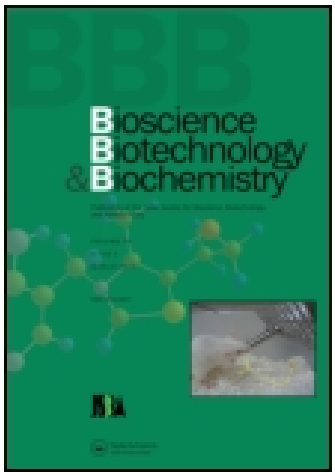

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Purification and Some Properties of a Protease from *Streptomyces limosus*

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Streptomyces limosus **was selected because it secreted a novel protease that catalyzed the synthetic reaction forming Pro-Pro-Pro from Pro-Pro. The protease was purified to an electrophoretically homogeneous state and an activity of more than about 20,000-fold that of the culture broth. The molecular mass of the enzyme was estimated to be 50 kDa by SDS–polyacrylamide gel electrophoresis. The enzyme was most active in alkaline pH for the synthetic reaction producing Pro-Pro-Pro from Pro-Pro, although for the hydrolytic reaction forming proline it was most active in neutral pH. The enzyme was inhibited by 1,2-epoxy-3-(*p*-nitrophenoxy)propane (EPNP) and diazoacetyl-DL-norleucine methyl ester (DAN). It can be considered that this enzyme belongs to the class of aspartic proteases. The substrate specificity indicates that this enzyme has a strong affinity for proline as a N-terminal amino acid of peptides.**

The reverse reaction of proteases, *i.e.*, peptide bond synthesis, can also be catalyzed by the same enzyme, as was first demonstrated by Bergmann and Fraenkel-Conrat[1] in 1937 with papain which catalyzed amide (anilide) bond synthesis. Since then, many reports have been appeared on reverse reactions of proteases. However, proteases that can catalyze the condensation or transfer reactions of proline residue are rare, except for proline-specific dipeptidylcarboxypeptidase,[2,3] although there are several enzymes having affinities for proline in the hydrolysis reaction such as prolyl endopeptidase, N-terminal exopeptidases (dipeptidyl aminopeptidase IV, proline iminopeptidase, and aminopeptidase P), C-terminal exopeptidases (prolyl carboxypeptidase and carboxypeptidase P), and dipeptidases (prolinase and prolidase).[4,5]

On the other hand, proline is a very unusual amino acid, in that the side-chain is cyclized back on to the backbone amide position. It was also reported that in all proteins with repetitive sequence containing proline, the proline-rich region serves to bind rapidly and reversibly to other proteins or polyphenol.[6] Therefore, it is worthwhile for elucidation of the proline-protein interaction to obtain enzymes with activities introducing proline into other peptides (or proteins).

To find an enzyme that efficiently catalyzes the condensation or transfer reactions of proline residues, we screened various microorganisms, using a high concentration of Pro-Pro solution as the substrate. A novel enzyme was prepared from a strain of *Streptomyces limosus* and this enzyme catalyzed the reaction forming Pro-Pro-Pro from Pro-Pro. This study deals with the purification and some properties of this protease from *S. limosus*.

Materials and Methods

Materials. Synthetic peptides were purchased from Bachem Feinchemikalien AG, Sigma Chemical Company, and the Peptide Institute, Inc. Osaka. Monoiodoacetic acid, *p*-chloromercuribenzoate, and dithiothreitol were purchased from Nacalai Tesque, Inc. E-64 was obtained from the Peptide Institute, Inc. Osaka. All other inhibitors of proteases were obtained from Sigma Chemical Company. All other chemicals used were of reagent grade.

Preparation of soybean protein hydrolysate (SPH). SPH was prepared from the hydrochloric acid-precipitated fraction of soybean protein which was incubated with pepsin, according to the method of Fujimaki *et al.*[7,8]

Screening for microorganisms that secrete proteases. Microorganisms that secreted protease were screened for from soils and stock cultures kept in our institute.

