J. Biochem. 121, 642-647 (1997)

Release of a Newly-Identified Cysteine Protease, Tetrain, from *Tetrahymena* into Culture Medium during the Cell Growth[1]

Kazu-Michi Suzuki,* Natsumi Hosoya,† Tadao Takahashi,* Toshikazu Kosaka,* and Hiroshi Hosoya*,[2]

*Department of Biological Science, Faculty of Science, Hiroshima University, Higashi-Hiroshima, Hiroshima 739; and †School of Social Information Studies, Otsuma Women's University, Karakida, Tama, Tokyo 206

Received for publication, October 4, 1996

Protease activity in the culture medium of *Tetrahymena pyriformis* markedly increased during the growth of the ciliate. The protease activity in the culture medium was purified by sequential column chromatographies. The purified protease had an apparent molecular mass of 28 kDa. N-terminal amino acid sequencing analysis suggested that the protease is a mature form of cysteine protease. Requirements of free sulfhydryl groups for activity and sensitivity to *N*-tosyl-L-phenylalanine chloromethyl ketone and *Nα*-*p*-tosyl-L-lysine chloromethyl ketone also indicated that the protease is a member of the papain family of cysteine proteases. The protease was designated as tetrain. Immunoblotting analyses showed that tetrain was present in higher amount in the culture medium in the stationary phase than in the logarithmic phase. Tetrain has high activities at neutral to alkaline pH values. This suggests that tetrain has functional roles in the culture medium in the stationary phase, because the pH of the culture medium became alkaline with the progress of *Tetrahymena* growth.

Key words: cysteine protease, growth phase, papain family, *Tetrahymena*, tetrain.

In recent years, many kinds of proteases have been suggested to have functional roles in cell growth and/or differentiation in various cells and tissues, in mammals and other higher vertebrates.

Thiol cathepsins are well characterized cysteine proteases. They are secreted in increased amounts by certain tumor cells and have been speculated to play an active extracellular role in cancer metastasis and tissue remodeling (*1*). They have also been suggested to function in the turnover of intracellular and secreted proteins involved in growth regulation (*2*). Many studies have been reported on the biochemical characterization of cysteine proteases and the cDNAs encoding these proteases in parasitic protozoa. Several independent lines of research have suggested that the cysteine proteases participate in host tissue damage or the supply of the nutritional requirements of the parasites (*3, 4*) directly by secretion from these parasites. However, little information is available on the biochemical and structural characters of these proteases in free-living protozoa, including their biological meanings, although there have been several reports on *Tetrahymena* proteases (*5-10*).

[1] This work was supported in part by grants for Scientific Research from The Narishige Zoological Science Award, The Uehara Memorial Foundation, and The Naito Foundation.

[2] To whom correspondence should be addressed. Tel: +81-824-24-7443, Fax: +81-0824-24-0734, E-mail: hhosoya@sci.hiroshima-u.ac.jp

Abbreviations: E-64, L-*trans*-epoxysuccinyl-leucylamido-(4-guanidinobutane); MES, 2-(*N*-morphorino)ethane sulfonic acid; PBS, phosphate-buffered saline; PMSF, phenylmethanesulfonyl fluoride; TCA, trichloroacetic acid; TLCK, *Nα*-*p*-tosyl-L-lysine chloromethyl ketone; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone.

This report describes the purification, identification, and characterization of the protease activity that increases during the growth of *Tetrahymena*. This is the first report on a *Tetrahymena* protease including these studies. The purified protease was designated as tetrain, a *Tetrahymena pyriformis* cysteine protease.

EXPERIMENTAL PROCEDURES

Materials—BSA (fraction V), myelin basic protein from bovine brain, PMSF, TLCK, TPCK, TPCK-treated trypsin from bovine pancreas (type VIII), and trypsin inhibitor from soybean were purchased from Sigma. E-64, leupeptin, and pepstatin A were obtained from Peptide Institute (Japan). Histone H1 from calf thymus, endoproteinase Glu-C from *Staphylococcus aureus* V8, and α2-macroglobulin from bovine plasma were purchased from Boehringer Mannheim. Casein (#032-01521) from milk, CNBr, and monoiodoacetic acid were purchased from Wako Pure Chemical Industries (Japan). Rabbit skeletal muscle actin was prepared by the method of Pollard and Mooseker (*11*). 10/20 Multi Gel, which has an acrylamide concentration gradient of 10 to 20%, was purchased from Daiichi Pure Chemicals (Japan).

