

Purification and Characterization of an Extracellular Protease from Culture Filtrate of *Salmonella schottmülleri*

Byoung Kuk Na and Chul Yong Song*

Department of Biology, College of Natural Sciences, Chung-Ang University,
Seoul 156-756, Korea

(Received July 3, 1995/Accepted September 4, 1995)

An extracellular protease of *Salmonella schottmülleri* was purified from culture filtrate by using 0~75% ammonium sulfate precipitation, DEAE Sepharose Fast Flow ion exchange chromatography, Ultrogel HA chromatography and Sephacryl S-200 HR molecular sieve chromatography. To measure enzyme activity, synthetic dipeptide substrate (CBZ-arg-arg-AFC) with low molecular weight was employed as substrate. The molecular weight of the purified enzyme was approximately 80 kDa when determined by gel filtration on Sephacryl S-200 HR and 73 kDa when estimated by SDS-PAGE. The isoelectric point was 5.45. The activity of the purified enzyme was inhibited by metal chelating agents such as EDTA and 1,10-phenanthroline. The divalent cations, such as Ca^{2+} , Zn^{2+} , Fe^{2+} , Mg^{2+} and Mn^{2+} enhanced its activity. These results suggested that it was a metalloprotease. It had a narrow pH optimum of 6.5~7.5 with a maximum at pH 7.0 and a temperature optimum of 40°C. It was stable at least for 1 week at 40°C and maintained its activity for 24 hours at 50°C, but it was rapidly inactivated at 65°C. This protease was shown to be sensitive to sodium dodecyl sulfate (SDS) and was inactivated in a dose-dependent manner. However, it was resistant to Triton X-100 and the activity was enhanced to 32.3% with treatment of 0.025% Triton X-100.

Key words: purification, characterization, extracellular protease, *S. schottmülleri*

Salmonella schottmülleri (formerly *Salmonella paratyphi* B) is a Gram-negative, facultative intracellular bacterium which is primarily infective in humans. The ability to adhere to and penetrate epithelial barriers of the host cells is essential for infection in humans (9). *Salmonella* spp. enter the host by invading the gastrointestinal mucosa (8, 9, 11). Once the *Salmonella* invade the intestinal epithelium, they enter deeper tissues, often reticuloendothelial cells (30). The contact between *Salmonella* spp. and the eukaryotic cell induces expression of new proteins, some of which appear to be required for invasion of host cells (10). The virulence factors of *Salmonella* which cause pathogenesis include cytotoxic proteins as well as Vi antigens and O antigens (3, 5, 8, 9, 11). Particularly, the cytotoxic factors such as enterotoxin, exotoxin and permeability factors are involved in pathogenesis of *Salmonella* (22, 25, 26). Bacterial protease attribute to bacterial pathogenesis by various molecular mechanisms; enhancements of vascular permeability and edema formation, degradation of defense oriented proteins inclu-

ding immunoglobulins as well as structural matrices such as fibronectin and collagen, inactivation of complement system and generated chemotactic factors, degradation of regulatory plasma protease inhibitors and destruction of intracellular integrity result in cell killing (17). The invasiveness of *S. schottmülleri* may be associated with some extracellular protease which degrade the mucous membrane of host defense barrier. However, the properties of the extracellular protease of *S. schottmülleri* has not been well understood. In this study, an extracellular protease of *S. schottmülleri* was purified and characterized.

Materials and Methods

Bacteria and culture condition

The strain of *Salmonella schottmülleri* used in this study was kindly provided by Dept. of Microbiology, College of Medicine, Chung-Ang Univ. in 1993 and maintained in our laboratory thereafter. For production of proteases, the organism was cultured in trypticase soy broth (Difco, USA) supplemented with 0.1% CaCl_2 at 37°C with

*To whom correspondence should be addressed.

vigorous shaking.

Assay of enzyme activity

Protease activity was estimated by synthetic dipeptide substrate with a fluorescent leaving group, carboxybenzoyl-arginine-arginine-7-amino-4-trifluoromethylcoumarin (CBZ-arg-arg-AFC; Enzyme System Products, USA). The assay mixture contained 50 mM sodium phosphate buffer (pH 7.0) and 10 μ g of CBZ-arg-arg-AFC dissolved in 10 μ l of DMSO in a total volume of 0.5 ml. After incubation at 37°C for 2 h with enzyme solution, the 7-amino-4-trifluoromethylcoumarin (AFC) liberated from the fluorogenic substrate was quantified with excitation at 400 nm and emission at 505 nm using a fluorometer (Model III, Sequoia-Turner Co., USA). One unit of the enzyme activity was defined as nmols of AFC produced per min under reaction condition.

Determination of protein concentration

Protein concentration was determined by the method of Lowry *et al.* (16) with BSA (Sigma, USA) as standard.

Purification of extracellular protease

Step 1. Culture and ammonium sulfate precipitation

A 5 ml sample of a late log phase culture of the organism was introduced into and cultured in 500 ml of trypticase soy broth (Difco, USA) in a 1 liter flask, with vigorous shaking at 37°C for 32 h. A 2 liter sample of the culture was centrifuged at 6,000 \times g for 15 min followed by filtration through a membrane filter (0.22 μ m), both at 4°C. Ammonium sulfate was added slowly to the supernatant, with gentle stirring, to a final concentration of 75% saturation. After standing overnight at 4°C, the precipitates were collected by centrifugation at 8,000 \times g for 15 min, dissolved in about 20 ml of 50 mM Tris-HCl buffer (pH 8.0), and dialyzed for 24 h against the same buffer.