For the first screening of protease-producing microorganisms, the plastein reaction, which is known as a reaction of peptide synthesis was adopted. Microorganisms were cultured in three media (A, B, and C) with shaking at 27°C for 2–4 days. Medium A was composed of 1% bonito extract, 1% peptone, and 0.5% NaCl (pH 7.0) for bacteria. Medium B was composed of 0.5% meat extract, 0.5% peptone, 1% glucose, and 0.5% NaCl (pH 7.0) for Actinomycetes. Medium C was composed of 1% yeast extract and 3% peptone (pH 6.0) for molds and yeast. After centrifugation of the culture broths, SPH (40 mg) was dissolved by each supernatant (0.2 ml) and then incubated at 37°C for 1–7 days. Here, we selected microorganisms forming turbidity or gelling the SPH solution. Then, these strains (bacteria and Actinomycetes) were cultured in two media (A and B) with shaking at 27°C for 2 or 4 days. After centrifugation of the culture broth, solid ammonium sulfate was added to the supernatant up to 80% saturation. The ammonium sulfate-precipitated enzymes, which were dialyzed in 0.01 M phosphate buffer (pH 7.0), were used in the second and third screenings.

For the second screening of microorganisms, we measured the hydrolytic activities of ammonium sulfate-precipitated enzymes, using 0.1% gelatin, which has an abundance of proline, as a substrate. After gelatin was hydrolyzed by each crude enzyme at 40°C in 0.02 M phosphate buffer (pH 7.0), the amounts of amino groups liberated were measured by the method of Yemm and Cocking.[9] Here, we chose microorganisms with strong hydrolytic activities.

For the third screening of microorganisms, their activities of peptide synthesis were checked by high voltage paper electrophoresis (HVPE), using Pro-Pro as a substrate. After each ammonium sulfate-precipitated enzyme was incubated with 0.3 M Pro-Pro at 40°C for 5 h in 0.015 M phosphate buffer (pH 7.0), products were analyzed by HVPE, using Toyo No. 50 filter paper (60 × 30 cm). Conditions of electrophoresis were 3000 V at 10°C for 105 min, using formic acid–acetic acid–water (3 : 9 : 8) as a buffer. After HVPE, reaction products were stained with ninhydrin reagent. Here, we chose microorganisms having synthetic activities.

Assay of the formation of Pro-Pro-Pro and proline. For each enzyme, 0.15 M Pro-Pro was incubated with the enzyme at 40°C in 20 mM phosphate buffer (pH 7.0). After an appropriate time of incubation, a mixture of 0.75 N HCl and 0.2 M phosphate buffer (pH 2.1) was added to the solution of the enzyme reaction. This solution was analyzed by reverse-phase high-performance liquid chromatography (HPLC) for measurement of the amounts of Pro-Pro-Pro and to an amino acid analyzer for that of proline.

HPLC was done with a Chemcosorb 5-ODS-UH column (0.46 × 25 cm) at a flow rate of 0.8 ml/min and 40°C, using 5% acetonitrile containing 0.05% trifluoroacetic acid as the mobile phase. The elution pattern was monitored by the absorbance at 220 nm. One unit of activity was defined as the amount of enzyme that produced 1 μmol of Pro-Pro-Pro per hour.

Assay of the hydrolytic activity of the enzyme against various peptides. A mixture of enzyme and each substrate (8 mM) was incubated at 40°C in 0.02 M phosphate buffer (pH 7.0) containing 10% methanol. After an appropriate time, the amounts of amino groups liberated in the reaction mixture were measured by the method of Yemm and Cocking.[9] The hydrolytic products were identified by thin-layer chromatography.[10]

Electrophoresis. Polyacrylamide gel electrophoresis (PAGE) was done on a 7.5% polyacrylamide gel as described by Davis.[11] Sodium dodecyl sulfate (SDS)–PAGE was done on a 15% gel by the method of Laemmli.[12] Protein bands on PAGE and SDS–PAGE were stained with Coomassie Brilliant Blue.

Protein measurement. Protein was measured spectrophotometrically by the absorbance at 280 nm, using a Shimadzu UV-210A spectrophotometer.

Field desorption-mass spectrum (FD-MS). FD-MS was done, using a JEOL JMS-DX303HF mass spectrometer. A lyophilized sample was analyzed.

