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Alkaline serine protease produced by *Streptomyces* sp. degrades PrP^{Sc} ☆

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Abstract

A PrP^{Sc}-degrading enzyme was isolated from the culture medium of *Streptomyces* sp. using perchloric acid-soluble protein (PSP) as a substrate. The media of 500 microbial species were screened to obtain the PSP-degrading enzyme. The medium containing the protease secreted from strain 99-GP-2D-5 showed the highest PSP-degrading activity. Strain 99-GP-2D-5 was assigned as the genus *Streptomyces* by its morphological and chemotaxonomic characteristics. When scrapie prion was used as the substrate, it was completely digested by the enzyme. The amino acid sequence of the enzyme was identical to that of the C-terminal region of alkaline serine protease (ASP) I. ASP I may be the precursor of the enzyme, and the enzyme seems to be the mature type of ASP I. The maximal activity of the enzyme was observed at 60 °C and pH 11, and the scrapie prion was degraded within 3 min under the optimum conditions.

Keywords: Protease; Prion; Transmissible spongiform encephalopathy; Perchloric acid-soluble protein; Scrapie

The prion protein (PrP) is closely associated with a group of related neurodegenerative diseases. It takes various forms that are manifested as sporadic, dominantly heritable, and transmissible disorders [1,2]. Normally, PrP is a cell-surface N-linked glycoprotein that is widely expressed and particularly abundant in the brain, and it may function as a signal transduction protein [3,4]. The underlying pathogenetic event in all prion diseases is conformational change of the normal or cellular prion protein (PrP^{C}) that is a soluble form with a predominant α-helical conformation to the pathogenic form that is aggregated, rich in β-sheets, and partially resistant to proteinase-K digestion (PrP^{Sc}) [5,6].

We have isolated and characterized a perchloric acid-soluble protein (PSP) from the cytosolic fraction of rat liver (RL-PSP) [7], rat kidney (RK-PSP) [8], rat brain (RB-PSP) [9], rat lung (RLu-PSP) [10], pig liver (PL-PSP) [11], and chick liver (CL-PSP) [12]. The cDNA of RL-PSP contains 411 bp and encodes a protein of 137 amino acid residues with a molecular mass of 14,149 Da [7]. The amino acid sequence of RL-PSP is completely identical to those of RK-PSP, RB-PSP, and RLu-PSP. Furthermore, a 14 kDa translational inhibitor protein that shows remarkable similarity to PSP has been characterized from human monocytes, mouse liver, and goat liver [13–15]. The expression of

☆ *Abbreviations:* PSP, perchloric acid-soluble protein; TSE, transmissible encephalopathy; PrP^{C}, cellular prion protein; PrP^{Sc}, disease-associated prion protein.

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the mRNA of p14.5, the human homologue of RL-PSP, is significantly up-regulated with the induction of differentiation into macrophages [13]. In addition, the synthesis of RK-PSP increases in the rat kidney from fetal day 17 to postnatal week 4, and then attains a steady-state level [8]. In contrast, the expression of RK-PSP in renal tumor cells was down-regulated [8]. Thus, PSP and PSP-like proteins appear to be involved in the growth and differentiation of different tissues. On the other hand, the cDNA sequences of PSP from mammals show a high similarity to members of a new hypothetical family (the YER057c/YJGF family) of small proteins whose functions are presently unknown. The high degree of evolutionary conservation of these proteins may reflect an involvement in basic cellular regulation.