Step 2. DEAE Sepharose Fast Flow ion exchange chromatography

The dialysate (50 ml) was applied to a column (2.6 by 18 cm) of DEAE Sepharose Fast Flow equilibrated with 50 mM Tris-HCl buffer (pH 8.0) and eluted with the same buffer at a flow rate of 45 ml/h. Adsorbed proteins were eluted with a 0.15, 0.2, 0.25, 0.5 M NaCl stepwise gradient. Eluate (6 ml/fractions) was collected for measurement of absorbance at 280 nm and enzyme activity. The active fractions were pooled, dialyzed against distilled water at 4°C and lyophilized.

Step 3. Ultrogel HA chromatography

The lyophilized preparation was resolved in 10 mM sodium phosphate buffer (pH 6.8) and applied to a column (1.6 by 11 cm) of Ultrogel HA equilibrated with

10 mM sodium phosphate buffer (pH 6.8). Adsorbed proteins were eluted with a linear gradient of 10~500 mM sodium phosphate buffer (pH 6.8). The active fractions were pooled, dialyzed against distilled water at 4°C and lyophilized.

Step 4. Sephacryl S-200 HR molecular sieve chromatography

For further purification, partially purified enzyme solution was dialyzed against 20 mM Tris-HCl buffer (pH 7.5) containing 0.15 M NaCl and applied to a column (1.6 by 50 cm) of Sephacryl S-200 HR equilibrated with 20 mM Tris-HCl buffer (pH 7.5) containing 0.15 M NaCl. Fractions (3 ml) of the eluate were collected similarly and dialyzed against distilled water followed by lyophilization. The lyophilized powder was stored at -80°C until used.

SDS-PAGE

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed in 10% (wt/v) slab gel as described by Laemmli (14). Protein samples were denatured by boiling for 2 min in 1% SDS and 0.1% β -mercaptoethanol. A current of 30 mA/gel was applied for 3.5 h. The gels were stained for 2 h with 0.1% Coomassie Brilliant Blue in methanol/acetic acid/H₂O (1:2:1, v/v) and destained in methanol/acetic acid/H₂O (5:1:4, v/v) at room temperature.

Substrate gel electrophoresis

The gelatin SDS-PAGE in slab gels containing SDS and gelatin as copolymerized substrate were used for the detection of the protease activity (13). Protein samples were mixed with equal volume of solution containing 2.5% SDS and 1% sucrose, and incubated at 37°C for 30 min. Electrophoresis was performed at 4°C at a constant current of 30 mA. After electrophoresis, the gels were washed in 2.5% Triton X-100 for 1 h at room temperature to remove the SDS and to restore enzyme activity. The gels were then transferred to 50 mM sodium phosphate buffer (pH 7.0) and incubated at 37°C for overnight. The gels were then fixed and stained by immersion for 1 h in 0.1% (wt/v) amido black and destained in methanol/acetic acid/H₂O (5:1:4, v/v). The bands of proteolytic activity were revealed as areas depleted of gelatin.

Determination of molecular weight

The molecular weight of the purified enzyme was determined by SDS-PAGE. The reference proteins were phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100) and α -lactalbumin (14,400) (Sigma,

USA). The native molecular weight of the purified enzyme was determined by molecular sieve chromatography. The purified enzyme was applied to a column (1.6 by 50 cm) of Sephacryl S-200 HR precalibrated with calibration standards. Standard proteins were aldolase (158,000), bovine serum albumin (67,000), ovalbumin (43,000), chymotrypsinogen A (25,000) and ribonuclease A (13,700) (Pharmacia, Sweden).

Determination of isoelectric point

Isoelectric focusing was carried out on a Pharmacia Phast gel (Pharmacia, Sweden) containing ampholine with a pH range of 3~10 and calibration kit proteins were used. After electrofocusing, the gel was stained and destained as described previously.

Effect of pH on enzyme activity

The pH effect was assayed at different pH values (pH range 5.5~10.0). 0.1 M sodium acetate, sodium phosphate, Tris-HCl, glycine-NaOH buffers were used for pH ranges of 5.5 to 6.0, 6.5 to 7.0, 7.5 to 8.5, 9.0 to 10.0, respectively.

Effect of temperature on enzyme activity

The enzyme reaction was carried out at different temperatures from 10°C to 60°C. After 2 h incubation, enzyme activities were measured.

Heat stability of enzyme

The purified enzyme was incubated for different time intervals in 50 mM sodium phosphate buffer (pH 7.0) at 10°C, 40°C, 50°C and 65°C. And then remaining activities were measured.

