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Catalytic Residues and Substrate Specificity of Recombinant Human Tripeptidyl Peptidase I (CLN2)

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Tripeptidyl peptidase I (TTP-I), also known as CLN2, a member of the family of serine-carboxyl proteinases (S53), plays a crucial role in lysosomal protein degradation and a deficiency in this enzyme leads to fatal neurodegenerative disease. Recombinant human TPP-I and its mutants were analyzed in order to clarify the biochemical role of TPP-I and its mechanism of activity. Ser280, Glu77, and Asp81 were identified as the catalytic residues based on mutational analyses, inhibition studies, and sequence similarities with other family members. TPP-I hydrolyzed most effectively the peptide Ala-Arg-Phe*Nph-Arg-Leu (*, cleavage site) (k_{cat}/K_m = 2.94 $\mu M^{-1} \cdot s^{-1}$). The k_{cat}/K_m value for this substrate was 40 times higher than that for Ala-Ala-Phe-MCA. Coupled with other data, these results strongly suggest that the substrate-binding cleft of TPP-I is composed of only six subsites (S_3-S_3'). TPP-I prefers bulky and hydrophobic amino acid residues at the P_1 position and Ala, Arg, or Asp at the P_2 position. Hydrophilic interactions at the S_2 subsite are necessary for TPP-I, and this feature is unique among serine-carboxyl proteinases. TPP-I might have evolved from an ancestral gene in order to cleave, in cooperation with cathepsins, useless proteins in the lysosomal compartment.

Key words: classical late-infantile neuronal ceroid lipofuscinosis, CLN2, neurodegenerative disease, sedolisin, serine-carboxyl proteinase.

Abbreviations: TPP-I, tripeptidyl peptidase I; mutein, mutated protein; CPd, cysteine proteinase gene deleted.

Neuronal ceroid lipofuscinoses (NCLs) are the most common inherited progressive neurodegenerative disorders of childhood (*1–3*), manifested by ceroid and lipofuscin-like autofluorescent deposits accumulating in different tissues. These diseases are characterized by vision loss, progressive neurodegeneration (seizures, ataxia, and psychomotor deterioration), and premature death (*2*). Classical late-infantile neuronal ceroid lipofuscinosis (LINCL), a progressive and fatal neurodegenerative disease of childhood, is caused by a variety of mutations in the *CLN2* gene (*1–4*).

The *CLN2* gene product (CLN2) was identified as a 46-kDa glycoprotein exhibiting sequence similarity with bacterial pepstatin-insensitive carboxyl proteinases (*4*). CLN2 shares 22% identity with sedolisin (*Pseudomonas* serine-carboxyl proteinase) (*5*), 29% identity with sedolisin-B (*Xanthomonas* serine-carboxyl proteinase) (*6*), 35% identity with kumamolisin (kumamolysin) (*7*), and 34% identity with kumamolisin-B (proteinase J-4) (*8*, *9*). CLN2 is involved in lysosomal protein degradation (*10*), and its deficiency results in the accumulation of subunit c of the mitochondorial ATP synthase complex (F_0F_1-ATPase) in lysosomal vesicles (*11*, *12*). Subunit c is a major component of an autofluorescent material, ceroid-lipofuscin, in lysosomal storage bodies (*11*).

CLN2 was identified in 1999 as tripeptidyl peptidase I [EC 3.4.14.9], based mainly on analysis of its sequence (*13*, *14*). A typical Gly-Thr-Ser motif, present in the region surrounding the catalytic serine in subtilisin-like serine peptidases, is also conserved in TPP-I/CLN2. However, no histidine or lysine residue, potentially acting as a general base, was found in the sequences of CLN2 or related bacterial sedolisins. We have previously reported that Asp170 and Asp328 in the sedolisin molecule appear to be essential for the catalytic function of this enzyme (*15*). On the other hand, it was reported that Ser280 in TPP-I is crucial for its catalytic function, based on mutational analysis and chemical modification (*16*). Thus, at that stage, the catalytic mechanism of these enzymes was still ambiguous. However, in 2001, we solved the crystal structure of sedolisin at high resolution and defined a novel family of serine-carboxyl peptidases (sedolisins, MEROPS S53) (*17*). A unique catalytic triad, Ser287-Glu80-Asp84, was shown to be responsible for the catalytic function, and it was postulated that Asp170 is involved in the formation of an oxyanion hole (these residues correspond to Ser280-Glu77-Asp81 and Asp165 in TPP-I, respectively) (*9*, *17–20*). In addition, Asp328

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(corresponding to Asp322 in TPP-I) was found to be involved in the creation of an essential Ca^{2+} binding site (*17*). The catalytic mechanism of this family was postulated to utilize glutamate as a general base, abstracting a proton from the serine that acts as a nucleophile.

TPP-I is a unique enzyme having both tripeptidyl peptidase activity for tridecapeptides and endopeptidase activity for decapeptides (*21*). The gene encoding TPP-I has been identified in a number of mammals, and the protein has been purified from a variety of sources, including human and mouse tissues (*21–27*). TPP-I is widely distributed among such higher organisms as mammals and fish, and may play important roles in their life cycles (*9*).

