Molecular cloning of a cystatin from parasitic intestinal nematode, *Nippostrongylus brasiliensis*

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Abstract : A novel member of the cystatin family, nippocystatin (NbCys), was identified from excretory-secretory (ES)-products of a nematode *Nippostrongylus brasiliensis*, and the cDNA was cloned and sequenced. The mRNA of NbCys was confirmed to be expressed in both larvae and adults of the parasite. NbCys was translated as a proform with a single domain for secretion and was detected as a 14-kDa mature form in ES-products of the adult worm. Recombinant protein of NbCys profoundly inhibited the activity of cysteine proteases such as cathepsin L and B, but not that of cathepsin D, an aspartic protease. Furthermore, the ES-products had also been confirmed to inhibit cysteine proteases. Taken together, NbCys may play a role in evasion of *N. brasiliensis* from host defense systems, since cysteine proteases are known to participate in immune systems of infected hosts.
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Keywords : *Cystatin, Nippostrongylus brasiliensis, cloning*

INTRODUCTION

Natural cysteine protease inhibitors are part of the cystatin superfamily, which is subdivided into families 1, 2 and 3, or the stefin family, cystatin family and kininogen family, respectively (1). There is high sequence homology among all of these families, particularly with respect to a highly conserved, proposed reactive site consisting of 5 amino acids, Gln-Val-Val-Ala-Gly (QVVAG) (2). Members of family 2, the cystatin family, are secretion-type proteins that have a single domain with a molecular weight of 13-15-kDa. In nematodes, members of this family have been identified from *Caenorhabditis elegans* (3) and some species of filaria (4-7).

Nippostrongylus brasiliensis, from the order Rhabditida, generally produce acute infections in rodents, and have life cycles similar to trichostrongyle parasites that infect the small intestines of humans and livestock (8). Although *N. brasiliensis* is supposed to express cystatin, there is no report of cystatin derived from this nematode.

We here describe a newly defined, 14-kDa nippocystatin (NbCys) belonging to the cystatin family 2 and derived from *N. brasiliensis*. This was a secretion-type cystatin, and its recombinant protein selectively inhibited cysteine proteases. Cysteine protease is indispensable for antigen-specific immunity because of its function in antigen processing and presentation (9,10). Therefore, this parasite may require this protease inhibitor to evade the host defense system via modulation of the host immune responses.

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MATERIALS AND METHODS

1. Parasites

The strain of *Nippostrongylus brasiliensis* was provided by Dr. M. Yamada, Kyoto Prefectural University of Medicine, Kyoto, Japan (11). Parasites were maintained by serial passage in SD rats. Excretory-secretory (ES)-products of adult *N. brasiliensis* were collected by the protocol described below. Adult worms of *N. brasiliensis*, collected from the small intestine of rats that had been infected 7 d previously with 4,000 infective-stage larvae per animal, were sterilized by

repeated washing with PBS containing penicillin and streptomycin. Worms were cultured with PBS at 37°C for 6 hr. Culture supernatant was collected and concentrated using Centricon Plus 20 PL-10 centrifuge (Millipore, Bedford, MA).