Results and Discussion

Screening of microorganisms

In the first screening, we obtained 27 strains the culture supernatants of which made the SPH solution turbid. These strains were only bacteria and Actinomycetes. In the second screening, we obtained 7 strains that secreted enzymes having strong hydrolytic activities on gelatin. In the third screening, it was found that the protease from *Streptomyces limosus*, which was a stock culture in our institute, produced unknown compounds (proline oligomers, as shown later) stained with ninhydrin reagent, aside from the hydrolytic product (proline) and the substrate (Pro-Pro). Therefore, to purify this enzyme, *S. limosus* was cultivated.

Cultivation and purification

S. limosus was cultivated at 27°C for 4 days in shaking flasks containing 70 ml of medium B. After centrifugation, the supernatant was used as the crude enzyme for purification.

Step 1. Ammonium sulfate fractionation. To the supernatant (4125 ml), solid ammonium sulfate was added up to 80% saturation. The resulting precipitate was collected by filtration and dissolved in a small amount of 0.01 M phosphate buffer, pH 7.0. The solution was dialyzed sufficiently against the same buffer.

Step 2. DEAE-cellulose column chromatography. The dialyzed solution (234 ml) was put on a DEAE-cellulose column (3.3 × 32 cm), which had been equilibrated with 0.01 M phosphate buffer, pH 7.0. The column was washed sufficiently with the same buffer and the protease was eluted by a linear gradient from 0 to 0.9 M NaCl. The active fractions were combined and dialyzed against 0.01 M phosphate buffer, pH 7.0, containing 0.1 M NaCl.

Step 3. Sephadex G-75 gel filtration. The dialyzed solution was concentrated with an ultrafiltration membrane. The concentrated solution was put on a Sephadex G-75 column (2.6 × 105 cm) that had been equilibrated with 0.01 M phosphate buffer, pH 7.0, containing 0.1 M NaCl. Elution was done with the same buffer. The active fractions were combined and concentrated to 8.6 ml.

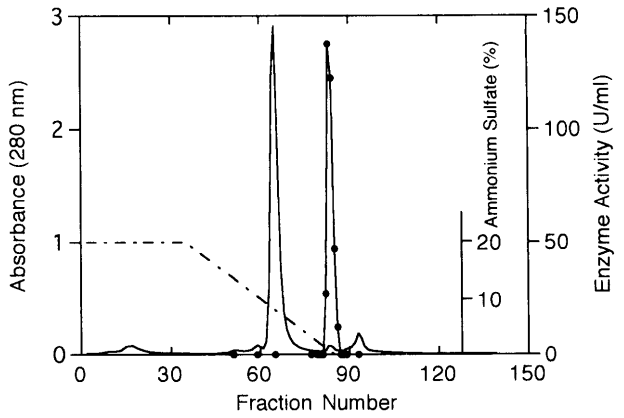

Fig. 1. Elution Pattern of the Protease from *S. limosus* from a Butyl-Toyopearl 650 M Column.

Elution was done with a gradient of ammonium sulfate (20 to 0% saturation) in phosphate buffer, pH 7.0. Fractions of 2 ml were collected.
●, enzyme activity; ——, absorbance at 280 nm; – – –, concentration of ammonium sulfate.

Table I. Purification Procedure of the Protease from *S. limosus*

Purification steps	Protein (A_{280})	Enzyme activity (U)	Specific activity (U/A_{280})	Yield (%)
Supernatant of broth	35,700	2590	0.073	100
Ammonium sulfate	3,220	3580	1.11	138
DEAE-cellulose	374	2110	5.65	81
Sephadex G-75	63.2	2220	35.1	85
Butyl-Toyopearl	1.05	1550	1480	60

Step 4. Butyl-Toyopearl column chromatography. The concentrated solution in the presence of 20% saturated ammonium sulfate was directly adsorbed on Butyl-Toyopearl 650 M column (1.6 × 10 cm) equilibrated with 20% saturated ammonium sulfate solution, and washed with the same solution. Elution was done with a gradient of ammonium sulfate (20% to 0% saturation) in phosphate buffer, pH 7.0 (Fig. 1). The active fractions were combined and used as the purified enzyme preparation. This preparation was purified about 20,000-fold from the culture broth and the specific activity of Pro-Pro-Pro formation was 1,480 units/Abs. at 280 nm. This purified enzyme was used in the following experiments. The results of the purification procedures are summarized in Table I.

