Characterization of Sodium Dodecyl Sulfate-Resistant Proteolytic Activity in the Hyperthermophilic Archaeabacterium

Pyrococcus furiosus

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Cell extracts from Pyrococcus furiosus were found to contain five proteases, two of which (S66 and S102) are resistant to sodium dodecyl sulfate (SDS) denaturation. Cell extracts incubated at 98°C in the presence of 1% SDS for 24 h exhibited substantial cellular proteolysis such that only four proteins could be visualized by amido black-Coomassie brilliant blue staining of SDS-polyacrylamide gels. The SDS-treated extract retained 19% of the initial proteolytic activity as represented by two proteases, S66 (66 kilodaltons [kDa]) and S102 (102 kDa). Immunoblot analysis with guinea pig sera containing antibodies against protease S66 indicated that S66 is related neither to S102 nor to the other proteases. The results of this analysis also suggest that S66 might be the hydrolysis product of a 200-kDa precursor which does not have proteolytic activity. The 24-h SDS-treated extract showed unusually thermostable proteolytic activity; the measured half-life at 98°C was found to be 33 h. Proteases S66 and S102 were also resistant to denaturation by 8 M urea, 80 mM dithiothreitol, and 5% β-mercaptoethanol. Purified protease S66 was inhibited by phenylmethylsulfonyl fluoride and disopropyl fluorophosphate but not by EDTA, ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, or iodoacetic acid. These results indicate that S66 is a serine protease. Amino acid ester hydrolysis studies showed that protease S66 was hydrolytically active towards N-benzoyl-L-arginine ethyl ester.

A number of bacteria capable of growing at or above 100°C, i.e., hyperthermophiles, have been isolated from several geothermic terrestrial and marine environments (12, 17). Among the many interesting features associated with these bacteria are their ability to grow and carry out biological functions at normally denaturing temperatures. An examination of enzymes from these hyperthermophilic bacteria and a comparison of these enzymes with those of their mesophilic counterparts should provide a better understanding of the structural basis for protein folding and stabilization. Work along these lines, however, is in its early stages since most of these bacteria have been isolated only in the last few years.

To understand how some organisms thrive in normally adverse environments, the relationship between their ecological niche and their metabolic characteristics should be considered. One aspect of this relationship is proteolysis, which can play a role in the turnover of proteins intracellularly as well as help in the acquisition of nutritional requirements extracellularly. For extremely thermophilic (optimum growth temperature of at least 80°C) and hyperthermophilic (optimum growth temperature of at least 100°C) bacteria, the role of proteolysis in the metabolisms of the organisms has not been examined to any extent. If hyperthermophiles are similar in some ways to mesophilic bacteria, then proteolysis in hyperthermophiles is likely to be an element of the response of the organism to nutrient availability.

Only a few proteases produced by extremely thermophilic bacteria have been characterized biochemically. Caldolysin, an extracellular protease produced by the extremely thermophilic bacterium Thermus aquaticus and characterized as a metal-chelator-sensitive enzyme, has a reported half-life (t½) of 1 h at 90°C but denatures rapidly at 100°C or in the absence of calcium ions (6, 11). Aqualysin I is an alkaline serine protease produced extracellularly by strain YT-1 of T. aquaticus and has a reported optimum temperature of 80°C (14). This subtilisinlike protease differs from subtilisins in that it contains disulfide bonds in the polypeptide chain (14, 15). The most thermostable protease thus far reported is archaelysin, an extracellular serine protease produced by a Desulfurococcus species (7). The reported t½ of this enzyme at 95°C is 75 to 90 min, and its optimum temperature is 98°C (7).

The purpose of this study was to examine the characteristics of proteolytic enzymes produced by the hyperthermophilic bacterium Pyrococcus furiosus. This anaerobic marine heterotroph was isolated from shallow solfataric muds off the coast of Vulcano Island, Italy (9), and grows optimally at 100°C. Several proteins from P. furiosus have been purified (1, 3, 5). In this work, we report the identification and initial characterization of unusually stable proteolytic activity in cell extracts of P. furiosus.