Cell Culture—The ciliate *Tetrahymena pyriformis* strain W (*12*) was kept in PY medium consisting of 1% (w/v) proteose peptone (Difco Labs., Michigan, USA) and 0.5% (w/v) yeast extract (Oriental Yeast, Japan) without shaking at 26°C. Logarithmically growing cells were seeded at an initial density of 25 cells per ml and kept at 26°C in 200-ml Erlenmeyer flasks containing 30 ml of the PY medium. Cell density was determined by removing aliquots

and counting cells under an optical microscope on each day indicated. Conditioned medium was prepared as follows: cells were seeded at an initial density of 25 cells per ml and kept at 26°C in 2-liter Erlenmeyer flasks containing 300 ml of the PY medium. The culture medium was centrifuged to remove cells at 1,200×*g* for 5 min. The supernatant was further filtered through a MILLEX-GS filter (0.22 μm; Millipore, USA) and stored at −80°C until use.

Purification of the Protease Activity in the Culture Medium in the Stationary Phase—Four days after start of culture, cells in the culture medium reached the stationary phase (approximately 3.0×10^5 cells per ml). The conditioned medium was applied to a column (2.5×25 cm) of DEAE-cellulose (Whatman DE 52) equilibrated with a buffer containing 10 mM HEPES (pH 7.5), 0.1 M KCl, 2 mM $MgCl_2$, and 1 mM EGTA (buffer A). The column was washed with buffer A, and the retained proteins were eluted with a linear gradient of 0.1-0.5 M KCl in 800 ml of buffer A. Each fraction was assayed for protease activity as described below. The protease activity was recovered in the flow-through fraction. The active fraction was collected and applied to a column (2.7×17.5 cm) of hydroxyapatite (Bio-Rad HTP) equilibrated with 10 mM potassium phosphate buffer (pH 7.0). The column was washed with the phosphate buffer, then retained proteins were eluted with a linear gradient of 10-500 mM potassium phosphate in 700 ml of the phosphate buffer. Each fraction was assayed for protease activity. Fractions containing protease activity were collected and concentrated with an Amicon Diaflo Ultrafiltration membrane (PM-10). This concentrated sample was applied to a gel filtration column (2×96 cm) of Sephacryl S-300 (Pharmacia) equilibrated with buffer A. Each fraction was assayed for protease activity. Fractions containing protease activity were combined, concentrated, and finally applied to the second gel filtration column (Simpack Diol-150; 0.79×50 cm; Shimadzu, Japan) of HPLC equilibrated with buffer A. Each fraction was assayed for protease activity. Fractions containing protease activity were pooled, designated as tetrain fraction, and stored at −80°C until use.

Assay for Protease Activity—Unless otherwise noted, protease activity was assayed as follows. The assay mixture contained 4 μg of BSA, 5 μl of 5×SDS sample buffer (see below), and 16 μl of protease fraction in a final volume of 25 μl. After a 30-min incubation at 25°C, the reaction was terminated by boiling for 2 min at 98°C and the mixture was analyzed by 10% SDS-PAGE. As the protease was activated in the presence of DTT and/or SDS (see below), the assay mixture contained 50 mM DTT and 1% SDS. One unit of protease activity was defined as the amount of enzyme that completely degraded of 4 μg of BSA per hour at 25°C under the assay conditions.

Characterization of Tetrain—To examine the effects of pH on the protease activity, the assay mixture contained 4 μg of BSA, 50 mM DTT, 0.6 unit of tetrain, and 25 mM buffer [MES (pH 5.5 to 6.5), HEPES (pH 7.0 to 8.0), or Tris (pH 8.5 to 10.0)] in a final volume of 20 μl. To examine the effects of activators on the protease activity, the assay mixture (pH 7.5) contained 4 μg of BSA, 2 units of tetrain, and various concentrations of activators in a final volume of 20 μl. To examine the effects of inhibitors on the protease activity, the assay mixture contained 4 μg of BSA, 2 units of tetrain, 5 μl of 5×SDS sample buffer (see below), and various concentrations of inhibitors in a final volume of 25 μl. These samples were assayed for protease activity as described above. In examining the effects of pH and activators, samples were mixed with 1/4 volume of 5× SDS sample buffer before electrophoresis.

SDS-PAGE—Unless otherwise noted, SDS-PAGE was carried out by the method of Blattler *et al.* (*13*). Before electrophoresis, protein samples were mixed with 1/4 volume of 5×SDS sample buffer containing 200 mM Tris-HCl (pH 6.8), 250 mM DTT, 5% SDS, and 22.5% glycerol with an adequate amount of bromophenol blue (*14*). (In the assay for protease activities, 5×SDS sample buffer was not further added, because samples already contained 5×SDS sample buffer.) Gels were stained and destained as previously described (*14*). The molecular weight of proteins was determined using Bio-Rad protein markers as a standard.