Recently, Volz [16] determined the structure of YJGF protein from *Escherichia coli* at a resolution of 1.2 Å. The YJGF molecule is a homodimer with exactly three-fold symmetry. Its tertiary and quaternary structures are related to those of *B. subtilis* chorismate mutase [17], although their active sites are completely different. Furthermore, we have crystallized RL-PSP [18] and determined the structure of the PSP protein (unpublished data). Its monomeric structure is the same as that of YJGF protein from *E. coli*. The monomeric structure of PSP consists of 2 α-helices and 6 β-sheets. Interestingly, the structure of PSP resembles the structure of PrP^{Sc} which consists of 2 α-helices and 4 β-sheets [5,6]. Furthermore, the chemical characters of PSP, which are heat-stability and proteinase-K resistance, are the same as those of PrP^{Sc} (unpublished data). These results suggest that PSP may become a model protein for PrP^{Sc} as a substrate of PrP^{Sc} protease. In this study, we screened microbial culture media to obtain the PrP^{Sc} protease. As a consequence, a *Streptomyces* species that secretes the PrP^{Sc} protease was screened out and the PrP^{Sc} protease was isolated and characterized.

Materials and methods

Purification of pig liver PSP as a substrate. Purification of PL-PSP was performed as described previously [11]. Isolated pig liver was homogenized in two volumes of cold 0.25 M sucrose in a buffer (50 mM Tris–HCl, pH 7.4, 25 mM KCl, and 10 mM $MgCl_2$) with an all-glass Potter–Elvehjem type homogenizer. After centrifugation at 10,000*g* for 30 min, the post-mitochondrial supernatant (PMS) was obtained and treated with an appropriate volume of 60% perchloric acid to make a 5% solution of the PMS, and then centrifuged again at 10,000*g* for 10 min. The supernatant was adjusted to 25% with respect to trichloroacetic acid. The precipitate was washed three times with cold acetone and dried. The dried material was adjusted to 0.9% with respect to acetic acid, and the supernatant was dialyzed overnight against 0.1 M sodium phosphate buffer (PBS; pH 7.4) and fractionated with saturated ammonium sulfate between 30% and 50% saturation. The precipitate was suspended in 0.1 M sodium phosphate buffer (pH 7.4) and dialyzed against the same buffer. The dialysate was applied through a 2 × 30 cm column of CM-Sephadex C-25 and the flow-through fractions were collected and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Finally, protein fractions that cross-reacted with a PL-PSP antibody were pooled and again subjected to SDS–PAGE. The corresponding protein fractions were used as the substrate in this study.

Screening of the microorganisms. Five hundred microbials were screened to obtain the PL-PSP-degrading protease. Ten microliter aliquots of media from the cultures were added to test tubes containing 1 μg PL-PSP in Tris–HCl buffer (pH 8.0) and incubated for 30 min. The samples were then applied to SDS–PAGE and subjected to Western blotting analysis with a PL-PSP antibody. The activity of the PL-PSP-degrading enzyme was estimated by the disappearance of the corresponding band with PL-PSP. After positive candidates had been obtained, all samples were compared with each other after gradual dilution. The medium that showed the highest activity was subjected to evaluation of the PrP^{Sc} degradation.

Microorganisms and growth conditions. The *Streptomycete* strain 99-GP-2D-5 was derived from the sediment of the Yatsuhigata tidal float in Narashino City, Chiba Prefecture, Japan, and isolated on 0.2% colloidal chitin agar (Colloidal chitin (Sigma) containing 0.07% K_2HPO_4, 0.03% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$, 0.0001% $ZnSO_4$, 0.0001% $MnCl_2$, and 2.0% agar (Difco), pH 7.0) in 50% artificial seawater (Jamarin Laboratories, Osaka, Japan). The pure culture was maintained on yeast-starch agar (1.0% soluble starch (Wako), 0.2% yeast extract (Difco), and 3.5% agar (Wako), pH 7.0). A slant culture of *Streptomyces* sp. 99-GP-2D-5 was inoculated into 500 ml Erlenmeyer flasks containing 100 ml of 1.0% seed medium (fish extract (Kyokuto Seiyaku), 2.0% glucose, 1.0% yeast extract (Difco), 0.05% $MgSO_4 \cdot 7H_2O$, and 0.32% $CaCO_3$, pH 7.0), and shaken on a rotary shaker (180 rpm) at 27 °C for 5 days. Five hundred microliters of the seed culture was inoculated into 100 ml of production medium and cultured at 27 °C for 7 days on a rotary shaker.

