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Purification and Properties of Serine Protease from *Halobacterium halobium*

L. S. IZOTOVA, A. Y. STRONGIN,* L. N. CHEKULAEVA, V. E. STERKIN, V. I. OSTOSLAVSKAYA, L. A. LYUBLINSKAYA, E. A. TIMOKHINA, AND V. M. STEPANOV

Institute of Genetics and Selection of Industrial Microorganisms, Moscow 113545, USSR

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Pure extracellular serine protease was isolated from the culture filtrate of *Halobacterium halobium* by bacitracin-Sepharose affinity chromatography. The enzyme activity was completely and irreversibly lost if the NaCl concentration fell below 2 M. The protease consists of one polypeptide chain with a molecular weight of 41,000. It is characteristically enriched in Asx and Glx content, whereas the level of basic amino acids in the enzyme molecule is unusually low. The protease shows a preference for leucine in the carboxylic side of the scissile bond of the substrate, cleaving the B-chain of oxidized bovine insulin only at the Leu_{15}–Tyr_{16} bond and liberating *p*-nitroaniline from L-pyroglutamyl-L-alanyl-L-alanyl-L-leucine-*p*-nitroanilide.

Halophilic bacteria of the genus *Halobacterium* belong to a separate bacterial kingdom, recently named archaebacteria (3). In their biochemistry and in the structure of certain macromolecules they differ from eucaryotes, as well as from other procaryotic bacteria (eubacteria) (3, 6). The enzymes from extremely halophilic bacteria represent a fascinating example of adaptation. These enzymes perform their functions in vivo and in vitro at 4 to 5 M NaCl, losing activity rapidly when exposed to low salt concentrations (6). The lability of halophilic proteins at low salt concentrations severely restricts the choice of purification methods, making the majority of the conventional procedures unsuitable. Until now only a few intracellular halophilic proteins have been isolated in a pure state and characterized (7, 9, 16, 17). Data on pure proteolytic enzymes from *Halobacterium* species are absent, although the production of proteases was reported to be common for these microorganisms (11).

We report here the purification of the extracellular serine protease from *Halobacterium halobium* and provide information on its molecular weight, amino acid composition, and substrate specificity.

MATERIALS AND METHODS

Enzyme assay. *Halobacterium* protease activity was determined by measuring *p*-nitroaniline liberation from the water-soluble substrate L-pyroglutamyl-L-alanyl-L-alanyl-L-leucine-*p*-nitroanilide (Pyr-Ala-Ala-Leu-*p*NA), synthesized in this laboratory. The standard assay mixture contained 1.25 ml of 50 mM Tris-hydrochloride–1 mM $CaCl_2$–3 M NaCl buffer (pH 7.2) (buffer A), 12.5 μl of the substrate from the stock solution of 10 mg/ml in dimethylformamide, and 5 to 50 μl of the enzyme solution. After 5 to 60 min of incubation at 37°C, the reaction was stopped with 0.25 ml of 2 M sodium citrate buffer (pH 5.0), and the released *p*-nitroaniline was assayed spectrophotometrically at 410 nm (14).

Proteolytic activity of the enzyme was measured in buffer A, using Azocasein (Serva) as the substrate (2).

Cleavage of bovine insulin B chain. Oxidized bovine insulin B-chain (Sigma Chemical Co.) was treated with purified *Halobacterium* protease (enzyme/substrate ratio, 1:1,000 [wt/wt]) in buffer A for 4 h at 37°C. The reaction was stopped by the addition of 1 N HCl to a final pH of 2. Peptides in the digest were analyzed by dansylation and manual Edman degradation, and the N-terminal residues of the peptides were identified as dansyl derivatives by thin-layer chromatography on polyamide sheets (8).

Electrophoresis. Polyacrylamide gel electrophoresis under nondenaturing conditions was performed in Tris-glycine buffer (pH 8.3) on 7.5 or 10% gels (1).