TCA Precipitation—Sodium deoxycholic acid was added to protein samples to a final concentration of 0.016% (w/v). After a 15-min incubation with cooling on ice, TCA was added to a final concentration of 6% (w/v). After a 10-min incubation at room temperature, the mixture was centrifuged at 1,770×*g* for 30 min at room temperature and the supernatant was removed. The precipitated proteins were mixed with 1×SDS sample buffer and immediately neutralized with 2.5 M Tris-HCl (pH 8.8).

Chemical and Enzymatic Cleavages—Proteins were separated on SDS-PAGE using a 15% SDS gel. After staining with Coomassie Brilliant Blue R-250, the 28- and 26-kDa bands were each cut out, soaked with water, and lyophilized. The 26-kDa component was then cleaved with 150 mM CNBr in 70% formic acid in the dark for 18 h at room temperature. The reaction mixture was evaporated, then lyophilized after addition of water. Then 2×SDS sample buffer was added, and the CNBr-cleaved peptides were finally separated on SDS-PAGE using a 16% SDS gel. The 28-kDa component was digested with *Staphylococcus aureus* V8 protease. Briefly, the 28-kDa band was placed on a stacking gel with 1.2 μg of the enzyme in 1×SDS sample buffer, and SDS-PAGE was carried out using a 16% SDS gel. After starting the electrophoresis, the electric current was turned off when a line of marker dye reached the top of the separation gel. After 30 min, the electrophoresis was resumed.

Amino Acid Sequencing Analysis—For amino acid sequencing, proteins were electrophoretically transferred to an Immobilon P^{SQ} membrane (ISEQ26260; Millipore). After staining with Coomassie Brilliant Blue R-250, the membrane was destained in a solution containing 90% methanol and 7% acetic acid. The protein bands were then cut out from the membrane and characterized by automated sequencing on a Perkin-Elmer Applied Biosystems amino acid sequencer model 473A or 477A. Sequence identity was analyzed using the Fasta program (DNA Data BANK of Japan).

Reduction and Carboxymethylation—Reduction and carboxymethylation of BSA were carried out essentially by the method of Takagi *et al.* (*15*). BSA (final concentration, 1 mg/ml) was mixed with the solution of 0.2 M Tris-HCl (pH 8.5), 6 M guanidine-HCl, 10 mM EDTA, and 10 mM DTT, and incubated for 1 h at room temperature. Then the reduced BSA was carboxymethylated by addition of monoiodoacetic acid to a final concentration of 15 mM. After incubation for 10 min at room temperature, the

carboxymethylated BSA was dialyzed against water overnight at 4°C, lyophilized, and dissolved in 10 mM Tris-HCl (pH 7.5) to a final concentration of 1 mg/ml.

Immunoblotting—Immunoblotting was performed by the method of Towbin *et al.* (*16*). Proteins were transferred to Immobilon P^{SQ} membranes. Membranes were treated with PBS (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, pH 7.3) containing 3% BSA and incubated with the anti-tetrain antiserum (obtained as below) at a dilution of 1:70 followed by a Vectastain ABC kit (Vector Laboratories, Burlingame, USA). The localization of peroxidase was detected by the reaction using *p*-dimethylaminoazobenzene according to the manufacturer's instructions.

Antibody—A peptide corresponding to the N-terminal end of tetrain with the sequence of APQVIDWTAKNVLPP was synthesized (Peptide Institute, Japan). The synthetic peptide was conjugated to keyhole limpet hemocyanin (Sigma) through an additional cysteine at its C-terminus using *N*-(*e*-maleimidocaproyloxy)-succinimide (Dojindo Labs., Japan), and the resulting conjugate (1.3 mg) was injected into a rabbit. After this initial immunization, immunity was boosted three times with the same quantity of the conjugate at about 2-week intervals. Antiserum was collected 8 days after the final immunization.

Other Procedures—Protein concentration was determined by the method of Bradford (*17*) using γ-globulin (Bio-Rad) as a standard.

RESULTS

Increase of Proteolytic Activities in the Culture Medium during the Cell Growth—Proteolytic activities in conditioned medium in the stationary phase of *Tetrahymena* growth were examined using BSA, myelin basic protein, histone H1, casein, and actin as substrates. The culture medium contained high proteolytic activities toward all proteins used in this experiment (data not shown). *Tetrahymena* cells seeded in fresh culture medium at 25 cells per ml grew logarithmically for 1–2 days, then stopped growing. As shown in Fig. 1, the proteolytic activities in the culture medium increased during the cell growth.