Taxonomy of the enzyme-producing strain. The international Streptomyces Project (ISP) media recommended by Shirling and Gottlieb, and media recommended by Waksman were used for the cultural and physiological characteristics [19,20]. The isomeric forms of diaminopimelic acid (DAP) isomers were determined by the method of Becker et al. [21].

Isolation and purification of the enzyme. All of the purification procedures were performed at 4 °C. The medium including the microbes was centrifuged at 10,000*g* for 30 min. Solid ammonium sulfate was added to 900 ml of the supernatant to a final concentration of 80% saturation. The resultant precipitate was collected by centrifugation at 10,000*g* for 15 min, re-dissolved in 25 ml buffer A (20 mM acetate buffer containing 2 mM calcium acetate (pH 6.0)), and dialyzed against the same buffer overnight. After dialysis, the clear supernatant was applied to a CM-Sephadex C-50 column which had been pre-equilibrated with buffer A. Elution was first performed with the running buffer (buffer A) followed by 0.5 M NaCl in the same buffer. The fraction of the second peak was collected and concentrated. The concentrated fraction was dialyzed against buffer A for 6 h, and the supernatant was applied to a Sephadex G-50 column pre-equilibrated with the same buffer. The fraction eluted from the Sephadex G-50 column was used as the purified enzyme.

The enzymatic activity of each fraction was evaluated by the disappearance of PL-PSP in 15% SDS–polyacrylamide gels. To detect PL-PSP, the proteins were subjected to Western blotting analysis with a PL-PSP antibody and peroxidase-conjugated goat anti-rabbit IgG as the secondary antibody. The protein concentration was estimated by a dye-binding method. In the case of cleavage of scrapie prion, a hamster brain homogenate including the scrapie prion was used as the substrate and ECL blotting analysis was performed with a prion protein monoclonal antibody (SAF-32) (Cayman Chemical).

Mass spectrometry and amino acid sequence analysis. Mass spectra of the purified enzyme were obtained by matrix-associated laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry with a Voyager mass spectrometer (Applied Biosystems) using α-cyano-4-hydroxycinnamic acid as the matrix. The mass signals were

in accord with the predicted molecular masses of the corresponding peptides. To determine the N-terminal amino acid sequence, peptide fragments were subjected to Edman degradation using a Procise 494cLC protein sequencer (Applied Biosystems).

Results

A PSP-degrading enzyme is secreted from Streptomycete strain 99-GP-2D-5

The media from 500 microbial species were screened to obtain a PSP-degrading enzyme. The media from 10 microbial cultures were selected as candidates for the PSP-degrading enzyme and compared with each other after gradual dilution. The medium secreted from strain 99-GP-2D-5 showed the highest activity after 10-fold dilution (Fig. 1). When scrapie prion was used as the substrate, it was completely digested (Fig. 2). Here, the PSP-degrading enzyme was named E77.

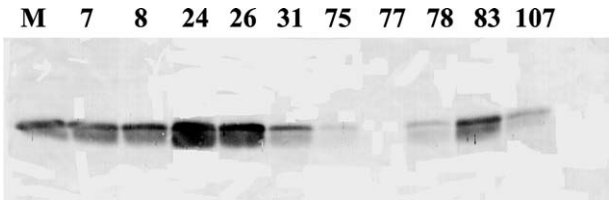

Fig. 1. Screening of a PL-PSP-degrading enzyme. Ten microbials which showed PSP-degrading activity were screened to obtain the PL-PSP protease. Ten microliter aliquots of 10-fold-diluted media from the cultures were added to test tubes containing 1 μg PL-PSP in Tris–HCl buffer (pH 8.0) and incubated at 37 °C for 30 min. The samples were subjected to SDS–PAGE and Western blotting analysis using a PL-PSP antibody. The activity of the PL-PSP-degrading enzyme was estimated by the disappearance of the corresponding band of PL-PSP. The sample numbers are shown on the top. E77 shows the highest activity.