2. cDNA cloning of NbCys

Total RNA was isolated from adult worms of *N. brasiliensis*, prepared by the method above, using Trizol reagent (Life Technologies, Rockville, MD). A fragment of NbCys cDNA was obtained by RT-PCR. The total RNA was reverse-transcribed using hexanucleotide random primers with Superscript II reverse transcriptase (Life Technologies). Then the cDNA was amplified with Taq DNA polymerase (Takara Shuzo, Shiga, Japan). The thermocycle conditions were 35 cycles of 94°C for 30 sec, 52°C for 30 sec and 72°C for 30 sec. The sense and antisense primer sequences were 5'-TCATCTCAAGTTGTCGCTGGT-3' and 5'-AAATTTTCCCATGGTTTCTCCCA-3', the designs of which were based on conserved sequences among previously defined cystatin from other nematodes, *Onchocerca volvulus* (4), *Brugia malayi* (7), *Acanthocheilonema viteae* (6) and *Caenorhabditis elegans* (3). Amplified DNAs were resolved by 2-3% agarose gel electrophoresis and stained with ethidium bromide. The DNAs were extracted using Qiaex II gel extraction kit (Qiagen, Hilden, Germany) and then subcloned into pGEM-T easy vector (Promega, Madison, WI). Plasmids were extracted using a Qiaprep spin miniprep kit (Qiagen). The DNA sequences were defined using ABI PRISM BigDye terminator cycle sequencing ready reaction kit (Perkin Elmer, Norwalk, CT) and ABI PRISM 377 DNA sequencer (Perkin Elmer). On the basis of the nucleotide sequence of this cDNA fragment, specific primers were then synthesized for 3'- and 5'-RACE. For 3'-RACE, total RNA from adult *N. brasiliensis* was reverse-transcribed using Not I primer-adapter with dT-polymer (Life Technologies). The adapter-added cDNA was amplified with Taq DNA polymerase. The thermocycle conditions were 35 cycles of 94°C for 1 min, 64°C for 1 min and 72°C for 1 min. The sense primer sequence was 5'-GCAAGCGAACTTACGGCGACGA-3', and the antisense primer was the adapter primer. For 5'-RACE, total RNA from adult *N. brasiliensis* was reverse-transcribed using the gene specific primer, 5'-TGGCAGTTCGTCGCCGTAAGTTCG-3'. The dC-polymer was added to the resultant RT-product with terminal deoxynucleotidyltransferase (TdT) and was amplified with Taq DNA polymerase. The thermocycle conditions were 30 cycles of 94°C for 1 min, 54°C for 1 min and 72°C for 1 min. The sense primer was the adapter primer with dG-polymer (Life Technologies) and the antisense primer was 5'-TCGCCGTAAGTTCGCTTGCAG-3'. The DNA sequences of the RACE-PCR products were defined as written above.

3. Evaluation of cystatin mRNA expression

Total RNA from adult worm and from third stage larvae were extracted with Trizol reagent and were reverse-transcribed using hexanucleotide random primers with Superscript II. Then the cDNAs were amplified with Taq DNA polymerase. The thermocycle conditions were 35 cycles of 94°C for 1 min, 54°C for 1 min and 72°C for 1 min. The sense and antisense primer sequences were 5'-ATGCCATCTGCGTTCGTTCTG-3' and 5'-TTAAACCTGCTCTCCTGGAGC-3'. The amplified DNAs were resolved by 2% agarose gel electrophoresis, stained with ethidium bromide and visualized with UV light.

4. Expression of rNbCys

The NbCys cDNA was subcloned into the expression plasmid vector pET and expressed in *E. coli* strain BL21 as a protein fused to a leader sequence of influenza virus HA epitope and six histidines. rNbCys was purified from *E. coli* lysate by affinity chromatography using TALON resin (Clontech Laboratories Inc., Palo Alto, CA). The purity of rNbCys was determined by SDS-PAGE and staining with Coomassie brilliant blue (CBB). Purified rNbCys was washed and dialyzed with PBS.

5. Measurement of protease activities and their inhibition

The activity of cysteine proteases was measured as follows. Protease was incubated with or without NbCys in 0.1 M sodium acetate buffer [pH 5.5] containing 1 mM EDTA, 8 mM cysteine and 20 μM Z-Phe-Arg-MCA (Peptide Institute, Osaka, Japan) as substrate at 37°C for 15 min. The reaction was stopped by addition of 100 mM monochloroacetate [pH4.3]. The amount of product was monitored fluorometrically with excitation at 370 nm and emission at 460 nm in a fluorescence spectrometer (Hitachi, Ibaraki, Japan). Activity of cathepsin D was measured in a Folin-Lowry reaction, as reported previously (12).

RESULTS

The cystatins identified from parasitic nematodes are all from species of the superfamily Filarioidea,

namely filaria. Although cystatin has also been identified in *C. elegans* by analysis of genomic sequence, this nematode is free living. All of the cysteine protease inhibitors from nematodes reported to date belong to the cystatin family. To obtain a homologue of cystatin from *N. brasiliensis*, we amplified cDNA by PCR using primers of a consensus sequence found among nematode cystatins. A PCR product of 174 bp was amplified as expected.