Effect of protease inhibitors and metal ions on enzyme activity

The effect of protease inhibitors on enzyme activity was examined. Purified enzyme was preincubated at 37°C for 30 min in 50 mM sodium phosphate buffer (pH 7.0) containing inhibitors. Substrate was then added. The reaction mixtures were incubated at 37°C for 2 h and enzyme activity was measured. The inhibitors used in this study were diisopropyl fluorophosphate (DFP, 100 μ M; Sigma, USA), phenylmethylsulfonyl fluoride (PMSF, 1 mM; Sigma, USA), L-trans-epoxy-succinyl-leucylamido-(4-guanidino)-butane (E-64, 10 μ M; Sigma, USA), leupeptin (100 μ M; Sigma, USA), pepstatin A (1 μ M; Sigma, USA), N- α -p-tosyl-L-lysine-chloromethyl ketone (TLCK, 100 μ M; Sigma, USA), N-tosyl-L-phenyl-alanine-chloromethyl ketone (TPCK, 100 μ M; Sigma, USA), iodoacetic acid (100 μ M; Sigma, USA), 1,10-phenanthroline (1 mM; Sigma, USA) and ethylenediaminetetraacetic acid (EDTA,

10 mM; Sigma, USA). The effect of metal ions on enzyme activity was examined by the following method. The purified enzyme was incubated in 50 mM sodium phosphate buffer (pH 7.0) containing 2 mM MgCl₂, MnCl₂, CaCl₂, ZnCl₂, HgCl₂, FeSO₄ at 37°C for 2 h. And then each enzyme activity was measured in comparison with the control which had no metal ions.

Reactivation of the EDTA-inactivated protease

Purified enzyme in 0.5 ml of 50 mM sodium phosphate buffer (pH 7.0) was inactivated by incubating with 5 mM EDTA at 37°C for 20 min. To this inactivated enzyme solution, 0.1 M MgCl₂, MnCl₂, CaCl₂, ZnCl₂, HgCl₂, FeSO₄ metal ion solutions were added to a final concentration of 2 and 5 mM. Each of these mixtures was further incubated at 37°C for 2 h and restored enzyme activity was measured in comparison to the EDTA-inactivated enzyme activity.

Effect of detergents on enzyme activity

To investigate the effect of detergents on enzyme activity, SDS and Triton X-100 were used. The purified enzyme was preincubated with detergents in 50 mM sodium phosphate buffer (pH 7.0) at 37°C for 2 h. And then substrate was added and each enzyme activity was measured in comparison with the control which had no detergents. The final concentrations of detergents were 0.0025, 0.005, 0.0125, 0.025, 0.05, 0.125, 0.25, 0.5, 1.25 and 2.5%.

Amino acid analysis

Amino acid analysis was performed by the method of Spackman *et al.* (27). Sample of purified enzyme (0.1 mg) was hydrolyzed at 110°C for 24 h with 1 ml of 6 N HCl *in vacuo*. The hydrolysate was dried *in vacuo* and amino acid analysis was performed with Beckman Model 6300 amino acid analyzer.

Results and Discussion

Protease production and cell growth

The time course of culture and the protease activity were shown in Fig. 1. Culture filtrate was harvested 48 h after incubation. The production of the proteases was highly increased when the growth reached late log phase, and total protease activity reached its maximum during the middle stationary phase. And then, protease activity decreased gradually. As cells become starved their overall metabolic rate decreases, but some level of endogenous metabolism is maintained. One function of endogenous metabolism is to maintain the ability to transport substrates into the cell. During starvation, the

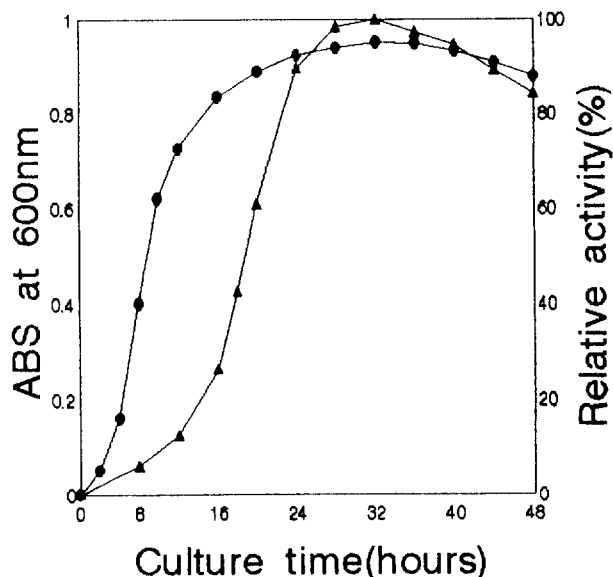


Fig. 1. Change of cell density and protease activity during cultivation of *S. schottmülleri* in TSB. *S. schottmülleri* were cultivated at 37°C with vigorous shaking. The aliquots taken out at the indicated time were used to measure the cell density (●) and protease activity (▲).

bulk of the protein synthesis is turning off (7, 12, 23). However, the synthesis of 30 to 50 proteins is induced in response to starvation (12, 15, 24, 28). These proteins involved in maintaining viability during prolonged starvation are necessary for cells to recover from starvation and to resume growth when nutrients become available. Therefore, this data proposes that when cells encounter nutritional starvation, they produce extracellular proteases which could be important in hydrolyzing environmental macromolecular protein sources to small, available substrates to overcome the nutritional starvation. But, when the available nutrient is not sufficient in the environment, growth ceases and cells enter the stationary phase.