TPP-I sequentially removes tripeptides from the N-terminus of peptide hormones such as angiotensin II, glucagons, and substance P (*21*, *27*, *28*). The subsite structure of TPP-I has not been analyzed yet. The enzyme activity is strongly inhibited by Ala-Ala-Phe-chloromethylketone (*27*). The hexahistidine-tagged TPP-I was completely inhibited by incubation for 70 min with 2 mM DFP at 25°C and pH 4.5 (*16*). In contrast, rat spleen TPP-I was not inhibited by incubation with 10 mM DFP and 1 mM PMSF for 10 min at 37°C and pH 4.0 (*21*). Sedolisin and kumamolisin are also insensitive to PMSF (*29*, *30*). Thus, the inhibitory activities of such serine-modifying regents for sedolisins are still ambiguous.

In this study, human TPP-I and its mutants were expressed in silkworm pupae and purified to a homogeneous state. The role of the putative catalytic residues of TPP-I was investigated using purified enzymes, and, in addition, the substrate specificity of TPP-I was analyzed by using synthetic peptide substrates.

EXPERIMENTAL PROCEDURES

Materials—pCLN2#17-GemT, including human CLN2 cDNA cloned by PCR, was a generous gift from Dr. J. Ezaki (Juntendo University, Tokyo, Japan). Restriction enzymes, calf intestine alkaline phosphatase, KOD-Dash polymerase, and kits for molecular biology were purchased from TOYOBO Co. (Osaka, Japan). PRISM dye terminator cycle sequencing kits were obtained from Perkin-Elmer (Chiba, Japan). RNase, AEBSF [4-(2-aminoethyl)benzene sulfonyl fluoride], Ala-Ala-Phe-chloromethylketone (AAF-CMK), and Ala-Ala-Phe-7-amido-4-methylcoumarin (Ala-Ala-Phe-MCA) were from Sigma (St. Louis, MO). Glycopeptidase F was obtained from Takara Bio (Kyoto, Japan). The BCA Protein Assay Reagent kit and Geneclean II kit were from Pierce (Rockford, IL) and Funakoshi (Tokyo, Japan), respectively. Oligonucleotides for cloning and sequencing were purchased from Kiko-Tech (Osaka, Japan). An antibody against CLN2 prepared using the carboxyl terminal fragment of human CLN2 (amino acid residues from Glu207 to Val323) (*11*), was a generous gift from Dr. E. Kominami and Dr. J. Ezaki (Juntendo University, Tokyo, Japan). CM-Sepharose, DEAE-Sepharose, Octyl-Sepharose, Superdex-200, and Mono-S were from Amersham Pharmacia Biotech. (Buckinghamshire, UK). Ac-IAF-CHO (*N*-acetyl-isoleucyl-alanyl-phenylalaninal) and Ac-IPF-CHO (*N*-acetyl-isoleucyl-prolyl-phenylalaninal) were kindly synthesized by Dr. K. Uchida (Teikyo University, Utsunomiya, Japan). Acetylpepstatin (*N*-acetyl-valyl-valyl-AHMHA-alanyl-AHMHA; AHMHA, 4-amino-3-hydroxy-6-methylheptanoic acid) and tyrostatin (*N*-isovaleryl-tyrosyl-leucyl-tyrosinal) were purified in our laboratory from microbial sources (*31*, *32*).

Bacterial Strains, Plasmids, and Media—E. coli JM109 (*endA1*, *recA1*, *gyrA96*, *thi*, *hsdR17*, *relA1*, *supE44*, (*lac-proAB*), F[*traD36*, *proA*$^+$*B*$^+$, *lacIq, lac*ΔM15] was used as a host. Plasmids pUC19 and pTZ were used for cloning and sequencing. The BmNPV-transfer vector, pYNG was used for the expression of TPP-I (*33*). *E. coli* JM109 cells were grown in Luria-Bertani broth (LB broth, 1% polypepton, 0.5% yeast extract, and 1% sodium chloride, pH 7.0) or super broth (1.2% polypepton, 2.4% yeast extract, 0.5% glycerol, 1.25% K_2HPO_4, and 0.38% KH_2PO_4, pH 7.0).

DNA manipulation—Plasmids were isolated by alkaline lysis and polyethylene glycol 6,000 precipitation (*34*). Competent cells for transformation were prepared by the method of Hanahan with a slight modification (*35*). *E. coli* JM109 cells were cultivated for 44 h at 18°C for high efficiency of transformation. The transformation buffer used here comprised 10 mM PIPES buffer, pH 6.7, containing 15 mM $CaCl_2$, 0.25 M KCl, and 55 mM $MnCl_2$. Nucleotide sequences were determined using a PRISM dye terminator cycle sequencing kit and an Applied Biosystems DNA sequencer model 373A. The reaction mixture was loaded on a 5.25% denaturing polyacrylamide gel. The nucleotide sequence was analyzed using the DNASIS software programs (Hitachi) for the prediction of an amino acid sequence.