On the basis of the sequence of this cloned cDNA fragment, the full length of the cDNA was cloned by using 3'-RACE and 5'-RACE (Fig. 1). The length of the open reading frame was 435 bp and the protein consisted of 144 amino acids. An amino acid alignment of the protein with cystatins from other nematodes using the BLASTP program with the sequence databases at NCBI revealed the following similarities : two cystatins from *C. elegans* (72% and 63%), onchocystatin from *Onchocerca volvulus* (55%), *Acanthocheilonema viteae* cystatin (33%), *and Brugia malayi* cystatin (30%). The amino acid sequence of NbCys showed a lesser similarity to rat cystatin C (27%). This cloned protein had a stretch of 5 amino acids, QVVAG, which is highly conserved in the cystatin superfamily, and had a hydrophobic signal peptide like other members of cystatin family 2 (Fig. 2). We concluded that this newly defined molecule belongs to family 2, and named this molecule nippocystatin (NbCys).

We evaluated whether NbCys mRNA is expressed in infective third stage larvae as well as in adult worms by RT-PCR. The open reading frame of NbCys cDNA was amplified as a 435 bp band from total cDNA of both larvae and adult worm (Fig. 3). These findings indicate that NbCys was expressed at the mRNA level at both developmental stages.

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        10         20         30         40         50         60
CTTTATGGGCGGGTGCGTTCAGTAAACATGCCATCTGCGTTCGTTCTGCGTATTGCTTTA
                                M  P  S  A  F  V  L  R  I  A  L

        70         80         90         100        110        120
GCATCGGTTGTGGTTACCAGCACAGTATCATCAATGGTTGGCGGTTTCACTCCTCAGGAT
 A  S  V  V  V  T  S  T  V  S  S  M  V  G  G  F  T  P  Q  D

        130        140        150        160        170        180
GTGTCTGACCCAGAGTACATGACACGAGCATGGAAGGCTGCGAAGGGAATTAATGATGAC
 V  S  D  P  E  Y  M  T  R  A  W  K  A  A  K  G  I  N  D  D

        190        200        210        220        230        240
GCGTCCAATGAGGGACCATATCATATGATTCCAGTCAAGATTTTAAACGCGAAGACTCAA
                                                PCR >>>>>>>>>
 A  S  N  E  G  P  Y  H  M  I  P  V  K  I  L  N  A  K  T  Q

        250        260        270        280        290        300
GTGGTCGCCGGAGTGAACCATGTTTTCGAAGTTCTTTTCGGAGAATCTAGCTGCAAGAAA
>>>>>>>>>>>>
 V  V  A  G  V  N  H  V  F  E  V  L  F  G  E  S  S  C  K  K

        310        320        330        340        350        360
GGCGACCTATCTGCAAGCGAACTTACGGCGACGAACTGCCAATTGAAGGAGGGAGGAAGG
           <<<<<<<<<<<<<<<<<<<< 5'RACE
    3'RACE >>>>>>>>>>>>>>>>>>>>>>
 G  D  L  S  A  S  E  L  T  A  T  N  C  Q  L  K  E  G  G  R

        370        380        390        400        410        420
AAAGTGATCTACGAAGTGCATCTATGGGAGAAACCGTGGGAGAACTTTGAGCAGTTCAAT
                          <<<<<<<<<<<<<<<<<<<<<<<< PCR
 K  V  I  Y  E  V  H  L  W  E  K  P  W  E  N  F  E  Q  F  N

        430        440        450        460        470        480
GTGAAGAAAGTTCGAACGCTTGCTCCAGGAGAGCAGGTTTAATAGACCGGCATACATATT
 V  K  K  V  R  T  L  A  P  G  E  Q  V  *