The molecular weight of the enzyme was determined by polyacrylamide gel electrophoresis under denaturing conditions in the presence of detergents: anionic, sodium dodecyl sulfate (SDS); or cationic, cetyltrimethylammonium bromide (Serva). SDS electrophoresis was performed according to standard procedures (15). Electrophoresis in the presence of cationic detergent was done as follows. The duration of electrophoresis on 12.5% gels in 0.1 M sodium phosphate buffer (pH 7.1) was 5 h at 8 mA per gel (6 by 100 mm). The gel and the buffer contained 0.1% cetyltrimethylammonium bromide. The polymerization of the gel was initiated with 6 μg of riboflavin per ml. Protease samples, inhibited with 5 mM diisopropyl fluorophosphate (see below) were denatured at 100°C for 2 to 3 min in 0.01 M sodium phosphate buffer (pH 7.1) containing 1% detergent and 1% 2-mercaptoethanol. In this system, the proteins migrated from anode to cathode. After a 30-min fixation of the gels in 30%

trichloroacetic acid, the protein bands appeared as opaque white zones. When necessary, the gels were additionally stained with Coomassie brilliant blue R-250 (Serva) in a 45% ethanol–10% acetic acid solution overnight and then destained with 7.5% acetic acid. The following proteins were used as markers for molecular weight estimation: cytochrome *c*, myoglobin, chymotrypsinogen, pepsin, ovalbumin, and bovine serum albumin (Serva).

Gel filtration. The molecular weight of the native enzyme was determined by gel filtration on Sephadex G-75 (1.5 by 100 cm) in buffer A. The fractions containing *Halobacterium* protease were identified by their enzyme activity.

Amino acid analysis. The protein samples were hydrolyzed in vacuo by being heated with 0.5 ml of 5.7 N HCl at 110°C for 24 h. The evaporated hydrolysates were chromatographed on a Durrum D500 amino acid analyzer. Trp was identified after hydrolysis of the protein with 3 M mercaptoethanesulfonic acid (Pierce) for 22 h at 115°C (12). Cysteine and methionine were identified as cysteic acid and methionine-sulfone after oxidation of the protein with performic acid (4).

Enzyme purification. *H. halobium* P-353 (a mutant containing no gas vacuoles) was grown without illumination in 0.5-liter flasks containing 100 ml of medium with the following content (per liter): 250 g of NaCl, 20 g of $MgCl_2$, 2 g of KCl, 3 g of sodium citrate, and 10 g of peptone (Organo-Therapeutic Plant, Tbilisi, Georgia, USSR). After 14 days of shaking at 37°C, the cells were removed by centrifugation, and 2 liters of the culture fluid was applied to a bacitracin-Sepharose 4B column (2.4 μmol of ligand per ml of wet gel; column size, 4 by 10 cm) (13). The column was then washed with buffer A, and the active protease was eluted with buffer A containing 25% isopropanol (Fig. 1). The low-molecular-weight impurities and the isopropanol were removed by additional gel filtration of the enzyme through a Sephadex G-25 column (2.6 by 30 cm) equilibrated in buffer A. The active fractions were pooled and finally purified by rechromatography through a bacitracin-Sepharose 4B column (1.8 by 8 cm) as above. The aliquots of the purified enzyme were inactivated with 5 mM diisopropyl fluorophosphate (1 h, 20°C) and exhaustively dialyzed against water containing 1 mM diisopropyl fluorophosphate to remove salts and isopropanol. The dialyzed samples were analyzed by electrophoresis and amino acid analysis.

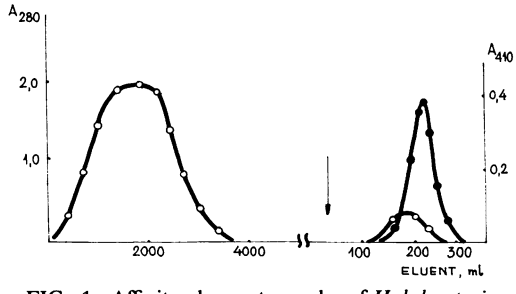

FIG. 1. Affinity chromatography of *Halobacterium* protease on bacitracin-Sepharose 4B. Contaminating proteins were eluted with buffer A; active protease was eluted with buffer A containing 25% isopropanol. The addition of isopropanol is shown by the arrow. Symbols: ○, absorbancy at 280 nm (A_{280}); ●, protease activity against Pyr-Ala-Ala-Leu-*p*NA.

RESULTS AND DISCUSSION

Active extracellular protease was isolated from the culture filtrate of *H. halobium* by affinity chromatography on Sepharose 4B containing the covalently bound antibiotic bacitracin and by Sephadex G-25 gel filtration. Bacitracin-Sepharose was previously widely applied in our laboratory for the isolation of various serine proteases (13). The purification of *Halobacterium* protease was performed in the presence of 3 M NaCl, required to obtain active enzyme possessing high specific activity against Azocasein and Pyr-Ala-Ala-Leu-*p*NA (Table 1). The binding of *Halobacterium* protease to the affinity sorbent was due to the hydrophobic affinity interaction between the enzyme and the ligand, imitating protein substrates.