Peptide Substrates—Lys-Pro-Ile(P_3)-Glu(P_2)-Phe*Nph-Arg(P_2')-Leu (*; cleavage site, Nph; *p*-nitrophenylalanine), with substitutions at the P_3 (K, E, I, T, Q, A, R, V, N, F, S, L, D, and Y), P_2 (K, I, T, Q, A, R, V, N, S, L, D, H, P, and Nle), and P_2' (K, E, I, A, V, N, S, L, D, and Nle) positions were used to analyze interactions in the substrate binding cleft (a total of 38 peptide substrates) (*36*, *37*). Ala-Ala-Ala-Phe*Nph-Arg-Leu and Ala-Ala-Phe*Nph-Arg-Leu were also used. In addition, combinatorial peptide substrates, Ala-Xaa-(Tyr/Nle/Leu/Trp/Phe)*Nph-Xaa-Leu (Xaa; 19 amino acid residues excluding Cys) were used to study the preference at the P_1 position. Lys-Pro-Xaa-Glu-P_1*Nph-Xaa-Leu were used to study preference at the P_1 position of sedolisin and kumamolisin (Xaa and P_1; 19 amino acid residues excluding Cys and Met, plus norleucine). The cleavage of the substrate between Phe (or P_1) and Nph was monitored at 37°C and pH 4.0 in 0.1 M sodium acetate buffer. The decrease in absorbance at 300 nm was monitored using a Beckman DU-7400 spectrophotometer.

Expression of Recombinant Enzyme in Silkworm Pupae—pCLN2#17-GemT was digested with *Xho*I and *Sal*I, and the resultant fragment was cloned into the *Sal*I-digested pTZ. The plasmid was digested with *Bam*HI and *Eco*RV, and the resultant fragment was cloned into the *Bgl*II and *Sma*I–digested plasmid pYNG. The constructed vector, pYNG-CLN2, was expressed in pupae using a baculovirus system (*33*).

Purification of the Recombinant TPP-I—Five virus-infected pupae were homogenized with 20 mM Tris-HCl buffer, pH 8.0, containing 10% glycerol, 0.1 M NaCl, 1 mM EDTA, and 10 mM benzamidine. Triton X-100 was

added to this preparation to a final concentration of 1%. After removal of insoluble materials by centrifugation (10,000 × *g*, 1 h, 4°C), a small amount of thiourea was added to the supernatant. This final preparation (pupal hemolymph) was stored at –30°C until use. The pupal hemolymph was dialyzed against 10 liters of 20 mM sodium acetate buffer, pH 4.0, containing 50 mM NaCl and 0.1% Triton X-100 (buffer A). The sample was loaded onto a column of CM-Sepharose (ϕ 2 × 25 cm) equilibrated with buffer A. The adsorbed enzyme was eluted with a linear gradient of 0.05 to 0.8 M NaCl in buffer A. The active fractions were mixed with 100 ml of octyl-Sepharose equilibrated with 20 mM sodium acetate buffer, pH 4.0, containing 0.4 M NaCl and 0.1% Triton X-100 (buffer B). The resin was packed into a glass column (ϕ 2 × 35 cm). The adsorbed enzyme was eluted with a linear gradient of 0 to 2% Triton X-100. The active fractions were dialyzed against 2 liters of buffer A. The sample was loaded onto a column of Mono-S (ϕ 0.5 × 5 cm) equilibrated with buffer A. The adsorbed enzyme was eluted with a linear gradient of 0.05 to 0.8 M NaCl in buffer A (flow rate: 0.5 ml/min). Active fractions were pooled and stored at –80°C until use. All procedures were carried out at 4°C, except for FPLC (room temperature) on the Mono-S column.

Kinetic Assays—Cleavage of each substrate between Phe and Nph was monitored at 37°C in 0.1 M sodium acetate buffer, pH 4.0. The decrease in absorbance at 300 nm was monitored using a Beckman DU-7400 Diode Array Spectrophotometer. For each kinetic assay, two tubes (tube 1: different concentration of substrate, tube 2: enzyme) were warmed for 5 min at 37°C. After the contents of the two tubes were mixed, the linear rate of absorbance change was measured to give the initial velocity. Kinetics constants were calculated using Lineweaver-Burk plots, with at least five initial substrate concentrations (15–100 μM).

Tripeptidyl Peptidase Activity and Protein Concentration—Tripeptidyl peptidase activity was assayed as described by Ezaki *et al*. with slight modification (*21*). Reaction mixtures comprised 0.1 μM Ala-Ala-Phe-MCA, 0.1 M sodium acetate buffer, pH 4.0, and the enzyme, in a final volume of 0.1 ml. After incubation at 37°C for 10 min, the reaction was terminated by adding 0.1 ml of 10% SDS and 2 ml of 0.1 M Tris-HCl, pH 9.0. One unit of activity was defined as the amount of enzyme that gives one unit of fluorescence intensity for 10 min (excitation, 380 nm; emission, 460 nm). Protein concentration was estimated with a BCA Protein Assay Reagent kit using bovine serum albumin as a standard. Each inhibitor was incubated with the enzyme at 37°C for 10 min, and then the remaining activity was assayed using Ala-Ala-Phe-MCA as a substrate.

Homogeneity—Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli in 12.5% polyacrylamide gels in 0.375 M Tris-HCl buffer, pH 8.8, containing 7.3% acrylamide, 0.2% *N,N′*-methylenebisacrylamide, and 0.1% SDS (*38*). Electrophoresis was carried out at a constant current of 20 mA at room temperature. Gels were stained with Coomassie Brilliant Blue R-250 in order to detect protein bands. Western blot analysis was carried out according to the method of Towbin *et al.*, using a mouse anti-CLN2 antibody and alkaline phosphate–conjugated mouse IgG antibody (*39*).