        490        500        510        520        530        540
TTAAATTCTACTCGCCAGTTATTCCTTTGTTGTGCTACTTTGAACACATGCAGTTACCGT

        550        560        570        580        590        600
TGTATCGGTCAACGTTTCAAGTGCGAACTGTACATTAGAGAATTCGTCTTATCTACTTAT

        610        620        630        640        650        660
CCGCACACATAAATTCGTTTCGTGTTTTGTACGCGTGAATGTCAAAGGGAAGATACAGGA

        670        680        690        700        710
TTGAAAAATTGTGAAAAAAATAAATATGAGGATTCCCGGGAAAAAAAAAA
```

Fig. 1. Sequence of nippocystatin cDNA. Conserved sequences of the cystatin superfamily are shaded. Gene-specific primers of PCR for cDNA cloning of nippocystatin are marked by (>>>) for the 5' primer and by (<<<) for the 3' primer. The signal peptide sequence predicted using the program SignalP v1.1 is underlined (13). The sequence data of nippocystatin are available from GenBank under accession number AB050883.

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NbCys       1 ----------------------------MPSAFVLRIALASVVVTSTVSSMVGGFTPQDV  32
                                           .  *     .        .  **   .*
CeCys         ----------------------------M-KAILVFALIAISIISVNAGMMTGGSVEQDA
OvCys         MLTIKDGTLLIHLLLFSVVALVQLQGAKSARAKNPSKMESKTGENQDRPVLLGGWEDRDP

Cys.C,rat     ---------------------------------VLAVAWAGTSRPPPRLLGAPQEADA

NbCys      33 SDPEYMTRAWKAAKGINDDASNEGPYHMIPVKILNAKTQVVAGVNHVFEVLFGESSCKKG  92
              .. *.   .... ...*.........*.  *.* ..... *****.  . .*....* ***.
CeCys         SQKEYSDKAWKAVKGINDQASNNGPYYYAPIKVTKASTQVVAGISTKLEVLVGESNCKKG
OvCys         KDEEILELLPSILMKVNEQSNDE--YHLMPIKLLKVSSQVVAGVKYKMDVQVARSQCKKS

Cys.C,rat     SEEGVQRALDFAVSEYNKGSNDA--YHSRAIQVVRARKQLVAGINYYLDVEMGRTTCTKS

NbCys      93 DLSASELTATNCQLKEGGRKVIYEVHLWEKPWENFEQFNVKKVRTLAPGEQV         144
               . . ..*  ... . ..    ..*  ********... . . . .   ..
CeCys         ELQAHEITSSNCQIKDGGSRALYQVTIWEKPWENFEQFTVEKIRDVTADEQF
OvCys         SNEKVDLTKCKKLEGHPEKVMTLEV--WEKPWENFMRVEILGTKEVIK----

Cys.C,rat     QTNLT-NCP-FHDQPHLMRKALCSFQIYSVPWKGTHTLTKSSCKNA------
```

Fig.2. Alignment of the amino acid sequence translated from cDNA of nippocystatin (NbCys), *C. elegans* cystatin (CeCys), onchocystatin from *O. volvulus* (OvCys) and rat cystatin C (Cys.C, rat). Conserved sequences of the cystatin superfamily are shaded. Among three sequences of cystatin from nematodes, completely conserved amino acids are marked by an asterisk (*), and the partially conserved amino acids are marked by a period (.).

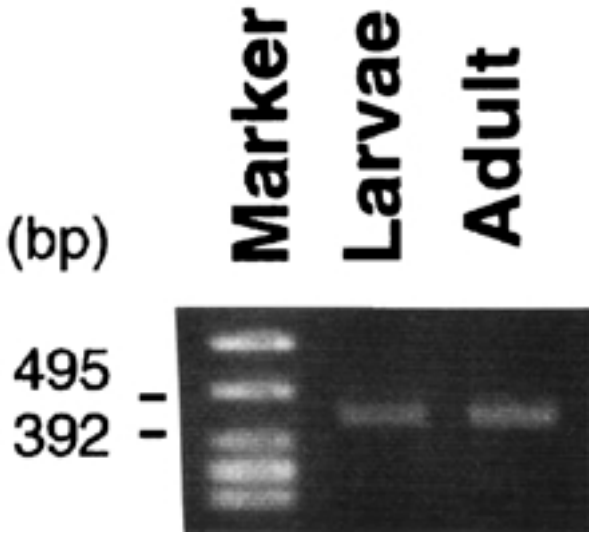

Fig.3. The mRNA expression of nippocystatin. The total RNA was extracted from adult and third stage larvae and the level of nippocystatin mRNA was determined by RT-PCR. The PCR products were resolved by 2% agarose gel electrophoresis, stained with ethidium bromide and visualized with UV light as a band with a size of 435 bp.