Homogeneity and molecular mass

Homogeneity of the purified enzyme preparation was tested by electrophoresis on polyacrylamide gel. The enzyme preparation was found to be homogeneous electrophoretically (Fig. 2). On SDS–PAGE, the enzyme preparation showed a single band corresponding to a molecular mass of 50 kDa, using phopshorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, and soybean trypsin inhibitor as standard proteins. Though we tried to estimate the molecular mass of this enzyme by gel filtration, we could not measure it owing to coagulation of the enzyme. It can be considered that this coagulation occurred because of the increase of hydrophobicity of the enzyme in the presence of ammonium sulfate on Butyl-Toyopearl chromatography. Further details of this will be investigated.

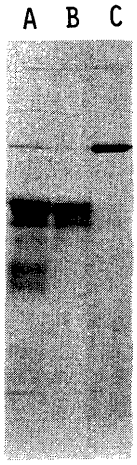

Fig. 2. Polyacrylamide Gel Electrophoresis of the Enzyme Preparations after Chromatographies on DEAE-cellulose (A), Sephadex G-75 (B), and Butyl-Toyopearl 650 M (C).

See the text for experimental details.

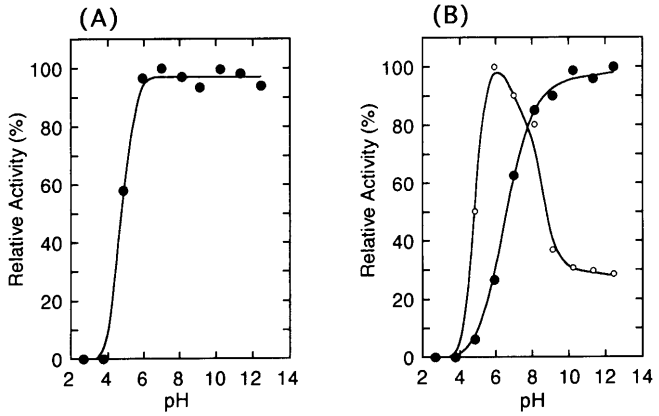

Fig. 3. Effects of pH on Stability (A) and Activity (B) of the Protease from *S. limosus*.

Conditions: (A) 40°C, 1 h (incubation); (B) 40°C, 1 h (reaction time), ●, Pro-Pro-Pro; ○, Pro.

Effects of pH on stability and activity

The enzyme was stable from pH 6.0 to 12.0 at 40°C for 1 h, as shown in Fig. 3(A). Using Pro-Pro as a substrate, the enzyme was most active from pH 10 to 12 in the reaction forming Pro-Pro-Pro (Fig. 3(B)). On the other hand, the optimum pH for the formation of proline was 6.0.

Effects of temperature on stability and activity

The enzyme was stable at temperatures below 50°C at pH 7.0 for 30 min (Fig. 4(A)). The optimum temperature for the formation of Pro-Pro-Pro was 65°C (Fig. 4(B)).

Effects of chemicals and metal ions

The enzyme was incubated with each inhibitor at 30°C for 15 min in 0.01 M phosphate buffer (pH 7.0), and with each metal ion at 30°C for 30 min in 0.05 M Tris–HCl buffer (pH 7.2). The synthetic activity was measured as described in Materials and Methods. As shown in Table II, the enzyme was markedly inhibited by EPNP and DAN, which are typical inhibitors of aspartic protease. Although PCMB also inhibited this enzyme, the enzyme was not inhibited by E-64, which is a typical inhibitor of cysteine protease. The inhibition by PCMB suggested that cysteine is near the active site. The chelators and the inhibitors of serine protease such as DFP and PMSF did not cause inhibition. From these results, it is considered that this enzyme is an aspartic protease. Metal ions at 1 mM including Mn^{2+}, Mg^{2+}, Ca^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Sr^{2+}, Cd^{2+}, and Ba^{2+} had no or partially inhibitory effects on this enzyme. Mercury ion inhibited the enzyme completely.