Purification of extracellular protease

Purification of an extracellular protease from culture filtrate of *S. schottmülleri* was performed with ammonium sulfate precipitation followed by a series of chromatographic steps. DEAE Sepharose Fast Flow chromatography of the ammonium sulfate precipitates yielded one peak of proteolytic activity (Fig. 2A). The active fractions were pooled, concentrated and applied to Ultrogel HA chromatography. Three peaks with proteolytic activity was obtained (Fig. 2B). The third peak which showed the highest proteolytic activity was pooled, concentrated and applied to Sephacryl S-200 HR chromatography. Four protein peaks were obtained and proteolytic activity was detected in the second peak (Fig. 2C). This active frac-

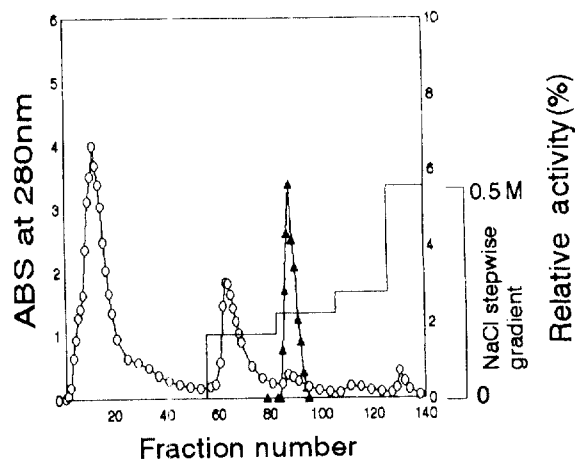


Fig. 2A. Elution profile of protease on the DEAE Sepharose Fast Flow ion exchange chromatography. NaCl stepwise gradient (—), protease activity (▲) and protein content at 280 nm (○).

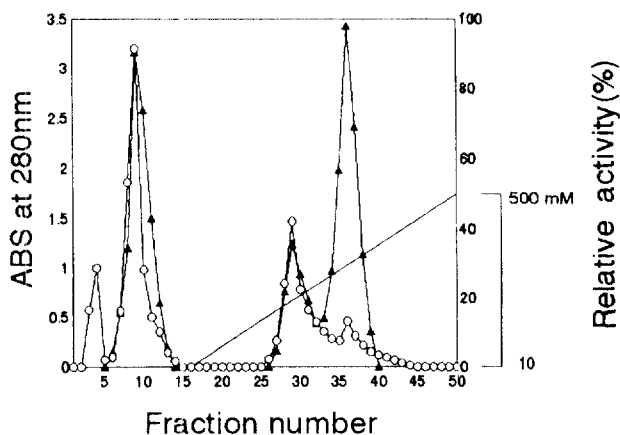


Fig. 2B. Elution profile of protease on the Ultrogel HA chromatography. Linear gradient elution using 10~500 mM sodium phosphate buffer (pH 6.8) (---), protease activity (▲) and protein content at 280 nm (○).

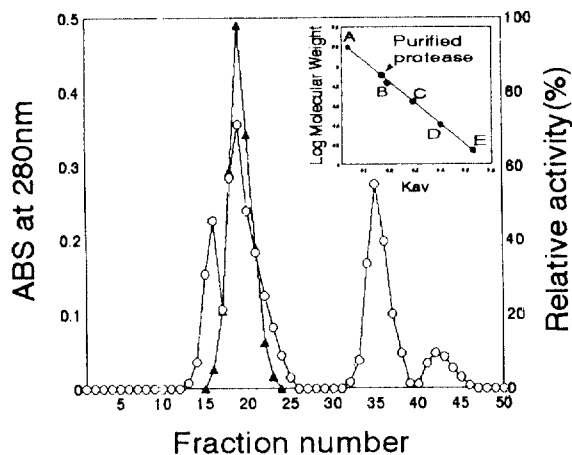


Fig. 2C. Elution profile of protease on the Sephacryl S-200 HR molecular sieve chromatography. Protease activity (▲) and protein content at 280 nm (○).

Table 1. Purification of protease from culture filtrate of *S. schottmülleri*.

Purification step	Total volume (ml)	Total protein (mg)	Total activity ^a (unit)	Specific activity ^a (U/mg)	Purification fold	Recovery (%)
Culture filtrate	1,500	3,110	3,865	1.3	1.0	100
Ammonium sulfate ^b	50	1,720	3,196	1.9	1.5	82.7
DEAE ^c	3.5	10.8	2,463	229.1	183.3	63.7
HA ^d	2.0	2.93	835	285.1	228.1	21.6
Sephacryl S-200 ^e	1.5	0.47	220	468.3	374.6	5.7

^aTotal and specific activity: nmoles of AFC production per min under reaction condition. ^b0~75% ammonium sulfate precipitation. ^cDEAE Sepharose Fast Flow ion exchange chromatography. ^dUltrigel HA chromatography. ^eSephacryl S-200 HR molecular sieve chromatography.