Deglycosylation with Glycopeptidase F—The reaction mixture comprised purified enzyme (10 μg), 0.1 M 2-mercaptoethanol, 0.5% SDS, and 0.5 M Tris-HCl, pH 8.6, in a final volume of 5 μl. After boiling for 3 min, 5 μl of 5% Nonidet P-40 and 13 μl of distilled water were added. Glycopeptidase F was added, and the sample was incubated at 37°C for 17 h. Removal of oligosaccharide chains was analyzed by SDS–polyacrylamide gel electrophoresis.

Construction of TPP-I Mutants—Polymerase chain reactions were carried out in order to introduce the E77A (GAG to GCG), D81A (GAT to GCT), D165A (GAC to GCC), S280A (TCG to GCG), and D322A (GAT to GCT) mutations into the human TPP-I cDNA sequence. Two outside primers covering either the 5′- or 3′-coding regions and complementary primers containing nucleotide changes were used for the first reaction. The reaction products were each purified using a Geneclean II kit and mixed with the outside primers. The products obtained by the second PCR were subcloned into pUC19 and the insert fragments were sequenced to confirm the presence of the desired mutations. pUC-E77A and pUC-D81A were digested with *Bst*II and *Xba*I, and each fragment was inserted into the *Bst*II and *Xba*I–digested pCLN2#17-GemT. pUC-D81A was digested with *Bst*EII and *Bsp*EI, and the fragment was inserted into the *Bst*EII and *Bsp*EI–digested pCLN2#17-GemT. pUC-D165A, pUC-S280A, and pUC-D322A were digested with *Sal*I and *Xba*I, and the resultant fragments were inserted into the *Sal*I and *Xba*I–digested pCLN2#17-GemT.

Purification of Muteins S280A and E77A—Muteins S280A and E77A were expressed in pupae. Each hemolymph (50 ml) was dialyzed against 1 liter of buffer A. As described in the section "Purification of the recombinant TPP-I," each mutant was purified by a series of CM-Sepharose, Octyl-Sepharose and Mono-S chromatographies. For further purification, each of the final preparations obtained here was concentrated and loaded onto a column of Superdex 200 (ϕ 1.0 × 30 cm) equilibrated with buffer A. Purified muteins, S280A and E77A, were stored at –80°C until use. All procedures were performed at 4°C, except for FPLC on Mono-S and Superdex 200 (room temperature). During purification, each mutein was monitored by Western blot analysis.

RESULTS

Purification and Properties of the Recombinant Enzyme—A recombinant CPd virus harboring pYNG-CLN2 was introduced into silkworm pupae. The infected pupae produced an immunoreactive protein. All the wild-type and mutant TPP-I proteins were synthesized as 66-kDa inactive precursors consisting of a 195 residue prepro-peptide and 368 residue mature enzyme. Most of the precursor proteins were converted to a 46-kDa form by post-translational proteolysis. It has been previously reported that, *in vivo*, the precursor of TPP-I is converted to the 46-kDa mature form by auto-activation in an intramolecular fashion under acidic conditions (*4*, *16*, *40–42*), and that, *in vitro*, the conversion is most likely due to the activity of endogenous proteinases in host cells during expression (*41*). We observed similar behavior during the expression

Table 1. **Purification of recombinant TPP-I expressed in pupae.**

Procedure	Total activity (unit)	Total protein (mg)	Specific activity (unit/mg)	Purification (-fold)	Yield (%)
Hemolymph	179,000	625	286	1	100
CM-Sepharose	113,000	92	1,230	4	63
Octyl-Sepharose	102,000	49	2,080	7	57
Mono-S	34,500	4.6	7,500	26	19

of TPP-I in silkworm pupae, since the purification procedure yielded the mature enzyme. As shown in Table 1, the purified wild-type TPP-I (4.6 mg), with a specific activity of 7,500 units/mg, was obtained from the pupal hemolymph. The specific activity corresponds to 228 nmol/min/mg according to the method of Ezaki, J. *et al.*, which coincides well with that of purified rat spleen TPP-I (*21*). The enzyme showed a single protein band (46 kDa) on SDS-PAGE (Fig. 1, line 2). After incubation with glycopeptidase F at 37°C for 17 h, the enzyme was converted to a 40-kDa protein (Fig. 1, line 1). Some properties of the recombinant enzyme were examined using Ala-Ala-Phe-MCA as a substrate. We found that the enzymatic activity was stable in a pH range from 2.5 to 5.0, with an optimum pH of 4.0; activity was lost above pH 7.

Inhibition Studies—The inhibitory activities of selected inhibitors of TPP-I are summarized in Table 2. TPP-I was strongly inhibited by 0.1 mM AAF-CMK (*26*, *27*). The enzyme inactivated by AAF-CMK was dialyzed twice against 100 volumes of 25 mM acetate buffer, pH 4.0, for 2 h at 4°C, but the treated enzyme did not recover its original activity. In contrast, sedolisin was not inhibited under the same conditions, or even after the inhibitor concentration was raised to 2 mM. TPP-I was also inhibited by 1 mM Ac-IAF-CHO, but was insensitive to acetyl-pepstatin and tyrostatin. In addition, TPP-I was not inhibited by AEBSF, even at a concentration of 20 mM. A similar lack of inhibition (at an inhibitor concentration of 10 mM) was also observed in our studies of sedolisin and kumamolisin (unpublished data).