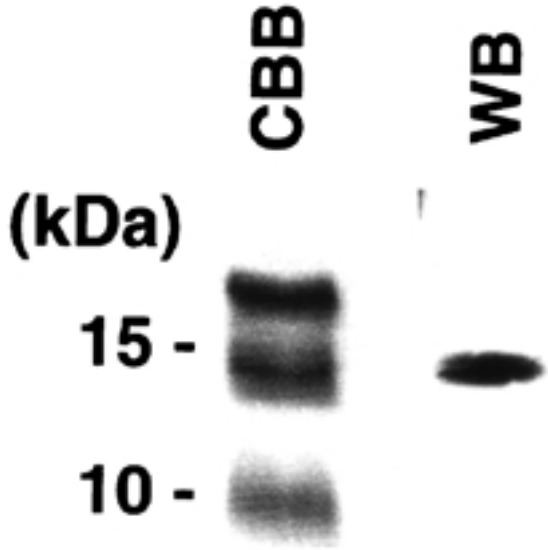

Fig.4. Nippocystatin contained in ES-products. ES-products were separated by SDS-PAGE [15%] and stained with CBB (left). Nippocystatin was detected by immunoblot analysis with rat anti-rNbCys antiserum (right).

Since the cDNA of NbCys encoded a hydrophobic signal peptide with 22 amino acids, NbCys was expected to be secreted as a 14-kDa mature form without a signal sequence. To confirm that NbCys is expressed as a mature form, we analyzed ES-products by immunoblotting with anti-recombinant NbCys (rNbCys) antiserum (Fig. 4). We detected NbCys as a 14-kDa band in ES-products, suggesting that NbCys is actually expressed as a protein of mature form.

To investigate the function of NbCys as a cystatin, we used rNbCys to examine the ability to inhibit several cathepsins (Fig. 5). rNbCys strongly inhibited the enzymatic activity of two cysteine proteases, cathepsin L and cathepsin B. Cathepsin L was inhibited at a lower concentration of rNbCys than cathepsin B. On the other hand, aspartic protease cathepsin D was

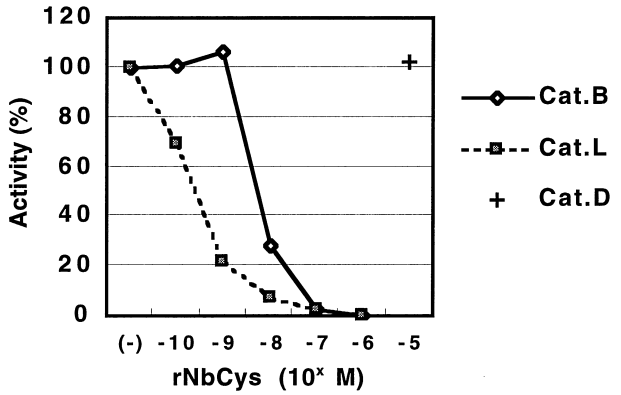

Fig.5. The inhibition of proteases by rNbCys. Purified cathepsin B (rhombus), cathepsin L (square) and cathepsin D (cross) were incubated with each of the substrates in the presence of various concentrations of rNbCys. Incubation of a cathepsin without rNbCys corresponds to 100% activity of the enzyme.

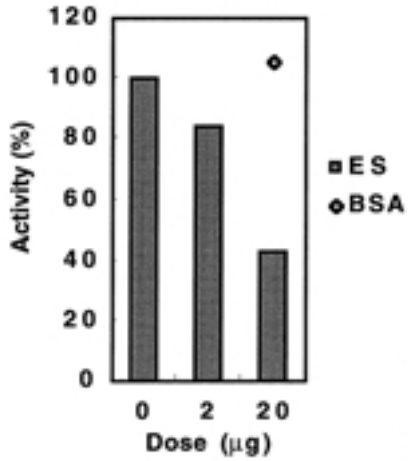

Fig.6. The inhibition of a cysteine protease by ES-products from *N. brasiliensis*. Purified cathepsin L was incubated with the synthetic substrate Z-Phe-Arg-MCA in the presence of various amounts of ES-products from *N. brasiliensis* or 20μg of BSA. Protease activity was assessed by subtraction of ES-derived activity. Incubation of cathepsin without ES-products corresponds to 100% activity of the enzyme.

not inhibited by rNbCys even at extremely high concentrations. These findings indicate that NbCys has the ability to inhibit cysteine proteases, like other members of the cystatin family.