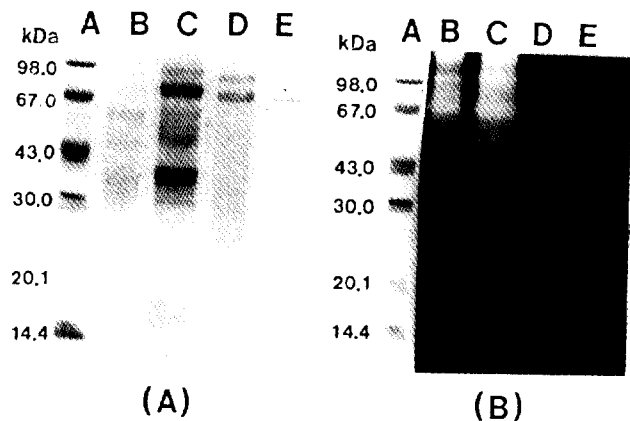


Fig. 3. PAGE analysis of purified protease from culture filtrate of *S. schottmülleri*. (A) 10% Polyacrylamide SDS-PAGE. (B) Substrate gel electrophoresis. Lane A, Standard marker protein; lane B, 0~75% ammonium sulfate precipitation; lane C, active peak from DEAE Sepharose Fast Flow ion exchange chromatography; lane D, active peak from Ultrigel HA chromatography; lane E, purified protease from Sephacryl S-200 HR molecular sieve chromatography (7.4 μ g). Molecular weight standard markers included the following proteins: phosphorylase B (94,000); bovine serum albumin (67,000); ovalbumin (43,000); carbonic anhydrase (30,000); trypsin inhibitor (20,100); α -lactalbumin (14,400).

tions were pooled, concentrated and used in a further study. A typical purification is shown in Table 1. The molecular weight of the enzyme was determined to be approximately 73 kDa on 10% SDS-polyacrylamide gel (Fig. 3A). The molecular weight of the enzyme was approximately 80 kDa when determined by gel filtration (Fig. 2C) and the isoelectric point was 5.45 (data not shown). This indicated that the purified enzyme was a slightly acidic protein with monomeric structure. This protease was examined further by substrate gel electrophoresis on 10% SDS-polyacrylamide gel containing 0.1% gelatin (Fig. 3B). The crude sample showed four major clearing smear bands. The result suggested that these proteases were either active in the presence of SDS during electrophoresis (4), or were binding to the substrate gelatin (1). However, the purified 73 kDa protease didn't show clearing smear bands (Fig. 3B, lanes D,

E). It indicated that this enzyme was sensitive to SDS; plus, its activity was restored after washing with Triton X-100. The effect of detergents at various concentrations on purified enzyme activity was determined. SDS, an ionic detergent, completely inactivated enzyme activity at a concentration of 0.5%. However, Triton X-100, a nonionic detergent, did not influence enzyme activity; moreover, the activity was enhanced to 32.3% with treatment of 0.025% Triton X-100 (data not shown). The fact that the activity of SDS-inactivated enzyme can be restored by washing with Triton X-100 in substrate gel indicates that SDS may inactivate the enzyme by modifying the active site or the conformation of the enzyme reversibly. This enzyme was more resistant to SDS than alkaline or neutral proteases of *B. subtilis* 6061, which were completely inactivated in the presence of 0.2% and 0.1% SDS, respectively (31). However, this enzyme is more sensitive to SDS than extracellular alkaline protease from *Bacillus subtilis* RM 615. The enzyme was reduced to 82% of maximal activity upon treatment with 1% SDS (20).

Effect of pH

The effect of varying the pH on protease activity was examined by using various buffers (Fig. 4). The enzyme was active over a narrow pH range of 6.5 to 7.5 with a pH optimum at 7.0. The activity decreased gradually below pH 6.0 and above pH 9.0. Thus, the protease has a neutral optimal pH. It corresponds to most bacterial proteases which are most active at neutral pH or at some alkaline pH (2, 6, 21, 32).

Effect of temperature and heat stability

The enzyme exhibited a broad temperature optimum with a maximum activity at 40°C and rapid inactivation above 45°C (Fig. 5). The enzyme was stable between 10°C and 40°C at least for 1 week. However, it was rather unstable at 50°C and was inactivated rapidly at 65°C (Fig. 6). This result was similar to peptidase N of *S. typhimurium* which was inactivated rapidly at 70°C (19).

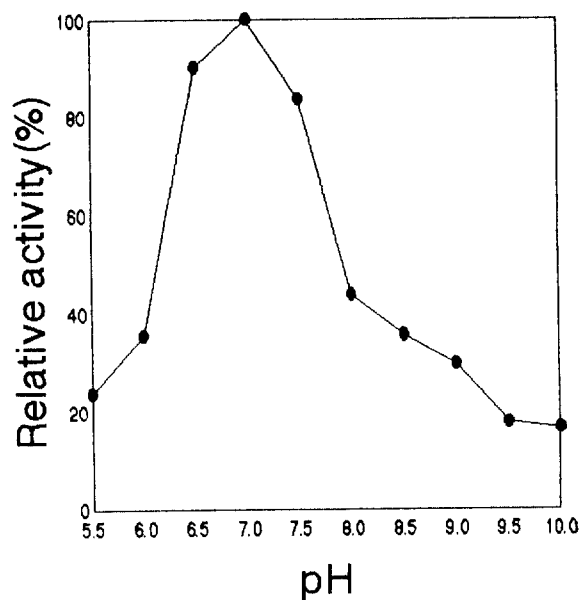


Fig. 4. Effect of pH on the activity of purified protease from culture filtrate of *S. schottmülleri*. The protease activity was assayed in 0.1 M sodium acetate buffer (pH 5.0~6.0), 0.1 M sodium phosphate buffer (pH 6.5~7.5), 0.1 M Tris-HCl buffer (pH 7.5~8.5), 0.1 M glycine-NaOH buffer (pH 9.0~10.0). Maximal activity was shown as 100%.