MATERIALS AND METHODS

Bacterial strain and culture conditions. P. furiosus DSM 3638 was obtained from the Deutsche Sammlung von Mikroorganismen, Braunschweig, Federal Republic of Germany. Bacteria were grown in a complex medium based on artificial seawater supplemented with 0.1% yeast extract, 0.5% tryptone, and 10 g of elemental sulfur per liter. The artificial seawater was a modification of the formulation of Kester et al. (13): equal volumes of solution A (47.8 g of NaCl, 8.0 g of Na2SO4, 1.4 g of KCl, 0.4 g of NaHCO3, 0.2 g of KBr, 0.06 g of H2BO3, each per liter) and solution B (10.8 g of MgCl2·6H2O, 0.025 g of SrCl2·6H2O, each per liter) were mixed, autoclaved, and then supplemented with 0.25 g of NH4Cl and 0.14 g of K2HPO4 per liter. Cells were grown at 98°C in a high-temperature fermentor (Bioengineering AG, Wald, Switzerland) in 4- or 8-liter batch runs, with continuous sparging with prepurified nitrogen gas (Linde

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Gases, Baltimore, Md.) at 200 ml/min to maintain anaerobic conditions and agitation at 100 rpm with a marine impeller. Cells were harvested at the end of the exponential growth phase. Typical cell yields were 0.3 to 0.5 g/liter (wet weight).

Preparation of cell extracts and SDS-resistant proteases. Bacteria were collected by centrifugation and washed with 50 mM sodium phosphate buffer (pH 7.5) at 4°C. Cell pellets (4 g, wet weight) were suspended in 20 ml of 50 mM sodium phosphate buffer (pH 7.5) and sonicated on ice for 3 min at 30-s intervals with a Tekmar sonic disruptor (Tekmar, Cincinnati, Ohio). Cell lysates were centrifuged for 30 min at 25,000 × g at 4°C to remove cell debris. The sodium dodecyl sulfate (SDS)-resistant proteases were obtained by incubation of the cell extract for 24 h in the presence of 1% SDS at 98°C and then dialyzed against 50 mM sodium phosphate buffer (pH 7.5) at room temperature for 24 h. The dialyzed fractions were then concentrated in a Pharmacia Omegacell stirred cell (nominal molecular mass cutoff, 10 kilodaltons [kDa]) (Pharmacia, Inc., Piscataway, N.J.) to a concentration of 2 to 3 mg of protein per ml. This procedure yielded 0.2 to 0.3 mg of protease S66 per g (wet weight) of cells. The S66 protease was purified by electroelution of the corresponding band after separation in SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Electrodution was performed in Tris-glycine buffer (3.03 g of Tris and 14.4 g of glycine per liter) for 12 h at 40 mA. After electrodution, the samples were dialyzed for 12 h against 50 mM sodium phosphate buffer (pH 7.5) at 4°C.

Enzyme assay. Proteolytic activity was measured by the hydrolysis of azocasein at 98°C unless otherwise noted. Assay mixtures contained 900 μl of 0.1% azocasein in 50 mM sodium phosphate buffer (pH 7.3 at 100°C) and 100 μl of enzyme sample (7). The reaction was terminated after 30 min by the addition of 500 μl of 15% trichloroacetic acid and then cooled on ice for 5 min. The precipitate formed was removed by centrifugation (12,000 × g for 3 min) in a microcentrifuge. Supernatant absorbance was measured at 440 nm. Linear response of the assay was obtained between 0.01 and 0.1 absorbance unit. One unit of activity is defined as the amount of protein which produces a change of 0.1 absorbance unit under the assay conditions. Total protein concentration was determined by using the Bio-Rad assay kit (Bio-Rad Laboratories, Richmond, Calif.) which is based on the binding of Coomassie brilliant blue dye to protein (2).

Activity towards synthetic substrates. N-benzoyl-l-arginine ethyl ester, N-benzoyl-l-tyrosine ethyl ester, N-α-benzoyl-DL-arginine-4-nitroanilide, and l-lysine-p-nitroanilide hydrolysers were performed in 50 mM sodium phosphate buffer (pH 7.5) at 90°C. Continuous spectrophotometric readings (253 nm) were taken with a Perkin-Elmer Lambda 3 spectrophotometer (The Perkin-Elmer Corp., Norwalk, Conn.) with a thermostatted six-cell transport system. A circulating water bath (VWR Scientific model 1130) containing a 1:1 mixture of ethylene glycol-water was used to maintain the temperature in the cell holder.