Purification of the Proteolytic Activities in the Culture Medium in the Stationary Phase—To characterize the proteolytic activities that increase during the cell growth, the protease activities were purified from the culture medium in the stationary phase. First, the conditioned medium in the stationary phase was applied to a DEAE-cellulose column. The protease activity was eluted in the flow-through fraction, which was collected and applied to a column of hydroxyapatite. The protease activity retained

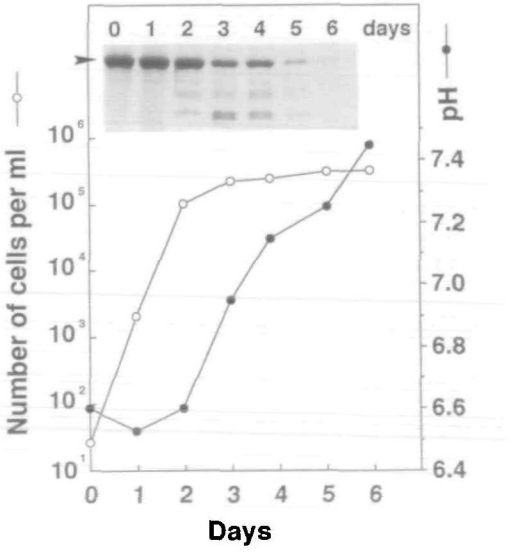

Fig. 1. **Increase of proteolytic activities in the culture medium during cell growth.** *Tetrahymena* cells were seeded at an initial density of 25 cells per ml and incubated at 26°C. Cell density (○) and pH (●) of the culture medium were determined daily. Inset, conditioned medium at each growth phase was assayed for proteolytic activities using BSA as a substrate. The arrowhead indicates the BSA band on SDS-PAGE gel.

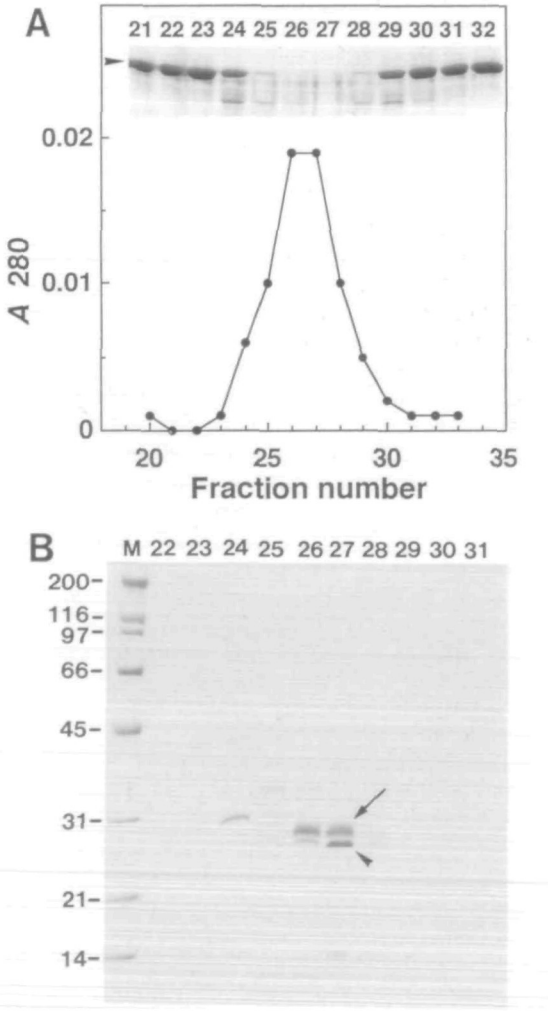

Fig. 2. **HPLC of the protease activity.** A: The active fractions (51 to 59) from the Sephacryl S-300 column were collected, concentrated and finally chromatographed on the second gel filtration column of HPLC. BSA degradation was analyzed by SDS-PAGE (fractions 21 to 32) and is shown in the upper part of the panel. The arrowhead indicates the BSA band on SDS-PAGE gel. B: Each fraction (500 μl) from panel A was TCA-precipitated and analyzed on SDS-PAGE using a 10/20 Multi Gel. The 28- and 26-kDa components are indicated by an arrow and an arrowhead, respectively. Fractions analyzed for protease activities are numbered on the top of the gels. Molecular mass markers (lane M) are indicated in kDa.