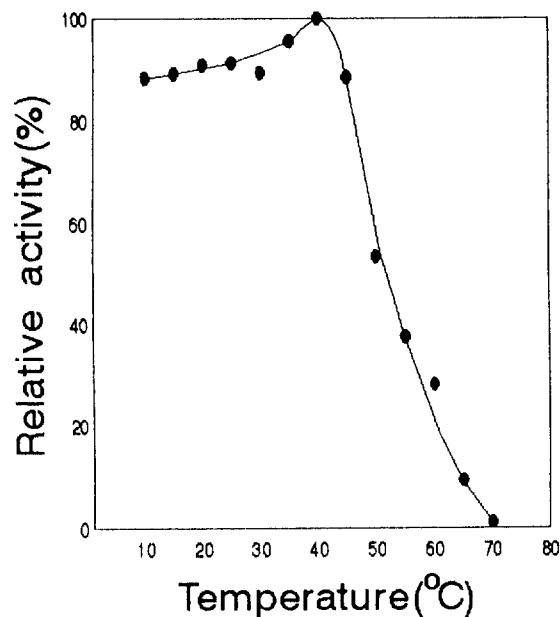


Fig. 5. Effect of temperature on the activity of purified protease from culture filtrate of *S. schottmülleri*. The purified protease was incubated at various temperature for 2 h and then the protease activity was assayed. Maximal activity was shown as 100%.

This result indicates that the enzyme can maintain its activity at a temperature similar to human body temperature for a long time. Therefore, if this enzyme is invol-

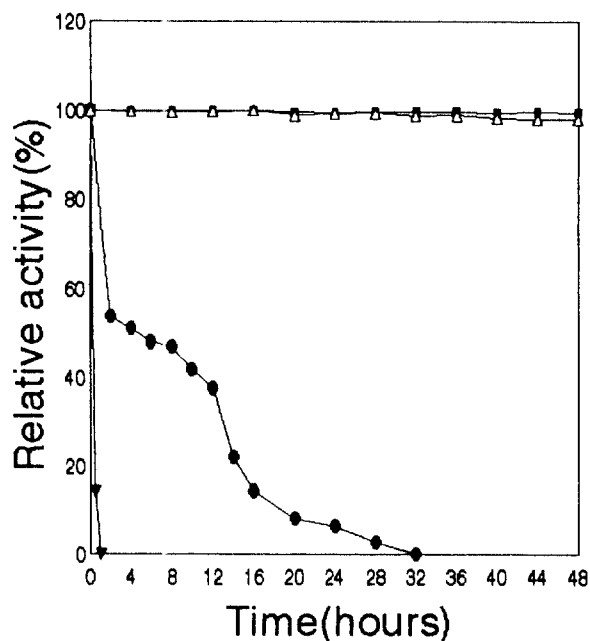


Fig. 6. Thermal stability of purified protease from culture filtrate of *S. schottmülleri*. The purified protease was incubated for various time intervals in 50 mM sodium phosphate buffer (pH 7.0) at 10°C (■), 40°C (△), 50°C (●), 65°C (▼) and then remaining activity was measured.

Table 2. Effect of inhibitors on the purified protease from culture filtrate of *S. schottmülleri*.

Inhibitors	Concentration (mM)	Relative activity ^b (%)
Control ^a		100.0
Serine class;		
DFP	0.1	87.0
PMSF	1	83.5
PMSF	1	94.7
Serine & Cysteine class;		
Leupeptin	0.1	97.5
TPCK	0.1	81.3
TLCK	0.1	91.8
Cysteine class;		
E-64	0.01	98.3
Iodoacetic acid	0.1	99.5
Aspartic class;		
Pepstatin A	0.001	88.5
Metallo class;		
EDTA	5	43.6
EDTA	10	5.2
1,10-Phenanthroline	1	22.6

^a Control represents the activity tested without any inhibitors.

^b % of control.

DFP: Di-isopropylfluorophosphate.

PMSF: Phenylmethylsulfonyl fluoride.

TPCK: N-tosyl-L-phenylalanine-chloromethyl ketone.

TLCK: N- α -p-tosyl-L-lysine-chloromethyl ketone.

E-64: L-trans-epoxysuccinyl-leucylamido-(4-guanidino)-butane.

EDTA: Ethylenediaminetetraacetic acid.

Table 3. The effect of divalent metal ions on the purified protease.

Metal ion ^a	Relative Activity (%) ^c
Control ^b	100.0 ± 2.5
MgCl ₂	119.8 ± 3.2
MnCl ₂	108.5 ± 3.1
CaCl ₂	125.3 ± 2.8
ZnCl ₂	123.7 ± 3.2
HgCl ₂	95.4 ± 3.3
FeSO ₄	105.7 ± 2.7

^aThe final concentrations of metal ions were 2 mM.