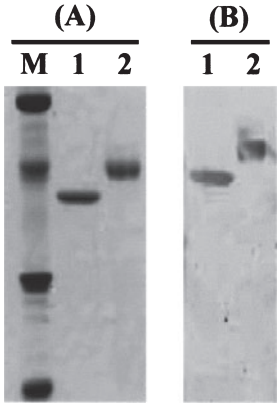

Fig. 1. **Homogeneity of the purified TPP-I.** Samples were subjected to 12.5% SDS–polyacrylamide gel electrophoresis (SDS-PAGE) after reducing treatment, stained with Coomassie Brilliant Blue R-250 (A), and analyzed by Western blotting using mouse anti–TPP-I/CLN2 antiserum (B). Lane 1, deglycosylated enzyme; 2, purified enzyme; lane M, molecular weight standards (bovine serum albumin, 66.2 kDa; ovalbumin, 45 kDa; bovine carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21.5 kDa).

Mutational Analysis—In order to identify the catalytic residues of TPP-I, we selected Glu77, Asp81, Asp165, and Ser280 for mutagenesis, based on their absolute sequence conservation among sedolisins (*9*). As mentioned above, recombinant TPP-I (wild-type) was produced in silkworm pupae mainly as the 46-kDa mature form (Fig. 2, lane 1) that showed normal tripeptidyl peptidase activity. The E77A and S280A mutant forms were expressed in the same manner as the wild-type enzyme (Fig. 2, lanes 2 and 5), but neither crude mutein showed enzymatic activity, even after acid activation. The D81A and D165A mutant forms were not produced in a soluble form (Fig. 2, lanes 3 and 4), although D165A was found in the insoluble material (data not shown). In contrast, the wild-type and D165A enzymes were expressed as soluble precursors in Sf-9 insect cells, although D165A was completely inactive (data not shown). It must be stressed that silkworm pupae (*Bombyx mori*) and Sf-9 cells (*Spodoptera frugiperda*) are very different types of host cells and that the culture conditions of Sf-9 cells might be suitable for normal folding of the D165A mutein. No D81A mutein was observed even in the insoluble material (data not shown). This lack of expression might be due to endogenous problems in the silkworm expression system.

Muteins S280A and E77A were purified from pupal hemolymphs as described under "EXPERIMENTAL PROCEDURES." As shown in Fig. 3, each enzyme showed a single band at around 46 kDa. The purified S280A could not cleave the substrate Ala-Arg-Phe*Nph-Arg-Leu, whereas the purified E77A showed very low, but measurable activity. Kinetic parameters of E77A for Ala-Arg-Phe*Nph-Arg-Leu were k_{cat} = 0.0124 s^{-1}, K_m = 123 μM, and k_{cat}/K_m = 0.0001 $\mu M^{-1} \cdot s^{-1}$. The k_{cat}/K_m value was four orders of magnitude lower than that of wild-type TPP-I

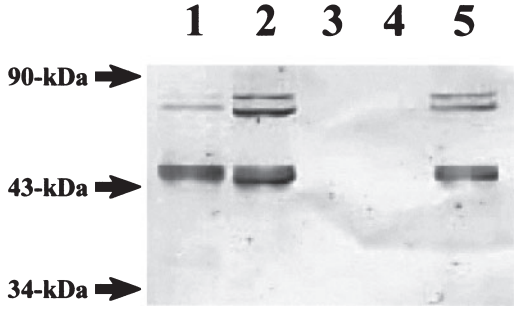

Fig. 2. **Western blot analysis of the TPP-I mutants.** The virus-infected pupae were homogenized. After the removal of insoluble materials, supernatants of wild-type (lane 1), E77A (lane 2), D81A (lane 3), D165 A (lane 4), and S280A (lane 5) were electrophoresed in 12.5% SDS–polyacrylamide gels and analyzed by Western blotting using a mouse anti-CLN2 antibody.

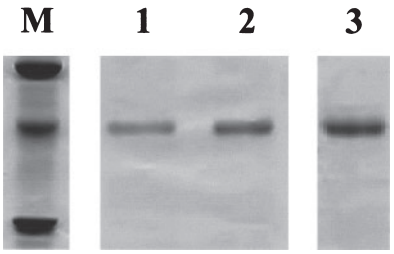

Fig. 3. **SDS-PAGE of the purified S280A and E77A muteins.** The purified enzymes were analyzed by SDS-PAGE under denaturing condition. After electrophoresis, the proteins were stained with Coomassie Brilliant Blue R-250. Lane 1, S280A; lane 2, E77A; lane 3, wild-type; lane M, molecular weight standards (bovine serum albumin, 66.2 kDa; ovalbumin, 45 kDa; bovine carbonic anhydrase, 31 kDa).

(k_{cat} = 16.4 s^{-1}, K_m = 22.5 μM, k_{cat}/K_m = 0.73 $\mu M^{-1}\cdot s^{-1}$) for the same substrate.