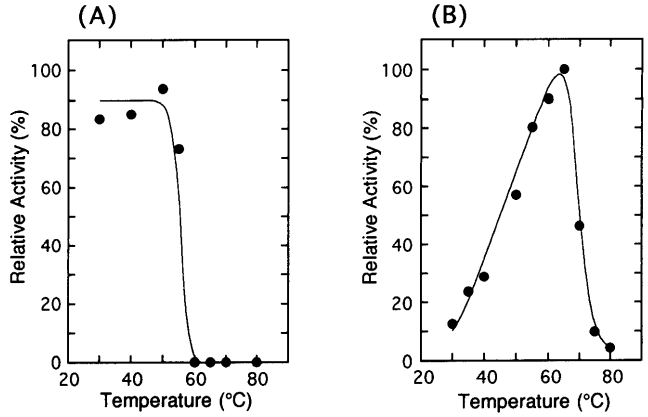

Fig. 4. Effects of Temperature on Stability (A) and Activity (B) of the Protease from *S. limosus*.

Conditions: (A) pH 7.0, 30 min (incubation); (B) pH 7.0, 1 h (reaction time).

Table II. Effects of Inhibitors on the Protease from *S. limosus*

Inhibitor	Concentration (mM)	Relative activity (%)
None		100.0
EPNP[a]	5 mg/ml	0.6
DAN[b]	2	9.0
E-64	2	83.2
PCMB	2	0.0
DTT	1	85.4
DFP	1	88.7
PMSF	4	116.1
EDTA	1	91.2
Phosphoramidon	2	104.8
o-Phenanthroline	1	92.0
Chloroquine	1	68.7
MIA	1	102.6
N-Ethylmaleimide	1	88.4
TPCK	4	114.8
TLCK	1	94.4
Aprotinin	119 U/ml	71.5
Chymostatin	0.25 mg/ml	92.9
Antipain	1.5	16.3
Leupeptin	1	39.9
Pepstatin A	1	8.7

[a] The reaction was done with 1 ml of enzyme solution and 5 mg of solid EPNP powder at 14°C and pH 7.0 for 72 h by the method of Tang.[13]

[b] The reaction was done in the presence of 2 mM $CuCl_2$.

EPNP, 1,2-epoxy-3-(*p*-nitrophenoxy)propane; DAN, diazoacetyl-DL-norleucine methyl ester; E-64, *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane; PCMB, *p*-chloromercuribenzoate; DTT, dithiothreitol; DFP, diisopropylfluorophosphate; PMSF, phenylmethanesulfonyl fluoride; EDTA, disodium ethylenediamine tetraacetate; MIA, monoiodoacetic acid; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; TLCK, *N*-tosyl-L-lysine chloromethyl ketone.

Hydrolytic action of the enzyme on peptides

To examine the hydrolytic action of the enzyme, it was

Table III. Hydrolytic Action of the Protease from *S. limosus* on Various Synthetic Peptides

Substrate	Relative activity (%)
Cbz-Pro-Phe-NH_2	0
Cbz-Pro-Phe	0
Pro-Phe-NH_2	236
Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg	0
Pro-Pro-Gly-Phe-Ser-Pro	280
Pro-Pro	100

The hydrolytic rate of Pro-Pro was taken as 100. Cbz, carbobenzoxy.

Table IV. Substrate Specificity of the Protease from *S. limosus*

Substrate	[Pro] (mM)	Unknown products
Pro-Pro	44.0	2
Pro-Hyp	16.4	2
Pro-Ala	10.7	1
Pro-Gly	15.5	3
Pro-Arg	16.1	4
Pro-Ser	13.6	3
Pro-Asp	19.8	0
Pro-Glu	22.9	1
Pro-Leu	14.0	0
Pro-Phe	14.5	1
Pro-Val	19.9	2
Ala-Pro	0.0	0
Gly-Pro	0.0	0
Arg-Pro	0.0	0
Ser-Pro	0.0	0
Tyr-Pro	0.0	0
Ile-Pro	0.0	0
Leu-Pro	0.0	0
Phe-Pro	0.0	0
Val-Pro	0.0	0

incubated with 8 mM peptides containing proline. As shown in Table III, this enzyme hydrolyzed N-unprotected peptides such as Pro-Phe-NH_2, and Pro-Pro. However, it was not active on N- and C-protected peptides or on N-protected peptides. Furthermore, this enzyme hydrolyzed Pro-Pro-Gly-Phe-Ser-Pro and then produced proline, although it could not hydrolyze Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg (bradykinin). Thus, it can be considered that this enzyme belongs to a class of aminopeptidases and attacks peptides containing proline as a N-terminal amino acid.