^bControl represents the activity tested without any metal ions.

^c% of control.

Table 4. Reactivation of the EDTA-inactivated protease by various divalent metal ions.

Metal ion	Concentration (mM)	Restored Activity (%) ^a
Control 1		100.0
Control 2		43.6
MgCl ₂	2	51.2
	5	58.7
MnCl ₂	2	61.7
	5	69.4
CaCl ₂	2	64.9
	5	87.3
ZnCl ₂	2	74.0
	5	93.4
HgCl ₂	2	45.1
	5	43.8
FeSO ₄	2	59.4
	5	65.5

Control 1: purified protease alone.

Control 2: purified protease+EDTA (5 mM).

^aTo the EDTA-inactivated enzyme solution, metal ions at 2 and 5 mM were added and incubated 50 mM sodium phosphate buffer (pH 7.0) at 37°C for 20 min. Substrate was then added.

ved in the pathogenesis of *S. schottmülleri*, it will exhibit its pathogenicity for a long time.

Effect of protease inhibitors and metal ions

The effect of a wide spectrum of inhibitors on the activity of enzyme was determined by measuring residual activity following preincubation of the enzyme with inhibitors in 50 mM sodium phosphate buffer (pH 7.0) at 37°C for 30 min (Table 2). The enzyme was inhibited by both EDTA and 1,10-phenanthroline. However, no significant inhibitions were observed with DFP and PMSF (inhibitors of serine proteases), E-64 and iodoacetic acid (inhibitors of cysteine proteases) and pepstatin A (inhibitor of aspartic proteases). Among metal ions, only Hg²⁺ exhibited some inhibition (Table 3). However, there was no indication of the sulfhydryl group involvement because no effect was observed by iodoacetic acid (Table 2). The addition of Mg²⁺, Mn²⁺, Ca²⁺, Zn²⁺ and

Table 5. Amino acid composition of the purified protease^a.

Amino acid	No. of amino acid residues	
	Obtained value	Nearest integer ^b
Cys	2.87	3
Asx	30.49	30
Thr	30.14	30
Ser	49.09	49
Glx	82.76	83
Pro	24.60	25
Gly	79.58	80
Ala	26.55	27
Val	42.85	43
Met	5.34	5
Ile	12.36	12
Leu	36.97	37
Tyr	3.23	3
Phe	19.60	20
Lys	40.91	41
His	82.99	83
Arg	6.07	6
Trp	1.98	2
Total		579

^aAmino acid composition was determined after acid hydrolysis for 24 h.

^bBased on the molecular weight of 73,000, the nearest integer was calculated which yielded 72,967.

Fe²⁺ enhanced the protease activity from 5.7% to 25.3% more than the control. Addition of metal ions to the EDTA-inactivated protease restored the activity except Hg²⁺ (Table 4). Particularly, Zn²⁺ and Ca²⁺ enhanced the enzyme activity in a high degree. Sensitivity to the metal chelating agent, EDTA, is typical of metalloproteases, many of which are stabilized or activated by Ca²⁺ and Mg²⁺ (18, 29). Moreover, the purified enzyme was inactivated significantly by 1,10-phenanthroline, a Zn²⁺-specific chelating agent. These results suggested that this enzyme can be classified as a Zn²⁺-dependent metalloprotease and Ca²⁺ is required for enzyme stability.

Amino acid analysis

The result of amino acid analysis of the enzyme is shown in Table 5. The protease contains 2 mol of tryptophan, 5 mol of methionine, 3 mol of cysteine per mol of protease.

References

1. Abbas, C.A., S. Groves, and I.E. Gonder, 1989. Isolation, purification, and properties of *Penicillium charlesii* alkaline protease. *J. Bacteriol.* **171**, 5630-5637.
2. Alichanidis, E. and A.T. Andrews, 1977. Some properties of the extracellular protease produced by the psychrotrophic bacterium *Pseudomonas fluorescens* strain AR-11. *Bio-*