To evaluate whether ES-products from *N. brasiliensis* actually have a component to inhibit cysteine proteases, we examined the ability of ES-products to inhibit cathepsin L as an index. When cathepsin L was incubated with ES-products, cathepsin L activity was inhibited in a dose-dependent manner (Fig. 6). This inhibitory activity in ES-products was not due to the large amount of protein, since an equivalent amount of BSA did not suppress the cathepsin L activity. NbCys was expressed in ES-products as a mature form (Fig. 4). These findings strongly suggest that the cysteine protease inhibitory component in ES-products is NbCys.

DISCUSSION

We newly cloned a cystatin from ES-products of intestinal nematode, *N. brasiliensis*. It consisted of 144 amino acids with a MW of 14-kDa and was named NbCys. NbCys was expressed in both third stage larvae and adult worm. Further, we synthesized its recombinant protein and confirmed that this protein exclusively inhibited cysteine proteases selectively. Immunoblot analysis showed that ES-products from *N. brasiliensis* contained NbCys. Furthermore, ES-products were confirmed to inhibit cysteine protease activity *in vitro*. These findings suggest that NbCys is secreted as a mature form and acts in the host.

Onchocystatin from the filarial parasite, *Onchocerca volvulus*, is the first cystatin to be identified in nematode (4). Onchocystatin belongs to cystatin family 2, having signal peptide and being expressed in third stage larvae and adults, suggesting that this protein is a secretion-type and has an extrasomatic function. Analysis of the genomic sequence in the free living nematode, *C. elegans*, indicated that this nematode has a cystatin encoded by two genes (3). The amino acid sequence of NbCys showed greater similarity to the sequence of two cystatins from *C. elegans* than to that of onchocystatin. These similarities do not contradict taxonomical findings, since both *N. brasiliensis* and *C. elegans* belong to the order Rhabditida, while *O. volvulus* belongs to the order Spiruria.

Recently there have been several reports that cystatin modulates immune responses. For example, chicken cystatin up-regulates NO-release from IFN-γ-activated mouse peritoneal macrophages via cytokine synthesis (14, 15). Cystatins from parasites have also been reported to show immunomodulatory effects. Recombinant cystatin from *Acanthocheilonema viteae* down-regulates T-lymphocyte proliferation and enhances IL-10 production *in vitro* (6). Furthermore, Garraud *et al*. reported that recombinant cystatin of *Onchocerca volvulus* induces either polyclonal or antigen-specific IgE and IgG4 antibodies *in vitro* in an IL-4-and/or IL-13-dependent manner (5). Thus, it is quite conceivable that NbCys also has immunomodulatory functions.

We previously reported that treatment of mice with CA074, a synthetic and specific inhibitor for cathepsin B, changes the immune response from a Th2- to Th1-type in BALB/c mice infected with *Leishmania major* (16) or immunized with ovalbumin (17). That is, treatment with CA074 suppresses IL-4-production

and augments IFN-γ-production. In general, host defense against helminth infection is mediated by Th2-type responses with IgE production, eosinophilia and mastocytosis (8). That NbCys has a similar effect as CA074 is quite reasonable since *N. brasiliensis* can defeat the host immunity by impairing the Th2-type responses.

ES-products of *N. brasiliensis* are well known as antigens that evoke an immune response in hosts infected with *N. brasiliensis* (11). Some functional proteins that induce the production of specific antibodies in infected rats have been isolated from ES-products of *N. brasiliensis* (18, 19). At present, however, although these products have been regarded as immunodominant antigens, their immunomodulatory effects have not been elucidated (18, 19). Our results suggest that NbCys is a novel candidate for the immunomodulatory effector secreted by *N. brasiliensis*.

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