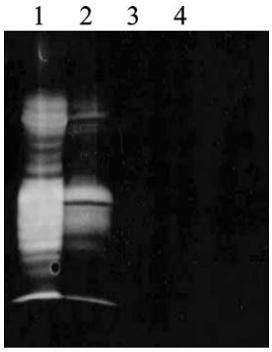

Fig. 2. Degradation of scrapie prion by E77. Forty micrograms of protein of a 15% brain homogenate including scrapie prion was incubated with the E77-containing medium for 30 min at 37 °C. The sample was subjected to 15% SDS–PAGE and ECL blotting analysis using a prion protein monoclonal antibody (SAF-32). Lane 1, control; lane 2, treated with proteinase K; lane 3, 10 μl E77 medium; and lane 4, 20 μl E77 medium.

Characterization of the microbial strain 99-GP-2D-5

Strain 99-GP-2D-5 was assigned as the genus *Streptomyces* by its morphological and chemotaxonomic characters, such as LL-diaminopimelic acid in whole cell hydrolysates. The complete 16S rDNA sequence of strain 99-GP-2D-5 was compared with those of other strains belonging to the Streptomycetaceae family. The sequence similarity was 95% and strain 99-GP-2D-5 was shown to be a *Streptomyces* sp. (data not shown). These results suggest that strain 99-GP-2D-5 belongs to a new species of the genus *Streptomyces*.

Isolation and purification of E77

E77 was purified as described in the Materials and methods. E77 was predominantly present in the medium but not in the cells (data not shown). E77 appeared in the 0.5 M NaCl-eluted fraction on CM-Sephadex C-50 chromatography and the Sephadex G-50 gel-filtered fraction was shown to be homogeneous by SDS–PAGE (Fig. 3). The molecular weight of E77 was about 19,000 Da. About 2.6 mg of the purified protein was obtained from 900 ml of the medium.

Mass spectrometry and amino acid sequence analysis

Mass spectra of the purified enzyme digested by trypsin were obtained by MALDI-TOF mass spectrometry. The protein was identified in the Genpept database through peptide mass fingerprinting (PMF) using the program MS-Fit in ProteinProspector (http://prospector.ucsf.edu). As a result, the enzyme was identified as

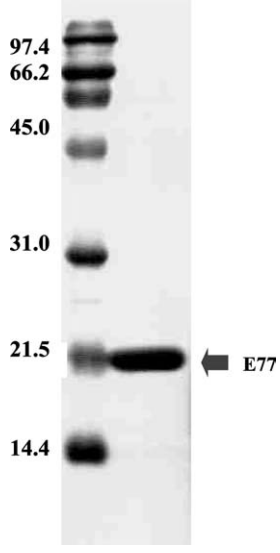

Fig. 3. SDS–PAGE of purified E77. E77 was purified as described in the Materials and methods. Ten micrograms of protein was subjected to 15% SDS–PAGE and stained with Coomassie brilliant blue R-250. Molecular mass standards (in kDa) are shown on the left.

Table 1
Determination of cleaved fragments of E77 enzyme

Fragments	Observed mass	Calculated mass	Assigned sequence
248–254	794.3897	794.4161	VSQGTFR
255–261	806.3532	806.3949	GSWFPGR
215–222	939.4555	939.4722	CSIGFSVR
262–279	2062.8992	2063.0020	DMAWVAVNSNWTPTSLVR
223–247	2494.0243	2494.1533	QGSTPGFVTAGHCGSVGNATTGFNR
310–332	2533.1322	2533.2469	CGTIQQHNTSVTYPQGTITGVTR