The enzyme was treated with 5 mM diisopropyl fluorophosphate in buffer A and then dialyzed against water containing 1 mM diisopropyl fluorophosphate. Homogeneity of the protease was confirmed by polyacrylamide gel electrophoresis at pH 8.3 (1), revealing a single protein band with an R_f of 0.3 against the marker dye bromophenol blue. It should be stressed that the native enzyme was completely autolyzed when dialyzed against water or buffers with a low salt concentration. The autolysis could not be prevented by phenylmethylsulfonyl fluoride, presumably owing to the partial restoration of the enzyme activity of phenylmethylsulfonyl fluoride-inhibited protease. It appears that even a minute amount of reactivated enzyme extensively cleaves the denatured protein at low ionic strength. Thus, only the treatment with the tightly bound, irreversible inhibitor diisopropyl fluorophosphate was suitable for the further analysis of the enzyme.

The active, purified *Halobacterium* protease was relatively unstable when stored even in 3 M NaCl, losing the activity completely in 10 days at 4°C.

The molecular weight of the active enzyme as estimated by Sephadex G-75 gel filtration in the presence of 3 M NaCl was 41,000 ± 2,000. On the contrary, the molecular weight of the enzyme when denatured in hot SDS-mercaptoethanol, derived from its mobility in SDS-polyacrylamide gel, was somewhat higher—56,000 ± 2,000. The difference between these two estimates was thought to be connected with the resistance of *Halobacterium* protease (an acidic protein) toward SDS denaturation. The enzyme presumably bound less anionic detergent as compared with the marker proteins isolated

TABLE 1. Purification of *Halobacterium* protease

Step	Protein (mg)	Total activity (U)	Sp act[a]	Purification (fold)	Yield (%)
Culture filtrate	21,000	500	0.023	1	100
Bacitracin-Sepharose chromatography	169	247	1.46	64	49
Sephadex G-25 gel filtration	74	168	2.3	100	34
Bacitracin-Sepharose rechromatography	33	174	5.2	230	35

[a] Micromoles of *p*-nitroaniline released per milligram of protein per minute. Pyr-Ala-Ala-Leu-*p*NA was used as the substrate.

from nonhalophilic organisms. This led to the reduced mobility of the SDS-protease complex and to an overestimation of the protein molecular weight. This explanation was confirmed by the data gained by electrophoresis in the presence of the cationic detergent cetyltrimethylammonium bromide. The value of the *Halobacterium* protease molecular weight determined by polyacrylamide gel electrophoresis in the presence of cetyltrimethylammonium bromide was 41,000, a value close to that found from gel filtration data. We conclude that the protease consists of one polypeptide chain with a molecular weight of about 41,000.

The most prominent feature of *Halobacterium* protease amino acid composition was the remarkably high content of dicarboxylic amino acids or their amides, or both (Table 2). The sum of these residues corresponds to 25% of the total number of residues in the enzyme molecule. On the other hand, the level of basic amino acid residues was reduced in the halophilic protease. This is apparent when *Halobacterium* protease is compared with nonhalophilic enzymes, e.g., *Bacillus amyloliquefaciens* proteases with similar substrate specificity (extracellular subtilisin BPN′ and intracellular serine protease) (Table 2). The high content of dicarboxylic amino acids appears to be a specific feature of many proteins from extremely halophilic microorganisms (6).

TABLE 2. Amino acid composition of *Halobacterium* protease and intracellular serine protease and subtilisin BPN′ from *B. amyloliquefaciens* (14)

Amino acid	Residues per molecule for:		
	Halobacterium protease	Intracellular serine protease	Subtilisin BPN′
Lys	5	20	11
His	3	6	6
Arg	11	6	2
Asx	69	36	28
Thr	32	13	13
Ser	43	25	37
Glx	34	33	15
Pro	18	13	14
Gly	56	36	33
Ala	47	32	37
Val	24	19	30
Met	2	5	5
Ile	16	13	13
Leu	28	25	15
Tyr	14	7	10
Phe	7	7	3
Trp	3	6	3
½ Cys	4	2	0

Dialysis of the protease solutions in buffer A against different buffers revealed that the enzyme activity was completely lost if the NaCl concentration fell below 2 M (Table 3). An increase in NaCl level from 2 to 4.3 M enhanced *Halobacterium* protease activity against Pyr-Ala-Ala-Leu-*p*NA severalfold. It was of interest that similar, although not so pronounced, effects were observed for the nonhalophilic proteases. The use of KCl instead of NaCl led to the same effects. Activation of the enzymes by high levels of salts was not revealed for Azocasein hydrolysis. *Halobacterium* protease activity remained practically the same with the increase in NaCl concentration, whereas the activity of the *Bacillus* proteases fell markedly.

