Research Articles

Intracellular proteases from the extremely thermophilic archaebacterium *Sulfolobus solfataricus*

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Received 18 January 1991; accepted 19 March 1991

Abstract. Proteolytic activities from the extremely thermoacidophilic archaebacterium *Sulfolobus solfataricus* were detected with the aid of synthetic substrates in a cell extract fractionated by gel filtration. Two aminopeptidases (aminopeptidase I and II), three endopeptidases (proteinase I, II and III) and one carboxypeptidase could be identified. Experiments carried out with protease inhibitors led to the identification of the exopeptidases as metalloproteases. Proteinases I and II behaved as chymotrypsin-like serine proteases, and proteinase III as a cysteine protease with a trypsin-like specificity. Molecular weight values assessed with the aid of marker proteins were as follows: aminopeptidase I, > 450 kDa; aminopeptidase II, 170 kDa; carboxypeptidase, 160 kDa; proteinase I, 115 kDa; proteinase II, 32 kDa; proteinase III, 27 kDa. On incubation for 15 min they retained most of their activity up to a temperature of 90 °C, with the sole exception of proteinase II, which was rapidly inactivated at 60 °C. Protease content was also determined in crude extracts from cells grown in a mineral medium both to the stationary and to the exponential phase, with glucose or with yeast extract as carbon sources. No dramatic change was detected depending on the growth phase; however, carboxypeptidase level was three- to four-fold higher when yeast extract was present in the medium instead of glucose; this might suggest an involvement of this enzyme in the digestion of extracellularly available peptides.
Key words. Proteases; thermostability; archaebacterium; *S. solfataricus.*

In recent years much research effort has been devoted to the isolation and characterization of enzymes from thermophilic microorganisms; the growing interest in such enzymes is justified both by their biotechnological potential [1] and by the opportunity they offer for a better understanding of the mechanisms by which biomolecules are stabilized at high temperatures. Among archaebacteria, *Sulfolobus solfataricus*, a thermoacidophilic microorganism isolated from hot springs, which grows optimally at 87 °C, has been subjected to extensive biochemical investigation: indeed, several enzymes have recently been identified and characterized from this source [2–5], including an aminopeptidase [6]. However, in spite of the remarkable biotechnological potential of thermophilic proteases [7], information about the proteolytic machinery of this archaebacterium is still scanty. Here we report the identification and some properties of several proteases from this source. Besides having possible biotechnological applications, this work might also aid the understanding of the mechanisms of intracellular proteolysis in *Sulfolobus solfataricus.*

Materials and methods

Cells of *Sulfolobus solfataricus* strain MT-4 (ATCC 49155) obtained under different growth conditions, were kindly supplied by the Servizio di Fermentazione del'Instituto per la Chimica di Molecole di Interesse Biologico del CNR, Arco Felice, Italy. Cells were grown aerobically in a mineral medium to the exponential or to the stationary phase, as reported [8], using yeast extract (2 g/l) or glucose (3 g/l) as the carbon source. Unless otherwise stated, experiments were carried out using extracts from cells grown to the stationary phase in the yeast extract-containing medium. Cells were stored frozen and thawed immediately before crude extract preparation. Cell samples (0.7 g wet wt) were thawed in 2.1 ml 0.1 M NaCl, 50 mM Tris-HCl, pH 7.3, and disrupted by vigorous shaking with 2 g of glass beads (0.25–0.30 mm diameter) in a Vortex mixer for 10 min at 4 °C. The homogenates were centrifuged for 30 min at 50000 × g. The supernatants were incubated for 15 min at room temperature in the presence of 0.15 mg of deoxyribonuclease and 0.40 mg of ribonuclease. The resulting samples were fractionated by gel filtration or ion-exchange chromatography (see below), or directly assayed to assess changes in enzyme levels under different growth conditions (see Results).