Substrate specificity of the enzyme against Pro-X (X, amino acid) and X-Pro

To elucidate the substrate specificity in high concentrations of dipeptides as substrates, the enzyme reaction was done with 0.15 M Pro-X and 0.15 M X-Pro in 0.02 M phosphate buffer (pH 7.0). After incubation for 1 h at 40°C, the amounts of hydrolytic products (amino acids) were measured with an amino acid analyzer. The reaction mixtures were analyzed by HPLC for the production of unknown compounds, aside from hydrolytic products (amino acids) and dipeptides as substrates. The enzyme hydrolyzed Pro-X, but not X-Pro, suggesting that the enzyme attacks peptides containing proline at the N-terminus (Table IV).

On the other hand, in the analysis of reaction products

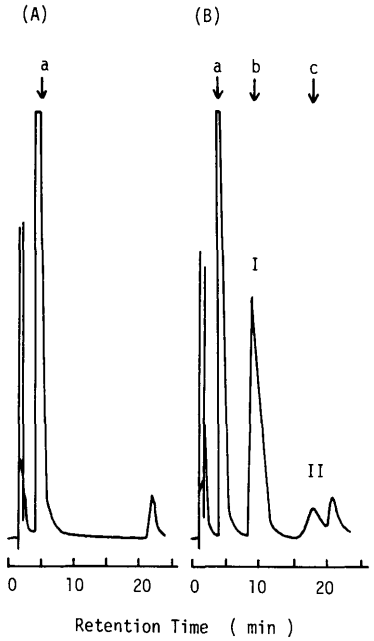

Fig. 5. Action of the Protease from *S. limosus* on 0.15 M Pro-Pro.
Reaction times were 0 h (A) and 1 h (B).
a, Pro-Pro; b, Pro-Pro-Pro; c, Pro-Pro-Pro-Pro; I and II, unknown products.

by HPLC, formation of unknown products was observed, using Pro-X as substrates. But there were no products in the case of X-Pro as substrates. Using Pro-Pro as a substrate, this enzyme formed two unknown products.

Analyses of products

Figure 5 shows the analysis of reaction products by HPLC. After 1 h of incubation, we observed the appearance of two new peaks. Retention times of product I and II corresponded to those of authentic peptides (Pro-Pro-Pro and Pro-Pro-Pro-Pro), respectively.

Their amino acid compositions were analyzed with the amino acid analyzer. In the cases of both products, only proline was found. Therefore, it can be considered that product I and II are oligomers of proline.

In the FD-MS analysis of product I, a molecular ion ($[M+H]^+$) peak was observed at *m/z* 310. A molecular weight of 309 was estimated, which corresponded to Pro-Pro-Pro. On the other hand, the analysis of product II is in progress, because it is now very difficult to obtain it.

From the above analyses, it became apparent that the protease from *S. limosus* catalyzed the synthetic reaction of Pro-Pro-Pro from Pro-Pro. The synthesized amounts of Pro-Pro-Pro was 12 mM under the conditions of 0.15 M Pro-Pro at pH 7.0 and 40°C for 1 h.

There are several proteases that catalyze transpeptidation reactions,[14-16] for example, α-chymotrypsin, trypsin, papain, and cathepsin C. However, there are no proteases that can catalyze the condensation or transfer reactions of proline residues, except for a proline-specific dipeptidylcarboxypeptidase.[3]

Compared with proline iminopeptidases so far reported, the enzyme from *S. limosus* has different points as follows. (1) This enzyme was inhibited by pepstatin and leupeptin. However, the enzymes from *Bacillus coagulans*[17] and *Bacillus megaterium*[18] were not affected by them. (2) The molecular mass of this enzyme, 50 kDa, is different from

those of the enzymes from *B. coagulans* (35 kDa)[17] and *B. megaterium* (60 kDa).[18] (3) Although the enzyme from *Escherichia coli*[19] was activated by Mn ion, this enzyme was not activated by any of the metal ions including Mn ion. These findings show that the protease from *S. limosus* is a novel enzyme having a strong affinity for proline in the synthetic reaction and forming Pro-Pro-Pro from Pro-Pro. This enzyme may be useful to introduce proline into other peptides or proteins.

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