- chim. Biophys. Acta* **485**, 424-433.
3. **Baltzer, I.M., K. Lindgren, B. Lindgren, and L. Edebo**, 1991. Endotoxin shedding by enterobacteria: Free and cell-bound endotoxin differ in *Limulus* activity. *Infect. Immun.* **59**, 689-695.
 4. **Blumentals, L.I., A.S. Robinson, and R.M. Kelly**, 1990. Characterization of sodium dodecyl sulfate-resistant proteolytic activity in the hyperthermophilic archaeobacterium *Pyrococcus furiosus*. *Appl. Environ. Microbiol.* **56**, 1992-1998.
 5. **Brubaker, R.R.**, 1985. Mechanisms of bacterial virulence. *Ann. Rev. Microbiol.* **39**, 21-50.
 6. **Chung, J.H. and A.L. Goldberg**, 1983. Purification and characterization of protease So, a cytoplasmic serine protease in *Escherichia coli*. *J. Bacteriol.* **154**, 231-238.
 7. **Davis, B.D., S.M. Luger, and P.C. Tai**, 1986. Role of ribosome degradation in the death of starved *Escherichia coli* cells. *J. Bacteriol.* **166**, 439-445.
 8. **Finlay, B.B. and S. Falkow**, 1988. Virulence factors associated with *Salmonella* species. *Microbiol. Sci.* **5**, 324-328.
 9. **Finlay, B.B. and S. Falkow**, 1989. Common themes in microbial pathogenicity. *Microbiol. Rev.* **53**, 210-230.
 10. **Finlay, B.B., F. Heffron, and S. Falkow**, 1989. Epithelial cell surfaces induce *Salmonella* proteins required for bacterial adherence and invasion. *Science* **243**, 940-943.
 11. **Giannella, R.A., O. Washington, P. Gemski, and S.B. Formal**, 1973. Invasion of HeLa cells by *Salmonella typhimurium*: A model for study of invasiveness of *Salmonella*. *J. Infect. Dis.* **128**, 69-75.
 12. **Groat, R.G., J.E. Schultz, E. Zychlinsky, A. Bockman, and A. Matin**, 1986. Starvation proteins in *Escherichia coli*: Kinetics of synthesis and role in starvation survival. *J. Bacteriol.* **168**, 486-493.
 13. **Heussen, C. and E.B. Dowdle**, 1980. Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulfate and copolymerized substances. *Anal. Biochem.* **102**, 196-202.
 14. **Laemmli, D.K.**, 1970. Cleavage of structural protein during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.
 15. **Lange, R. and R. Hengge-Aronis**, 1991. Identification of a central regulator of stationary phase gene expression in *E. coli*. *Mol. Microbiol.* **5**, 49-59.
 16. **Lowry, O.H., N.J. Rosebrugh, A.L. Farr, and R.J. Randall**, 1951. Protein measurement with the foline phenol reagent. *J. Biol. Chem.* **193**, 265-275.
 17. **Maeda, H. and A. Molla**, 1989. Pathogenic potentials of bacterial protease. *Clin. Chem. Acta.* **185**, 357-368.
 18. **Matsumoto, K., H. Maeda, K. Takata, R. Kamata, and R. Okamura**, 1984. Purification and characterization of four proteases from a clinical isolate of *Serratia marcescens* kums 3958. *J. Bacteriol.* **157**, 225-232.
 19. **Miller, C.G. and K. Mackinnon**, 1974. Peptidase mutants of *Salmonella typhimurium*. *J. Bacteriol.* **120**, 355-363.
 20. **Moon, S.Y., T.K. Oh, and H.M. Rho**, 1994. Purification and characterization of an extracellular alkaline protease from *Bacillus subtilis* RM 615. *Korean. Biochem. J.* **27**, 323-329.
 21. **Morihara, K.**, 1964. Production of elastase and proteinase by *Pseudomonas aeruginosa*. *J. Bacteriol.* **88**, 745-757.
 22. **Na, B.K. and C.Y. Song**, 1994. Purification and partial characterization of cytotoxic protein from culture filtrates of *Salmonella schottmüllerii*. *Kor. J. Microbiol.* **32**, 417-423.
 23. **Nystrom, T., P. Marden, and S. Kjelleberg**, 1986. Relative changes in incorporation rates of leucine and methionine during starvation survival of two bacteria isolated from marine waters. *FEMS Microbiol. Ecol.* **38**, 285-292.
 24. **Nystrom, T., P. Marden, and S. Kjelleberg**, 1990. Responses to multiple nutrient starvation in marine *Vibrio* sp. strain CCUG 15956. *J. Bacteriol.* **172**, 7085-7097.
 25. **Peterson, J.W. and P.D. Sandefur**, 1979. Evidence of a role for permeability factors in the pathogenesis of Salmonellosis. *Amer. J. Clin. Nutr.* **32**, 197-209.
 26. **Sandefur, P.D. and J.W. Peterson**, 1976. Isolation of skin permeability factors from culture filtrates of *Salmonella typhimurium*. *Infect. Immun.* **14**, 671-679.
 27. **Spackman, D.W., W. Stein, and S. Moore**, 1958. Automatic recording apparatus for use in the chromatography of amino acids. *Anal. Chem.* **30**, 1190-1206.
 28. **Spector, M.P., Z. Aliabadi, T. Gonzalez, and J.W. Foster**, 1986. Global control in *Salmonella typhimurium*: Two-dimensional electrophoretic analysis of starvation-, anaerobiosis- and heat shock-induced proteins. *J. Bacteriol.* **168**, 420-424.
 29. **Stepaniak, L., P.F. Fox, and C. Daly**, 1982. Isolation and general characterization of a heat-stable proteinase from *Pseudomonas fluorescens* AFT 36. *Biochim. Biophys. Acta* **717**, 376-383.
 30. **Takeuchi, A.**, 1967. Electron microscopic studies of experimental *Salmonella* infection: 1. Penetration into the intestinal epithelium by *Salmonella typhimurium*. *Am. J. Pathol.* **50**, 109-136.
 31. **Uehara, H., Y. Yoneda, K. Yamane, and B. Mamo**, 1974. Isolation and characterization of alkaline and neutral protease of *Bacillus subtilis* 6061. *J. Bacteriol.* **199**, 82-91.
 32. **Yamamoto, N., A. Akino, and T. Takano**, 1993. Purification and specificity of a cell-associated proteinase from *Lactobacillus helveticus* CP 790. *J. Biochem.* **114**, 740-745.