For gel filtration, 0.8 ml of crude extract (15 mg protein) was applied to a Sephacryl S-300 Superfine column (1.5 × 44 cm) preequilibrated with 0.1 M NaCl, 20 mM Tris-HCl, pH 7.3. Elution was carried out with the same buffer at a flow rate of 5.5 ml/h. Eluted fractions (1.13 ml) were tested for enzyme activity. Molecular weight marker proteins were run under the same conditions. For a preliminary resolution of proteinase II from proteinase I, a crude extract (15 mg protein) was loaded onto an anion Mono Q HR 5/5 exchange column of an FPLC apparatus (Pharmacia, Uppsala, Sweden), preequilibrated with 20 mM Tris-HCl, pH 8.1. After washing the column, elution was performed with a linear gradient, from 0 to 0.5 M KCl, in the same buffer at a flow rate of 0.5 ml/min. Proteinase II was eluted in the range

of 0.35–0.40 M KCl and was completely devoid of proteinase I activity. The active fractions were pooled, concentrated to 0.8 ml in Centricon-10 microconcentrators (Amicon, Danvers, MA) and subjected to gel filtration as described above.

Aminopeptidases and endopeptidases were assayed at 70 °C (except for proteinase II, tested at 60 °C) in 0.1 M potassium pipes (pipes: 1,4-piperazinediethanesulfonic acid), pH 7.0, using aminoacyl- or peptidyl-p-nitroanilides as substrates. The assay volume was 0.7 ml. The absorbance of the p-nitroaniline released was measured at 410 nm at suitable incubation times against a blank sample with no extract added. A molar absorption coefficient of 9500 $M^{-1} cm^{-1}$ was determined for p-nitroaniline under the assay conditions. Carboxypeptidase assays were carried out at 70 °C in 0.1 M potassium Mes (Mes: 4-morpholineethanesulfonic acid), pH 6.5, in a total volume of 0.1 ml, using Cbz-Asp (Cbz: benzyloxycarbonyl) as a substrate. The amount of amino acid released was detected by a Cd-ninhydrin colorimetric method at 505 nm[9] (method D) against a blank sample with no extract added. One unit of enzyme activity is defined as the amount of enzyme which hydrolyzes 1 µmol of substrate/min in the standard assay conditions, i.e. those reported in figure 1.

Protein was assayed by the Bio-Rad protein assay kit from Bio-Rad (Richmond, VA, USA), following the standard procedure, and using bovine plasma immunoglobulin G as standard protein.

Results

A cell extract from *Sulfolobus solfataricus* was fractionated by gel filtration on Sephacryl S-300 Superfine. In the eluted fractions proteolytic activities were tested using a number of synthetic substrates (fig. 1); based on the type of substrate hydrolyzed six different peaks of activity could be identified, i.e. two aminopeptidases, one carboxypeptidase and three endopeptidases. The aminopeptidases were referred to as aminopeptidase I and II, and the endopeptidases as proteinase I, II and III, in order of elution. Since proteinase I slightly hydrolyzed the substrate preferentially attacked by proteinase II, this latter enzyme was subjected to ion-exchange chromatography prior to gel filtration to remove proteinase I (see Materials and methods); thus, the peak of proteinase II shown in figure 1 was not obtained from the same elution as all the other peaks presented in the same figure.

Based on the elution volumes of the individual proteases and those of marker proteins, the following molecular weights could be assessed: aminopeptidase I, > 450 kDa; aminopeptidase II, 170 kDa; carboxypeptidase, 160 kDa; proteinase I, 115 kDa; proteinase II, 32 kDa; proteinase III, 27 kDa.

The activity peaks corresponding to the different proteases resolved by gel filtration (fig. 1) were characterized as regards their sensitivity to different inhibitors (table 1). These assays allowed the classification of the