alkaline serine protease I (ASP I: Accession No. S395197) from *Streptomyces* sp. (Table 1). Although there are two isoforms of ASP (ASP I and ASP II), E77 was identical to ASP I. However, the molecular mass of ASP I is 39,567 Da, which is much larger than that of E77. Therefore, E77 was digested with trypsin, and the products were separated by reverse-phased HPLC. Finally, 10 peptides were obtained and five of these (Nos. 1, 4, 5, 8, and 10) were directly sequenced. The amino acid sequences of these five peptides were aligned with ASP I and ASP II (Table 2). The amino acid sequences of the trypsin-digested peptides were identical with the sequence of ASP I. Since the N-terminal amino acid sequence of E77 was YDLVG, peak 8 was the N-terminal fragment derived from the trypsin-digested peptides. Thus, the N-terminal amino acid of E77 started from the 200th amino acid of ASP I. These results suggest that ASP I may be the precursor of E77.

Table 2
Alignment of ASP I, ASP II, and amino acid sequences of tryptic fragments of E77

Peptide	Enzyme	Amino acid sequences
Peak 1	ASP I	SGSTTGWR
	ASP II	SGSTTGWH
	E77	SGSTTGWR
Peak 4	ASP I	GSWFPGR
	ASP II	ESSFPGD
	E77	GSWFPGR
Peak 5	ASP I	CGTIQQHNT SVTYPQGTITGVTR
	ASP II	CGTIQQHNTSVTYPEGTITGVTR
	E77	CGTIQQHNTSVTYPQGTITGVTR
Peak 8	ASP I	YDLVGGDAYYIGNGR
	ASP II	YDLVGGDAYYMGGGR
	E77	YDLVGGDAYYIGNSR
Peak 10	ASP I	DMAWVAVNSNWTPTSLVR
	ASP II	DMAWVSVNSDWNTTPTV
	E77	DMAWVAVNSNWTPTSLVR

Effects of temperature and pH on the activity of E77

The effects of temperature and pH on the enzyme are shown in Fig. 4. The maximum activity of E77 was observed at 60–80 °C (Fig. 4A), and the optimum pH was >10 (Fig. 4B). When scrapie prion was used as a substrate, it was completely digested by E77 within 3 min under the optimum conditions of 60 °C and pH 10 (Fig. 5).

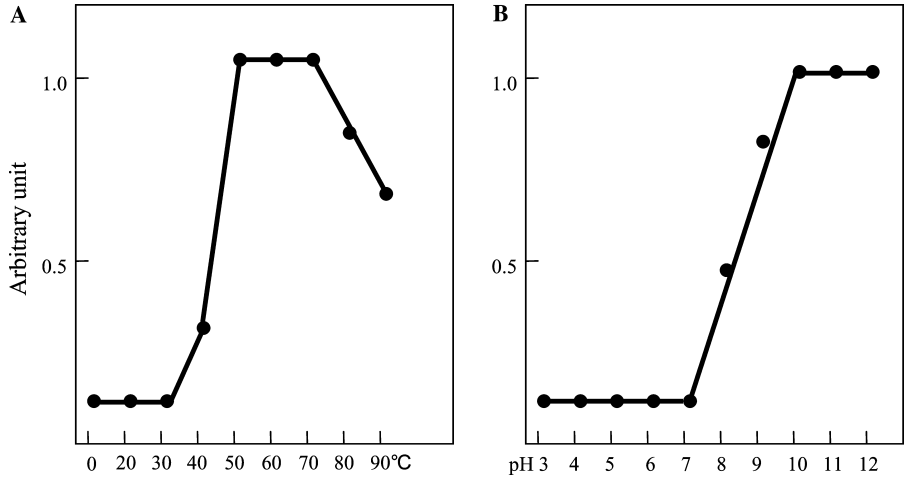

Fig. 4. Effects of temperature and pH on the activity of E77. Forty micrograms of a 15% hamster brain homogenate containing scrapie prion was incubated with 2 μg E77 at the indicated temperatures and pH. The samples were subjected to 15% SDS–PAGE and ECL blotting analysis using a prion protein monoclonal antibody (SAF-32). Photographic films of the ECL blots were recorded with a scanner and the resulting densities were further processed with NIH Image 1.62.