TABLE I. **Purification of protease activity from *Tetrahymena* conditioned medium of the stationary phase.**

Step	Volume (ml)	Protein concentration (μg/ml)	Total proteins (μg)	Total activity (U)	Specific activity (U/μg)	Purification (fold)	Yield (%)
Conditioned medium	495	66.6	32,967	35,357	1.1	1	100
DEAE-cellulose	495	15.1	7,475	35,357	4.7	4	100
Hydroxyapatite	37	25.6	947	7,929	8.4	8	22
Sephacryl S-300	26	10.8	281	3,714	13.2	12	11
Sim-pack Diol 150	25	4.8	120	3,571	29.8	27	10

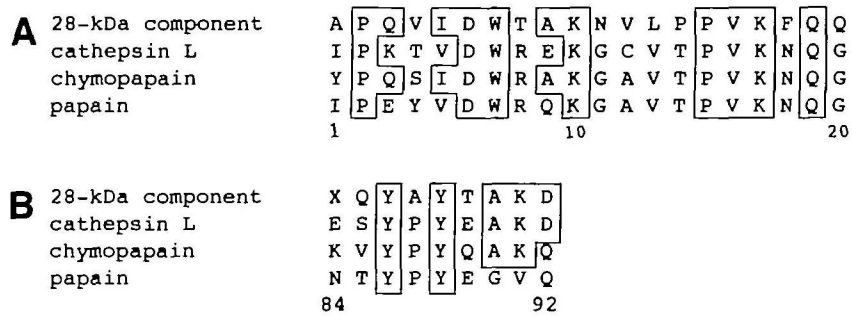

Fig. 3. **Partial amino acid sequence analysis of the 28-kDa component.** Partial amino acid sequences of the 28-kDa component are aligned with those of three related cysteine proteases, rat cathepsin L (*18*), chymopapain (*25*), and papain (*19*). The numbering system for mature papain (*19*) is shown. Amino acid identities are shown by boxes. A, N-terminus of original peptide; B, N-terminus of digested peptide.

in the column was eluted at 150-250 mM potassium phosphate. Active fractions were collected, concentrated, and applied to a gel filtration column of Sephacryl S-300. The protease activity was eluted in fractions 51 to 59. Finally, the active fractions from Sephacryl S-300 were collected, concentrated, and separated on the second filtration gel (Sim-pack Diol-150) by HPLC (Fig. 2A). Table I summarizes the purification procedure. These steps resulted in a specific enzyme activity of 29.8 units/μg protein, representing a 27-fold purification over the culture medium in the stationary phase.

Identification of the Protease Activity—Each fraction (fractions 22 to 31 in Fig. 2A) was concentrated by using TCA precipitation and analyzed on SDS-PAGE (Fig. 2B). In fractions 26 and 27, which showed the peak of protease activity (Fig. 2A), two components with apparent molecular masses of 28 and 26 kDa were detected.

Partial amino acid sequencing was conducted on four preparations of the 28-kDa component obtained from different batches of the conditioned medium. All sequences at the N-terminal end except residue 7 matched completely: residue 7 was Trp in three preparations out of four (Fig. 3A) and Ser in the remaining one. In a preliminary experiment in which we cloned and sequenced the cDNA encoding the 28-kDa component, residue 7 deduced from the nucleotide sequence was Trp (data not shown). Next, the 28-kDa component was digested with *Staphylococcus aureus* V8 protease. The N-terminal amino acid sequence of a selected 8-kDa peptide from the digest was analyzed and determined as shown in Fig. 3B. In a search by use of the Fasta program, these sequences were highly homologous to those of the mature forms of cysteine proteases such as cathepsin L, chymopapain, and papain (Fig. 3). The partial N-terminal and internal amino acid sequences of the 26-kDa component were also determined as XPAIVHPHXGXAL (in sequencer cycles 1, 9, and 11, amino acids residues were not determined) and DSAFDYILD, respectively. In a search by use of the same program, however, no sequences homologous to such proteases were detected. From these results, we here focused on the 28-kDa component as a

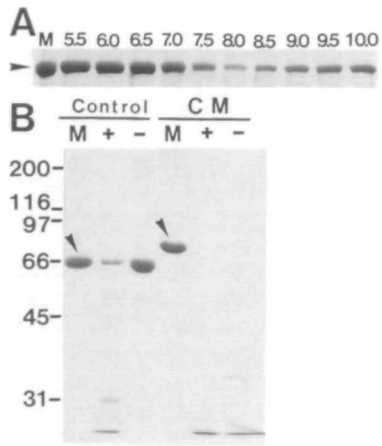

Fig. 4. **Characterization of tetrain.** A: Effect of pH on the activity of tetrain. BSA was incubated with tetrain in the pH range of 5.5-10.0. The pH values are indicated at the top of the gel. M indicates BSA as a marker. The arrowhead shows the BSA band on SDS-PAGE gel. B: Effect of DTT on the activity of tetrain. BSA was incubated with the protease in the presence (+) or absence (−) of 50 mM DTT. BSA degradations are shown for non-carboxymethylated (Control) or carboxymethylated (CM) preparations with or without DTT. M indicates BSA as a marker. Arrowheads show BSA bands on SDS-PAGE gels.