The Sizes of Subsites—The substrate specificity of TPP-I was investigated using the octapeptides described in "MATERIALS AND METHODS," based on the parent substrate Lys-Pro-Ile-Glu-Phe*Nph-Arg-Leu (Nph, *p*-nitrophenylalanine; *, cleavage site). TPP-I hydrolyzed the S_5-S_3' substrate Lys-Pro-Ile-Ala-Phe*Nph-Arg-Leu most effectively among them, but its k_{cat}/K_m value was only 0.0024 $\mu M^{-1}\cdot s^{-1}$ (k_{cat} = 0.68 s^{-1}; K_m = 288 μM). TPP-I showed higher activities against the S_4-S_3' substrate Ala-Ala-Ala-Phe*Nph-Arg-Leu and the S_3-S_3' substrate Ala-Ala-Phe*Nph-Arg-Leu. k_{cat}/K_m values of TPP-I for the hepta- and hexapeptide substrates were 0.15 and 2.33 $\mu M^{-1}\cdot s^{-1}$, respectively. These values are 63 and 970 times higher than that for the octapeptide substrate described above. Tripeptidyl peptidase activities of bacterial homologues such as sedolisin, sedolisin-B, kumamolisin, and kumamolisin-B for Ala-Ala-Phe-MCA were less than 1% that of TPP-I.

Substrate Specificity—(1) Effects of the P_1 residue of the substrate: The P_1 preference of TPP-I was studied using combinatorial peptide substrates Ala-Xaa-(Tyr/Nle/Leu/Trp/Phe)*Nph-Xaa-Leu. TPP-I cleaved all of the P_1-substituted substrates (Table 3). TPP-I preferred the following amino acid residues at the P_1 position: Phe (100%), Nle (97%), Tyr (82%), Leu (78%), and Trp (67%). By contrast, sedolisin preferentially cleaves peptides having a Glu or Asp at the P_1 position, whereas kumamolisin prefers Arg at the P_1 position (Table 3). Both enzymes also hydrolyzed P_1-Phe or P_1-Tyr derivatives.

Table 2. **Effect of inhibitors on tripeptidyl peptidase activity.**

Inhibitor	Inhibition (%)		
	100 μM	1 mM	20 mM
AAF-CMK	100	100	ND
acetylpepstatin	0	14	ND
tyrostatin	0	19	ND
Ac-IAF-CHO	30	88	ND
Ac-IPF-CHO	7	38	ND
chymostatin	3	47	ND
AEBSF	ND	ND	0

Each inhibitor was incubated with the enzyme at 37°C for 10 min at pH 4.0, and then the remaining activity was measured using Ala-Ala-Phe-MCA as a substrate. ND, not determined.

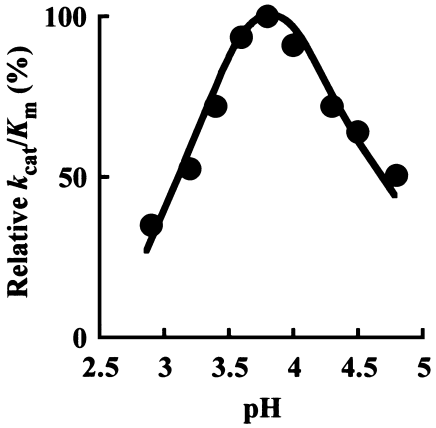

Fig. 4. **pH dependence of k_{cat}/K_m for TPP-I–catalyzed hydrolysis of Ala-Arg-Phe*Nph-Arg-Leu.** Values were obtained from the Lineweaver-Burk plot based on five initial substrate concentrations.

(2) Effects of the P_2 residue of the substrate: TPP-I preferentially cleaved peptides having an Arg, Ala or Asp at the P_2 position (Table 4). The best P_2 replacement was arginine. The k_{cat}/K_m value was 2.94 $\mu M^{-1}\cdot s^{-1}$, which was the highest value among the substrates tested in this study. This value is 40 times higher than that for Ala-Ala-Phe-MCA (k_{cat}/K_m = 0.068 $\mu M^{-1}\cdot s^{-1}$), the general assay substrate for TPP-I (*21*). Replacement of Arg by Ser or His decreased the k_{cat}/K_m value in this order.

(3) Effects of the P_3 residue of the substrate: TPP-I preferentially cleaved peptides with an Ala residue at the P_3 position (Table 4). Replacement of Ala by Asp, His, Arg, or Ser significantly decreased the k_{cat}/K_m value in this order. The specificity constants of Asp, His, Arg, or Ser derivatives were 14, 28, 50, and 53 times lower than that of Ala-Ala-Phe*Nph-Arg-Leu, respectively.

(4) pH profile: As shown in Fig. 4, the pH profile of the k_{cat}/K_m values of TPP-I for Ala-Arg-Phe*Nph-Arg-Leu is bell-shaped with an optimum pH of 3.9. In addition, the pK_a values were 3.5 and 4.1, respectively. Based on these data, it is suggested that carboxyl or imidazole residues are involved in the catalytic function of TPP-I.

DISCUSSION

TPP-I (CLN2) is a lysosomal proteinase that participates in protein degradation (*10*). A deficiency of this enzyme results in a fatal neurodegenerative disease (Batten's disease) (*4*). In order to clarify the biological implications of a TPP-I deficiency, recombinant human TPP-I and its muteins were characterized by molecular and biochemical approaches.