The pH optimum of the enzyme activity against Pyr-Ala-Ala-Leu-*p*NA was between pH 8 and 9, similar to that of the *Bacillus* serine proteases (14). In the presence of 3 M NaCl, *Halobacterium* protease showed maximal activity for Pyr-Ala-Ala-Leu-*p*NA hydrolysis at 37°C and pH 7.2.

Phenylmethylsulfonyl fluoride and diisopropyl fluorophosphate, inhibitors of serine proteases, at a concentration of 1 mM inactivated the enzyme completely. Hence, extracellular protease from *H. halobium* may be classified as a serine protease.

The specific inhibitor of subtilisin (a structural homolog of the well-known *Streptomyces* subtilisin inhibitor [5]) inactivated *Halobacterium* protease completely, whereas soy bean trypsin inhibitor was without any effect. *p*-Chloromercuribenzoate (1 mM) inhibited 50% of the en-

TABLE 3. Effect of NaCl on activities of *Halobacterium* protease and intracellular serine protease and subtilisin BPN' from *B. amyloliquefaciens* against Azocasein and Pyr-Ala-Ala-Leu-*p*NA

Enzyme	Sp act[a] with the following substrate and NaCl concn (M):									
	Pyr-Ala-Ala-Leu-*p*NA					Azocasein				
	0	1	2	3	4	0	1	2	3	4
Halobacterium protease	0	0	3	5	15	0	0	0.39	0.43	0.46
Intracellular serine protease	16	22	27	32		0.4	0.44	0.38	0.32	
Subtilisin BPN'	10	20	24	30		1.6	1.45	1.1	1.0	

[a] The specific activity with Pyr-Ala-Leu-*p*NA is expressed in arbitrary units; the specific activity with Azocasein is micromoles of *p*-nitroaniline released per milligram of protein per minute.

zyme activity, indicating the presence of SH groups in the enzyme. EDTA and ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*-tetraacetic acid, chelating agents for Ca^{2+} ions, at a concentration of 5 mM led to 70% inhibition of the enzyme. It appears that Ca^{2+} ions are required for maintaining the structure of halophilic protease. The addition of 5% dimethylformamide to the assay mixture inhibited at least 30% of the enzyme activity, whereas in the presence of 16.6% dimethylformamide, the enzyme activity against Pyr-Ala-Ala-Leu-*p*NA was completely lost (Table 4). The synthetic substrates (e.g., Z-Ala-Ala-Leu-*p*NA), which require dimethylformamide for solubilization, were therefore unsuitable for the assay of *Halobacterium* protease activity.

The enzyme did not cleave the leucine aminopeptidase and trypsin substrates L-Leu-*p*NA and Bz-L-Arg-*p*NA, respectively. *Halobacterium* protease possessed a rather high esterolytic activity, comparable with that of *Bacilli* proteases and hydrolyzed ethyl and methyl esters of Bz-L-tyrosine. The esters of L-arginine were not cleaved by the enzyme.

Purified *Halobacterium* protease in buffer A cleaved only one peptide bond, Leu_{15}–Tyr_{16}, in the oxidized bovine insulin B-chain, thus showing a strong preference for leucine in the carboxylic side of the scissile bond of the substrate, comparable with that of *Bacillus* intracellular serine protease (14). On the contrary, subtilisin BPN' cleaves five bonds in the B-chain (10).

In summary, we isolated pure extracellular serine protease from extremely halophilic *H. halobium*, which belongs to the archaebacteria.

TABLE 4. Effect of dimethylformamide on specific activity of *Halobacterium* protease against Pyr-Ala-Ala-Leu-*p*NA

Dimethylformamide concn (%)	Sp act (U/mg)
1	5
5	3.5
10	2.5
16.6	0

The substrate specificity and certain physicochemical parameters of the *Halobacterium* protease revealed common features with serine proteases of sporulating nonhalophilic *Bacillus* spp., typical members of the eubacteria. To our knowledge, the *Halobacterium* protease is the first secretory enzyme isolated in a pure state from extremely halophilic microorganisms. Additional study of structural and functional features of *Halobacterium* protease might reveal a possible evolutionary relationship among functionally related proteolytic enzymes from eubacteria and archaebacteria.

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