Tetrahymena protease, which was designated as tetrain, a *Tetrahymena pyriformis* cysteine protease.

Effects of pH on the Protease Activity of Tetrain—In *Tetrahymena* culture, the pH of the culture medium increases during cell growth (Fig. 1). Therefore, the effects of pH on the protease activity of tetrain were examined (Fig. 4A). BSA was incubated with tetrain at a pH of 5.5-10.0. The degradation of BSA was most significant at pH 8.0, suggesting that the optimum pH for protease activity is 8.0.

Effects of DTT on the Protease Activity of Tetrain—The effects of DTT on the protease activity of tetrain were examined. BSA was degraded by tetrain in the presence of 30-100 mM DTT, but not in its absence (Fig. 4B, Control).

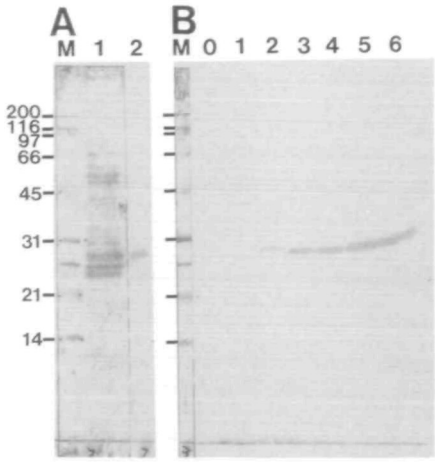

Fig. 5. **Release of tetrain into culture medium during the cell growth.** Crude conditioned medium was separated on SDS-PAGE using a 15% SDS gel, transferred to Immobilon P^{SQ} membrane, and stained with Coomassie Brilliant Blue R-250 (panel A, lane 1). The transferred membrane was also used for immunoblotting with antiserum against tetrain (panel A, lane 2, and panel B, lanes 0 to 6). A: Culture medium 4 days after start of the cell culture was used for immunoblotting. Note that the antiserum reacts specifically with tetrain (lane 2). B: Tetrain is detected 2 days after start of cell culture. The lane numbers 0 to 6 refer to the days after start of cell culture. Molecular mass markers (lane M in panels A and B) are indicated in kDa.

Similar effects were observed with 5-20% 2-mercaptoethanol and 0.1-3% SDS, but not urea and EDTA (data not shown).

As the protease activity was not detected in the absence of DTT, the assay mixtures employed in this study contained DTT. To examine the effects of DTT, BSA that had been denatured irreversibly by carboxymethylation was used as a substrate for tetrain in the presence or absence of DTT. Electrophoretic mobility of the carboxymethylated BSA was slower than that of non-carboxymethylated BSA (Fig. 4B). The carboxymethylated BSA was degraded even in the absence of DTT, whereas the non-carboxymethylated BSA was not (Fig. 4B, CM). These findings suggest that DTT acts on tetrain to give free sulfhydryl groups in its active site, and/or on the substrates to give a *flexible* polypeptide chain which is attacked by tetrain.

Effects of Inhibitors on the Protease Activity of Tetrain—The effects of protease inhibitors on the activity of tetrain were examined. Monoiodoacetic acid and E-64, which are blocking reagents of sulfhydryl groups, effectively inhibited the protease activity at 10 and 0.1 mM, respectively, suggesting that tetrain requires sulfhydryl groups for its activity. In addition, the protease activity of tetrain was inhibited by 0.1 mM TPCK or 10 mM TLCK. Other protease inhibitors (trypsin inhibitor, PMSF, leupeptin, pepstatin A, EDTA, and α2-macroglobulin) did not inhibit the protease activity of tetrain.

Release of Tetrain into the Culture Medium during the Cell Growth—Using a synthetic peptide corresponding to the N-terminal end of tetrain as an antigen, an antiserum against tetrain was produced. Immunoblotting analysis showed that the antiserum reacted only with the 28-kDa component in the crude conditioned medium in the stationary phase (Fig. 5A). The partial N-terminal amino acid sequence of the protein was completely identical with that of tetrain (data not shown), suggesting that the antiserum is highly specific for tetrain. Immunoblotting analysis with the antiserum revealed that the amount of tetrain gradually increased in the culture medium from 2 days after the start of cell culture (Fig. 5B). This is consistent with the increase of protease activity in the culture medium as shown in Fig. 1.

DISCUSSION

Although there have been several reports on *Tetrahymena* protease activities (*5-10*), little information is available concerning the biochemical and structural characters of these activities. In this study, we have purified, identified, and characterized the protease activity in the *Tetrahymena* culture medium in the stationary phase. This is the first report on the *Tetrahymena* protease including these studies.