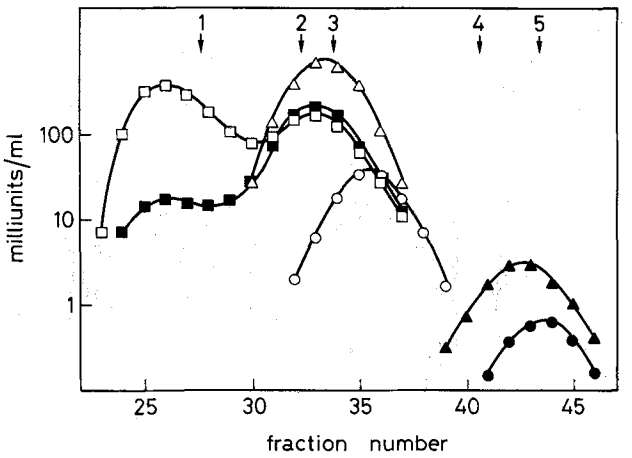

Figure 1. Proteolytic activities in a cell extract supernatant from cells of *Sulfolobus solfataricus* fractionated by Sephacryl S-300 Superfine gel filtration. Aminopeptidase and endopeptidase activities were tested using the following substrates at the indicated concentrations: (□) 1 mM Leu-pNA (aminopeptidases I and II); (■) 1 mM Arg-pNA (aminopeptidases I and II); (△) 1 mM Cbz-Asp (carboxypeptidase); (○) 1 mM Suc-Phe-pNA (proteinase I); (▲) 0.4 mM Suc-Ala-Ala-Pro-Phe-pNA (proteinase II); (●) 1 mM Bz-Arg-pNA (proteinase III). The tests were performed as described in Materials and Methods. The arrows indicate the following marker proteins; 1, apoferritin (M_r 450 kDa); 2, β-amylase (M_r 200 kDa); 3, alcohol dehydrogenase (M_r 150 kDa); 4, ovalbumin (M_r 45 kDa); 5, carbonic anhydrase (M_r 29 kDa).

exopeptidases as metallo-proteases, since their activity was completely suppressed by o-phenanthroline and by EDTA; furthermore, the two aminopeptidases were also inhibited by bestatin, a typical inhibitor of aminopeptidases[10]. Proteinase I and proteinase II behaved as chymotrypsin-like serine proteases in that they were sensitive to PMSF (PMSF: phenylmethanesulfonyl fluoride), TPCK (TPCK: tosyl-phenylalanine chlormethyl ketone) and chymostatin, a microbial chymotrypsin inhibitor[10,11]; this is consistent with their specificity for Suc-Phe-pNA (Suc: succinyl; pNA: p-nitroanilide) and for Suc-Ala-Ala-Pro-Phe-pNA, respectively. In contrast, proteinase III was inhibited by pCMBS (pCMBS: p-(chloromercuri)benzenesulfonate), iodoacetamide and TLCK (TLCK: tosyl-lysine chloromethyl ketone) but not by PMSF and TPCK, which led to its identification as a cysteine protease with a trypsin-like substrate specificity (table 1). This kind of specificity is that expected on the basis of the substrate preferentially hydrolyzed, i.e. Bz-Arg-pNA (Bz: benzoyl).

Protease thermostability was investigated by enzyme incubation for 15 min at pH 7.0 at different temperatures and subsequent determination of the residual activity under the standard assay conditions (fig. 2): except for proteinase II the proteases did not undergo any significant inactivation up to 90 °C. This demonstrates the remarkable thermostability of these enzymes. Furthermore, the much lower stability of proteinase II might be a consequence of autolysis as substantiated by the following findings: 1) the enzyme showed a second order decay kinetics; 2) 4 M urea was inhibitory towards the

Table 1. Effect of different inhibitors on the activity of the proteases from *Sulfolobus solfataricus*. Enzymes were preincubated for 15 min at room temperature in the presence of the indicated inhibitors at the respective concentrations, in 0.1 M potassium pipes, pH 7.0. Then, samples were withdrawn and the residual activities determined in the respective standard assay mixtures. Leu-pNA was used as a substrate for both aminopeptidases. Values are expressed as percentage relative to a control sample (no effector added).