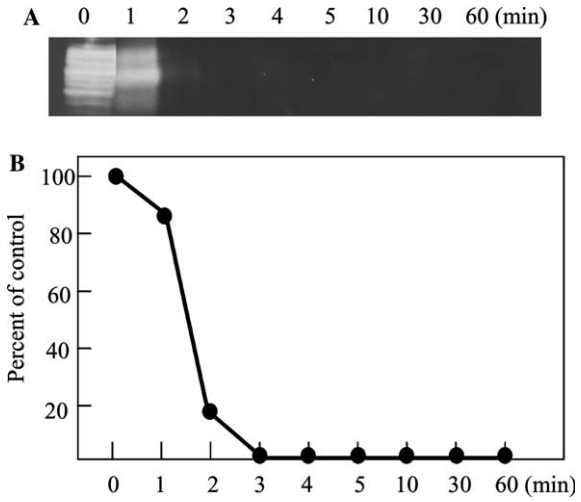

Fig. 5. Time course of the degradation of scrapie prion by E77. Forty micrograms of a 15% hamster brain homogenate containing scrapie prion was incubated with 2 μg E77 at 60 °C and pH 11. The samples were processed as described in the legend for Fig. 4.

Discussion

A PrP^{SC}-degrading enzyme was isolated from the medium of *Streptomyces* sp. using PL-PSP as a substrate. The monomer structure of PSP consists of 2 α-helices and 6 β-sheets. Interestingly, the structure of PSP resembles the structure of PrP^{Sc} which consists of 2 α-helices and 4 β-sheets. Furthermore, the properties of PSP, such as heat-stability and proteinase K resistance, are very similar to those of PrP^{Sc}. Thus, PSP could be used as a safe substrate for screening for PrP^{Sc}-degrading enzymes.

The amino acid sequence of E77 was identical to that of the C-terminal region of ASP I. ASP I may be the precursor of E77, and E77 seems to be the mature type of ASP I. At present, investigations into the mechanism by which E77 is processed from ASP I are actively in progress. There are two isoforms of ASP (ASP I and ASP II). The cDNA of the C-terminal region of mature-type ASP II was amplified by PCR and an expression vector was constructed (unpublished data). The recombinant ASP II was expressed in *E. coli* and also showed the PrP^{Sc}-degrading activity. Although the homology between ASP I and ASP II is only 70%, both enzymes have PrP^{Sc}-degrading activity. Yum et al. [22] reported an alkaline serine protease from an alkalophilic *Streptomyces* sp. This enzyme seems to be derived from ASP II according to the N-terminal amino acid sequence.

In this study, it was shown that scrapie prion was digested by E77. Prion protein derived from CJD was also digested by E77 (data not shown). It is highly possible that BSE prion would also be digested by E77 based on the similarity of the three-dimensional structures. The activity of E77 was completely inhibited by DFP and PMSF, but not by EDTA. Thus, the sensitivity of the enzyme to inhibitors is identical to those of other serine proteases.

Recently, Langeveld et al. [23] studied a keratinase from *Bacillus licheniformis* using BSE-infected brain material. However, heat pretreatment to >100 °C was required for extensive enzymatic break down of PrP^{Sc} to a state where it was immunochemically undetectable in the presence of detergents. Furthermore, McLeod et al. [24] reported that the subtilisin-enzyme Properase showed a significant extension in the incubation period in mouse bioassays following a 30 min incubation at 60 °C and pH 12. Since E77 degrades PrP^{Sc} within 3 min under the optimum conditions and does not require preincubation, the activity of E77 seems to be the highest among these enzymes. However, mouse bioassays need to be tested to demonstrate the final effectiveness of prion inactivation by E77. Mouse bioassays using E77 are actively in progress.

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