Table 3. **Relative hydrolysis rates of TPP-I, sedolisin, and kumamolisin for P_1-combinatorial peptide substrates.**

TPPI	Phe (100) > Nle (97) > Tyr (82) > Leu (78) > Trp (67)
sedolisin	Glu (100) > Asp (92) > Gly (77) > Asn (77) > Phe (66)
kumamolisin	Arg (100) > His (63) > Leu (48) > Trp (46) > Tyr (41)

Numbers in parentheses are relative activity, %.

Table 4. **P_2 and P_3 preferences of TPP-I for hexa-peptides.**

Substrate						K_{cat} (s^{-1})	K_m (μM)	K_{cat}/K_m ($\mu M^{-1}\cdot s^{-1}$)
P_3	P_2	P_1	P_1'	P_2'	P_3'			
Ala	**Ala**	Phe	Nph	Arg	Leu	34.5	14.8	2.33
Ala	**Arg**	Phe	Nph	Arg	Leu	11.8	4.02	2.94
Ala	**Asp**	Phe	Nph	Arg	Leu	11.0	5.09	2.16
Ala	**Ser**	Phe	Nph	Arg	Leu	30.7	48.3	0.64
Ala	**His**	Phe	Nph	Arg	Leu	26.4	42.2	0.63
Asp	Ala	Phe	Nph	Arg	Leu	18.5	110	0.17
His	Ala	Phe	Nph	Arg	Leu	13.7	167	0.082
Arg	Ala	Phe	Nph	Arg	Leu	0.91	19.3	0.047
Ser	Ala	Phe	Nph	Arg	Leu	4.7	108	0.044

The recombinant TPP-I showed an optimum pH of 4.0, and was stable in the acidic pH region. The optimum pH of the human brain enzyme was determined to be 3.5, using Gly-Phe-Phe-Leu-amino-trifluoromethyl coumarin as substrate (*43*). The bovine brain enzyme was stable at acidic pH but not at neutral or alkaline pH (*27*). Thus, the enzymatic properties of the recombinant TPP-I are similar to those reported for naturally occurring enzymes.

The molecular mass of the recombinant TPP-I was estimated to be 46 kDa (Fig. 1, lane 2). TPP-I is translated as a 66-kDa precursor protein, and then post-translationally glycosylated and processed to a 46-kDa mature enzyme (*4*, *16*). Based on the DNA sequence, the molecular mass of the mature enzyme is predicted to be 39.7 kDa, with five putative *N*-glycosylation sites (*4*, *41*). After deglycosylation of the recombinant TPP-I, the mature enzyme migrated as a single 40-kDa band on SDS-PAGE (Fig. 1, lane 1), suggesting that the enzyme expressed in pupae is glycosylated by a typical Man_3-$GlcNAc_2$ motif, as observed in insect cells (*44*).

Lin *et al.* reported that Ser280 in TPP-I can be labeled by 5 mM DFP (*16*). In contrast, recombinant TPP-I is not inhibited by 20 mM AEBSF. Rat spleen and bovine brain enzymes are also insensitive to DFP, PMSF, and AEBSF (*26*, *27*). In addition, sedolisin and kumamolisin are not inhibited by 10 mM AEBSF. Thus, such chemical modifiers as DFP are not effective for inhibiting sedolisins, even though a serine residue is involved in the catalytic function.

The active site serine residue in serine proteases reacts with the carbonyl of a chloromethyl ketone to give a tetrahedral structure, followed by alkylation of the nitrogen in the imidazole ring of the active site histidine residue (*45*). As shown in Table 2 and reported previously (*12*, *14*, *16*, *21*, *24*, *27*), TPP-I was inactivated by AAF-CMK, but the treated enzyme did not recover its activity by dialysis, suggesting that this type of inhibition is irreversible. In addition, TPP-I was inactivated by Ac-IAF-CHO. In kumamolisin, Ac-IAF-CHO was found to bind covalently to the catalytic serine residue (*19*). The results described here suggest to us that a Ser residue in TPP-I is also crucial for the catalytic function.

In order to elucidate the identity of the catalytic residues of TPP-I, mutational analysis was carried out. In silkworm pupae, a 46-kDa mature form appeared without acidic activation. This might be due to the cleavage of the precursor protein by endogenous silkworm TPP-I or by other proteinases from infected virus and/or silkworm pupae, as observed in Chinese hamster ovary cells (*41*, *42*). The muteins S280A and E77A did not show any significant enzymatic activities. Based on the results of inhibition studies and on the sequence similarity with other sedolisins, the involvement of Ser280, Glu77, and Asp81 in TPP-I in the catalytic function of this enzyme is strongly indicated. The involvement of Ser280 and Asp81 has been shown directly before (*9*, *17*), although Glu77 has not previously been shown experimentally to be one of the catalytic residues in TPP-I. Thus, this study provides the first clear experimental evidence that Glu77 in TPP-I is involved in the catalytic function, most likely as a general base.