Effector added		Amino-peptidase I	Amino-peptidase II	Carboxy-peptidase	Proteinase I	Proteinase II	Proteinase III
PMSF	(1 mM)	102	95	123	7	0	127
pCMBS	(10 mM)	94	73	13	87	73	6
Iodoacetamide	(5 mM)	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	0
Pepstatin	(8 µM)	103	92	95	95	108	102
EDTA	(1 mM)	15	3	97 (4)[b]	100	91	100
o-Phenanthroline	(10 mM)	2	1	2	97	94	115
TPCK	(0.5 mM)	90	103	103	0	18	97
TLCK	(0.25 mM)	105	100	116	98	94	4
Chymostatin	(0.25 mg/ml)	n.d.[a]	n.d.[a]	n.d.[a]	0	0	100
Leupeptin	(10 µM)	105	94	117	92	100	103
Phosphoramidon	(10 mM)	103	102	121	94	93	105
Bestatin	(30 µM)	3	15	96	n.d.[a]	n.d.[a]	n.d.[a]

[a] Not determined. [b] 5 mM.

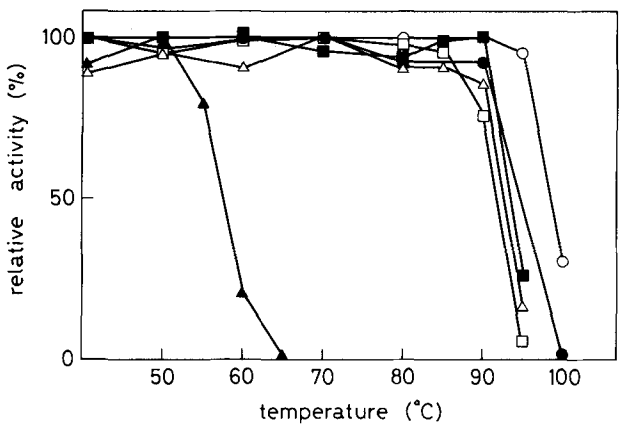

Figure 2. Thermal stability of the proteases from *Sulfolobus solfataricus*. Proteolytic activities resolved by gel filtration were preincubated for 15 min at the indicated temperatures in 0.1 M potassium Pipes, pH 7.0. Then, samples were withdrawn, chilled and the residual activity determined according the respective standard assays. (□) aminopeptidase I; (■) aminopeptidase II; (△) carboxypeptidase; (○) proteinase I; (▲) proteinase II; (●) proteinase III. Activities are expressed as percentage relative to zero-time preincubation.

proteinase, and also distinctly retarded its inactivation; 3) in the presence of 4 M urea the enzyme displayed first-order inactivation kinetics (data not shown).

To check whether the proteases were subject to variations in their intracellular levels under different growth conditions, their activity was tested in crude extracts from cells grown with either glucose or yeast extract as a carbon source, both in the exponential and in the stationary phase. The enzyme levels did not display dramatic changes depending on the growth phase. In contrast, the carbon source exerted a significant effect on proteinase II and carboxypeptidase, the activity of proteinase II being two-fold higher in cells grown in the presence of glucose, and that of carboxypeptidase being three- to four-fold higher in the presence of yeast extract (table 2). Carboxypeptidase might be involved in the digestion of extracellularly available peptides after their internalization. We cannot, however, find any obvious explanation to account for the reduced levels of proteinase II in the presence of yeast extract as a carbon source: for this, a better understanding of its physiological role is necessary.

Discussion

The experiments reported in this paper led to the identification in *Sulfolobus solfataricus* of at least five different intracellular proteases representing almost all of the known classes, namely serine, cysteine and metalloproteases. No aspartic protease could be found, and as yet no extracellular proteolytic activity could be demonstrated. As regards their substrate specificity, these enzymes could be classified as amino-, carboxy- and endopeptidases; to our knowledge, the carboxypeptidase reported here is the only one so far identified in thermophilic microorganisms. The exopeptidases could be

Table 2. Proteolytic activities in crude extracts from cells of *Sulfolobus solfataricus* obtained under different growth conditions. Cells were grown in a mineral medium[8] to the exponential or to the stationary phase with glucose or with yeast extract as a carbon source. Cell samples were collected, disrupted and the activity of each protease was determined in crude extracts using the respective standard assays. Leu-pNA was used to measure aminopeptidase activity. Values of proteinase II activity are corrected for the slight Suc-Ala-Ala-Pro-Phe-pNA-hydrolyzing activity of proteinase I.