The purified protease activity contained a major and a minor component, with molecular masses of 28 kDa (tetrain) and 26 kDa, respectively. Two lines of evidence revealed that the 28-kDa component contributes to the protease activity and we here focused on the 28-kDa component. First, its N-terminal and internal amino acid sequences aligned well with those of cysteine proteases such as cathepsin L, chymopapain, and papain. Second, no such sequence homologies were detected for the 26-kDa component. The members of the papain family are known to be translated as a preproenzyme with a signal sequence followed by a propeptide and a mature protein (*4, 10, 18-20*). As the N-terminal sequence of tetrain is homologous to those of the mature forms of cysteine proteases, tetrain is a mature protease. Furthermore, the specific antibody detected mature tetrain exclusively in the culture medium. Although we have not yet purified the preproenzyme of tetrain, in a preliminary experiment the antibody detected a 45-kDa component together with a 28-kDa component in the *Tetrahymena* extract (data not shown). These findings suggest that tetrain is synthesized in the cells as a preproenzyme, then released into the culture medium as a mature form.

Requirements of free sulfhydryl groups for activity and sensitivity to TPCK and TLCK also indicate that tetrain is a member of the papain family of cysteine proteases. Tetrain seems to prefer both *N*-benzyloxycarbonyl-L-arginyl-L-arginine-7-amido-4-methylcoumarin and *N*-benzyloxycarbonyl-L-phenylalanyl-L-arginine-7-amido-4-methylcoumarin but not L-arginine-7-amido-4-methylcoumarin as fluorogenic substrates (manuscript in preparation). So tetrain resembles the mammalian lysosomal cysteine protease, cathepsin B, in its substrate specificity. Interestingly, tetrain is not inhibited by leupeptin and is active in neutral to alkaline conditions. The optimum pH for tetrain is similar to those of the trematode protease (*20*) and other *Tetrahymena* cysteine proteases (*5, 8, 9*). However, animal cathepsins B, H, and L of the papain family optimally function at pH 6.0, 5.5-6.8, and 5.0-5.5, respectively (*21*). Therefore, tetrain has unusual features as a member of the papain family.

DTT was required to detect the protease activity of tetrain. This suggests that DTT acts on tetrain to give free sulfhydryl groups in its active site, and/or on the substrates

to give a *flexible* polypeptide chain which is attacked by tetrain. Interestingly, tetrain degraded myelin basic protein (our unpublished data) and carboxymethylated BSA in the absence of DTT. Because BSA (*22*), but not myelin basic protein (*23*), has many intramolecular disulfide bonds, it may have a *stable* and *flexible* three-dimensional structure. These findings suggest that a *flexible* polypeptide chain like myelin basic protein or carboxymethylated BSA can be easily degraded by tetrain. Further works are in progress to elucidate the mechanism of tetrain activation.

In immunoblotting analyses, tetrain was detected in higher amount in the culture medium in the stationary phase than in the logarithmic phase. It might be speculated that tetrain accumulates in the culture medium due to release from dying cells in the stationary phase. However, as no cell death was observed throughout the period of culture in this study, tetrain must be released from living cells rather than accumulate in the medium due to leakage from cells damaged by cytolysis. Since it showed high activity at neutral to alkaline pH values, tetrain must have functional roles in the culture medium in the stationary phase, because the pH of the culture medium became alkaline with passage of *Tetrahymena* growth. In *Tetrahymena*, extracellular digestion of phosphorylcholine enabled the cells to grow and multiply in a nutrient medium with organic phosphate compounds as the only phosphate source (*24*). Similarly, released tetrain may digest nutrients to enable the cells to grow and multiply.

We thank Dr. T. Takagi of Tohoku University for his helpful suggestion. We are grateful to Drs. M. Sasaki (Nagoya City University), Y. Nozawa (Gifu University), and Y. Watanabe (Joubu University) for helpful discussions. We also thank members of our laboratory for encouragement and discussions throughout this work.