TPP-I hydrolyzed Ala-Arg-Phe*Nph-Arg-Leu most effectively among all substrates tested. Kinetic parameters of TPP-I for this peptide were K_m = 4.02 μM, k_{cat} = 11.8 s^{-1}, and k_{cat}/K_m = 2.94 $\mu M^{-1}\cdot s^{-1}$. The k_{cat}/K_m value was 40 times higher than that for Ala-Ala-Phe-MCA, which has been used as a general substrate for TPP-I (*21*). TPP-I showed lower activities for octa- (S_5-S_3') and hepta-pep-

Table 5. **Substrate specificity of authentic TPP-I for various substrates.**

Substrate										Total residues	Bovine brain[27]	Rat liver[24]
		P_3	P_2	P_1	↓	P_1'	P_2'	P_3'				
1	ATP synthase subunit C fragment	D	I	D		T	A	A	–	6	O	ND
2	angiotensin II	D	R	V		Y	I	H	–	8	O	×
3	angiotensin III	R	V	Y		I	H	P	–	7	ND	O
4	bradykinin	R	P	P		G	F	S	–	9	ND	×
5	dynorphin A 1-8	Y	G	G		F	L	R	–	8	ND	×
6	des-[1]Tyr-dynorphin	G	G	F		L	R	R	–	7	ND	O
7	γ-endorphin	Y	G	G		F	M	T	–	17	×	×
8	substance P	R	P	K		P	Q	Q	–	11	×	×

O, hydrolysis; ×, not hydrolysis; ND, not determined.

tides (S_4-S_3') than for hexa-peptides (S_3-S_3'). Sedolisin and kumamolisin did not exhibit any significant activities against Ala-Ala-Phe-MCA. In addition, sedolisin was not inhibited by AAF-CMK. These results suggest that the substrate-binding cleft of TPP-I is shorter than those of other sedolisins, and that it is composed of three non-prime subsites (S_3-S_1) and at least three prime side subsites (S_1'-S_3'). Homology modeling of the structure of CLN2 based on the experimentally-determined structures of sedolisin and kumamolisin (*46*) lead to analogous conclusions.

TPP-I preferred bulky and hydrophobic amino acid residues at the P_1 position of a substrate, suggesting that the S_1 subsite of TPP-I is large enough to accommodate such bulky amino acid residues, and that it is composed of hydrophobic residues (Table 3). The substrate specificity of authentic TPP-I for natural substrates reported so far is summarized in Table 5. TPP-I hydrolyzes its known physiological substrate, ATP synthase subunit c, between an Asp3 and a Thr4 residue (P_1-P_1'). TPP-I does not cleave peptides having a Lys at the P_1 position. According to a homology model of TPP-I, it has been predicted that the S_1 pocket of TPP-I has a less polar character than that of sedolisin (*46*). The results described above strongly support this prediction.

TPP-I preferentially cleaved peptides having Arg, Ala, or Asp residue at the P_2 position (Table 4). TPP-I also cleaved natural peptides having Ile, Arg, Val, or Gly at this position (Table 5). In contrast, sedolisin and kumamolisin did not exhibit any preference for peptides having such charged amino acid residues at the P_2 position (*36*, *37*, *47*). These results suggest that the electrostatic state of the S_2 pocket in TPP-I is different from those of sedolisin and kumamolisin. The human lysosomal aspartic proteinase, cathepsin D, is involved in the normal degradation of intracellular and endocytosed proteins (*48*). Cathepsin D accommodates large hydrophobic residues in the P_2 position of the substrate, but does not prefer such positively charged residues as Lys or Arg in this position (*47*, *49*, *50*).

As shown in Table 4, TPP-I preferentially hydrolyzed a peptide with a P_3-Ala substitution. In addition, TPP-I cleaved natural peptides having Asp, Arg or Gly at the P_3 position (Table 5). TPP-I also hydrolyzed a collagen-related peptide (*23*). In contrast, TPP-I did not cleave natural peptides having Tyr at this position (Table 5). These results suggest that TPP-I prefers small amino acid residues at the P_3 position. On the other hand, sedolisin and cathepsin D prefer large and hydrophobic residues at this position (*36*, *47*, *49*, *50*). Thus, the S_3 subsite of TPP-I may be smaller than those of sedolisin and cathepsin D.

As shown in Table 2, TPP-I was inhibited by Ac-IAF-CHO. However, the inhibitory activity was weaker than that of AAF-CMK. In contrast, Ac-IAF-CHO showed inhibition for sedolisin and kumamolisin in the micromolar range [K_i = 0.6 μM) (*18*) and (K_i = 0.9 μM) (*7*), respectively]. These results suggest that electrostatic interactions between the amino-terminal group at the P_3 position and a carboxyl group in the S_3 pocket is important for the catalytic reaction. In the homology model of TPP-I, it was predicted that the carboxyl group of Asp132 would extend out into the active site cleft and act as an anchor for the N terminus of the substrate (*46*). The experimental result is consistent with that prediction. The pH profile of TPP-I is similar to that of kumamolisin (*30*). In kumamolisin, the active site cleft shows a strong negative overall charge (*19*). This suggests that the catalytic machinery of TPP-I is identical to that of kumamolisin.

TPP-I is the only member of the family of sedolisins that has been shown to exhibit tripeptidyl peptidase activity. In addition, the P_2 and P_3 preferences of TPP-I are quite different from those of bacterial homologues and cathepsin D. The substrate-binding cleft of TPP-I might have evolved in order to share function with cathepsin D in the lysosomal compartment. To describe the catalytic function in detail, three-dimensional structural analyses of TPP-I complexes with and without new tripeptide-based inhibitors are currently under way.

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