Carbon source	Growth phase	Milliunits/mg protein				
		Aminopeptidase	Carboxy-peptidase	Proteinase I	Proteinase II	Proteinase III
Glucose	Exponential	103	22	10.4	11.0	0.27
Glucose	Stationary	122	29	10.2	9.0	0.22
Yeast extract	Exponential	116	95	5.7	4.9	0.26
Yeast extract	Stationary	139	79	9.4	5.4	0.23

classified as metallo-proteases because of their sensitivity to o-phenanthroline and EDTA. Furthermore, dialysis of the carboxypeptidase at pH 5.5 first in the presence and then in the absence of EDTA completely abolished enzyme activity, which could be restored by micromolar amounts of Zn^{2+} and Co^{2+} but not of other divalent cations (data not shown).

As mentioned above, cell extract fractionation could resolve two aminopeptidase activities, I and II, the former being mainly active against Leu-pNA, the latter against both Leu-pNA and Arg-pNA. We tentatively propose, however, that these two activities represent different forms of the same enzyme. This is substantiated by the fact that they displayed practically identical inhibition patterns (table 1), stability curves (fig. 2), pH optima and activation energies (data not shown): furthermore, Hanner et al. [6] could isolate only one form of aminopeptidase from *Sulfolobus solfataricus*, and its properties compare well with those of both forms described in this paper.

Determinations of protease levels in crude extracts evidenced substantially higher specific activities for the exopeptidases as compared to those of the endopeptidases: this suggests that the strategy of intracellular protein degradation of this microorganism might involve aspecific roles for the former enzymes and more specific ones for the latter. This is also supported by the fact that the exopeptidases proved to be active against several synthetic substrates, whereas the endopeptidases could only attack a limited number of them (data not shown).

In conclusion, the data presented here show that *Sulfolobus solfataricus* represents a rich source of proteolytic enzymes, most of which are endowed with a remarkable thermostability. Purification of some of them is currently being carried on in our laboratory, and their possible biotechnological applications are under investigation.

Acknowledgments. We thank V. Calandrelli, E. Esposito and I. Romano (Servizio di Fermentazione dell'Istituto per la Chimica di Molecole di Interesse Biologico del CNR, Arco Felice, Italy) for supplying biomasses of *Sulfolobus solfataricus*. We are grateful to Dr A. Gambacorta (from the same Institute) for her constant interest and helpful discussion. This work was supported by grants from the Italian Ministero della Pubblica Istruzione, D.P.R. 382/80.

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0014-4754/91/101057-04$1.50 + 0.20/0

Copper metabolism in the LEC rat: Involvement of induction of metallothionein and disposition of zinc and iron

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Received 3 December 1990; accepted 27 March 1991

Abstract. The Cu concentration was about 40 times higher in the liver of LEC (Long-Evans with a cinnamon-like coat color) rats aged 77 days (227.5 ± 21.6 μg/g liver) than in Fischer rats (5.2 ± 0.1 μg/g liver). However, in the kidney and brain of the LEC rats, Cu concentrations were lower than in these organs of the Fischer rats. Cu concentration in the hepatic metallothionein fraction was about 130 times higher in the LEC rats than in the Fischer rats. The LEC rats showed markedly low concentrations of Cu in the serum and bile. It seems likely that excretion of Cu from the liver into the bile and blood (as ceruloplasmin) is inherently lacking in the LEC rat.
Key words. LEC rat; copper; metallothionein; ceruloplasmin; zinc; iron.

The LEC (Long-Evans with a cinnamon-like coat color) rat is an inbred strain which shows fulminant liver damage at three to four months after birth [1]. This hepatitis, characterized by sub-massive necrosis of hepatocytes with few inflammatory cell responses, occurs in all rats of this strain [2]. Furthermore, in rats which survive for a long time, there is a high incidence of hepatocellular carcinomas [3]. Although several hypotheses regarding the fulminant hepatitis and spontaneous carcinomas in the LEC rat have been proposed [4], the pathogenic mecha-