGR-1 suggests that the protein might be glycosylated. The predicted molecular mass of mature TLGR-1 is 25.3 kDa, while the protein appeared to have an approximate mass of 33 kDa, suggesting the presence of sugar residues in the predicted glycosylation site on the polypeptide chain. Glycosylation of granzymes is critical, because they have been shown to be targeted to the lytic granules by the mannose-6-phosphate receptor (Griffiths and Isaacs 1993).

Granzymes are characterized by their very narrow substrate specificity. The requirement for P<sub>1</sub> residue varies significantly between different classes of mammalian granzymes (Kam et al. 2000). In most cases, the substrate specificity of individual granzymes can be predicted based on the architecture of its substrate-binding pocket (Wouters et al. 2003). Such prediction algorithms suggested that TLGR-1 had a preference for bulkier P<sub>1</sub> residues than that for tryptases, Asp-ases, or Met-ases. Using synthetic substrates designed with a bulky Phe residue at the P<sub>1</sub> site, the activity of TLGR-1 was shown to be similar to that of mammalian chymases (Figs. 9 and 10).

In conclusion, the novel serine protease identified from tilapia NCC was shown to have chymase activity. It is not clear whether these cells do express other granzymes and other components of granule exocytosis pathway. The TLGR-1 gene is highly inducible with various stimuli. Compared to the expression pattern of similar granzymes in channel catfish NCC, tilapia granzymes require longer activation for the transcription and translation of cytotoxic molecules, as determined by the protease activity of supernatants from granule exocytosis assay (data not shown). Studies are underway to uncover the physiological roles of this protease in these cytotoxic cells and also to identify other granule components in tilapia NCC.

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