REFERENCES

1. Erickson, A.H. (1989) Biosynthesis of lysosomal endopeptidases. *J. Cell. Biochem.* **40**, 31-41
2. Kane, S.E. and Gottesman, M.M. (1990) The role of cathepsin L in malignant transformation. *Semin. Cancer Biol.* **1**, 127-136
3. Murta, A.C.M., Persechini, P.M., Souto-Padron, T., de Souza, W., Guimaraes, J.A., and Scharfstein, J. (1990) Structural and functional identification of GP57/51 antigen of *Trypanosoma cruzi* as a cysteine proteinase. *Mol. Biochem. Parasitol.* **43**, 27-38
4. Eakin, A.E., Mills, A.A., Harth, G., McKerrow, J.H., and Craik, C.S. (1992) The sequence, organization, and expression of the major cysteine protease (cruzain) from *Trypanosoma cruzi*. *J. Biol. Chem.* **267**, 7411-7420
5. Dickie, N. and Liener, I.E. (1962) A study of the proteolytic system of *Tetrahymena pyriformis* W. I. Purification and partial characterization of the constituent proteinases. *Biochim. Biophys. Acta* **64**, 41-45
6. Muller, M. (1972) Secretion of acid hydrolases and its intracellular source in *Tetrahymena pyriformis*. *J. Cell Biol.* **52**, 478-487
7. Blum, J.J. (1975) Effects of metabolites present during growth of *Tetrahymena pyriformis* on the subsequent secretion of lysosomal hydrolases. *J. Cell. Physiol.* **86**, 131-142
8. Levy, M.R., Sisskin, E.E., and McConkey, C.L. (1976) A protease that increases during a period of enzymic and metabolic adjustment in *Tetrahymena*. *Arch. Biochem. Biophys.* **172**, 634-647
9. Banno, Y., Yano, K., and Nozawa, Y. (1982) Biochemical characterization of secreted proteases during growth in *Tetrahymena pyriformis* WH-14: Comparison of extracellular with intracellular proteases. *J. Protozool.* **29**, 91-98
10. Karrer, K.M., Peiffer, S.L., and Ditomas, M.E. (1993) Two distinct gene subfamilies within the family of cysteine protease genes. *Proc. Natl. Acad. Sci. USA* **90**, 3063-3067
11. Pollard, T.D. and Mooseker, M.S. (1981) Direct measurement of actin polymerization rate constants by electron microscopy of actin filaments nucleated by isolated microvillus cores. *J. Cell Biol.* **88**, 654-659
12. Hosoya, H., Matsuoka, T., Hosoya, N., Takahashi, T., and Kosaka, T. (1995) Presence of a *Tetrahymena* growth promoting activity in fetal bovine serum. *Dev. Growth Differ.* **37**, 347-353
13. Blattler, D.P., Garner, F., Van Slyke, K., and Bradley, A. (1972) Quantitative electrophoresis in polyacrylamide gels of 2-40%. *J. Chromatogr.* **64**, 147-155
14. Hosoya, H., Yamashiro, S., and Matsumura, F. (1991) Mitosis-specific phosphorylation of myosin light chain kinase. *J. Biol. Chem.* **266**, 22173-22178
15. Takagi, T., Konishi, K., and Mabuchi, I. (1988) Amino acid sequence of starfish oocyte depactin. *J. Biol. Chem.* **263**, 3097-3102
16. Towbin, H., Staehelin, T., and Gordon, T. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**, 4350-4354
17. Bradford, M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254
18. Ishidoh, K., Towatari, T., Imajoh, S., Kawasaki, H., Kominami, E., Katunuma, N., and Suzuki, K. (1987) Molecular cloning and sequencing of cDNA for rat cathepsin L. *FEBS Lett.* **223**, 69-73
19. Cohen, L.W., Coghlan, V.M., and Dihel, L.C. (1986) Cloning and sequencing of papain-encoding cDNA. *Gene* **48**, 219-227
20. Wijffels, G.L., Panaccio, M., Salvatore, L., Wilson, L., Walker, I.D., and Spithill, T.W. (1994) The secreted cathepsin L-like proteinases of the trematode, *Fasciola hepatica*, contain 3-hydroxyproline residues. *Biochem. J.* **299**, 781-790
21. Barrett, A.J. and Kirschke, H. (1981) Cathepsin B, cathepsin H, and cathepsin L. *Methods Enzymol.* **80**, 535-561
22. Hirayama, K., Akashi, S., Furuya, M., and Fukuhara, K. (1990) Rapid confirmation and revision of the primary structure of bovine serum albumin by ESIMS and Frit-FAB LC/MS. *Biochem. Biophys. Res. Commun.* **173**, 639-646
23. Brostoff, S.W., Reuter, W., Hichens, M., and Eylar, E.H. (1974) Specific cleavage of the A1 protein from myelin with cathepsin D. *J. Biol. Chem.* **249**, 559-567
24. Tiedtke, A. and Rasmussen, L. (1988) Lysosomal enzymes in extracellular digestion in the unicellular eukaryote *Tetrahymena*. *J. Cell. Physiol.* **136**, 554-556
25. Watson, D.C., Yaguchi, M., and Lynn, K.R. (1990) The amino acid sequence of chymopapain from *Carica papaya*. *Biochem. J.* **266**, 75-81