Inhibition studies. Protease samples were preincubated at 37°C for 1 h in the presence of 10 mM concentrations of the following protease inhibitors: diisopropylfluorophosphate, phenylmethylsulfonyl fluoride, iodoacetate, and EDTA, and EGTA [ethylene glycol-bis(β-aminoethil ether)-N,N,N',N'-tetraacetic acid]. Remaining proteolytic activity was determined by azocasein hydrolysis at 98°C for 30 min in the presence of the inhibitors (10 mM). Thermostability studies. Thermostability of proteolytic activity was determined in cell extracts and in the 24-h SDS-treated extracts. For this, samples were incubated at 98°C in the absence of substrate in 50 mM sodium phosphate buffer (pH 7.5). Samples were removed periodically, and remaining proteolytic activity was measured by azocasein hydrolysis.

Effect of pH on proteolytic activity. The effect of pH on proteolytic activity was determined by using the 24-h SDS-treated cell extract. Activity was measured by the hydrolysis of azocasein (1 g/liter) dissolved in 100 mM sodium phosphate buffer (adjusted to the desired pH value) and following the procedure described earlier.

Gelatin-PAGE protease assay. Substrate-PAGE electrophoresis and substrate-overlay gels were used to determine proteolytic activity of individual protein bands. In the first procedure, samples containing 1.0 to 3.0 μg of total protein were boiled for 2 min in the presence of 1% SDS, 80 mM dithiothreitol, 100 mM Tris (pH 6.8), and 15% glycerol, and electrophoresed onto an SDS-polyacrylamide gel containing 0.5% copolymerized gelatin (gelatin-PAGE) (10). After electrophoresis, the gels were washed for 1 h at room temperature in 50 mM sodium phosphate buffer (pH 7.5) containing 2.5% Triton X-100 and incubated at 75°C for 5 h in 50 mM sodium phosphate buffer (pH 7.5). The gels were then cooled and stained with 1.8 g of amido black-0.04 g of Coomassie brilliant blue per liter in 35% methanol-7% acetic acid. Proteolytic activity was visualized by clearing zones resulting from gelatin hydrolysis. Substrate-overlay gel analysis was done by using the procedure described by Deane et al. (8). First, protein samples (total protein, 1 to 3 μg) were separated by conventional SDS-PAGE. The gel was then washed in 2.5% Triton X-100-50 mM sodium phosphate buffer (pH 7.5) for 1 h at room temperature to remove the SDS. A second polyacrylamide gel containing 0.5% (wt/vol) gelatin was placed on top of the original gel, and both were pressed together to remove air bubbles. The gels were kept moist with 50 mM sodium phosphate buffer (pH 7.5) during the transfer. The transfer step was performed in a 75°C oven for 6 to 12 h. After this, the gelatin containing gel was stained with amido black-Coomassie brilliant blue staining solution as described before.

Western blot (immunoblot) analysis. Protein samples were electrophoresed on 10% polyacrylamide gels and blotted onto nitrocellulose membranes (Bio-Rad) by using transfer buffer containing 20 mM Tris and 150 mM glycine in 20% methanol (18) in a Bio-Rad transblot unit. After electrophoretic blotting (16 h, 40 V, 4°C), the nitrocellulose membranes were immersed in blocking solution (3% gelatin, 20 mM Tris, 500 mM NaCl (pH 7.5)) for 30 min and then exposed to guinea pig serum containing antibodies against protease S66 (1:200 dilution in 1% gelatin, 20 mM Tris, 500 mM NaCl, 0.05% Tween-20 (pH 7.5)) for 2 h at room temperature. The membranes were then washed and exposed to an anti-guinea-pig-horseradish-peroxidase complex (1:2,000 dilution; Bio-Rad) for 1 h at room temperature. Finally, the membranes were immersed in the color-developing solution containing 4-chloro-1-naphthol. All steps were performed according to Bio-Rad recommendations. To prepare the antibodies, the protein band corresponding to protease S66 was excised from polyacrylamide gels after electrophoretic separation and the gel piece was crushed and homogenized in 50 mM sodium phosphate buffer. This preparation was then used to immunize the guinea pigs.

RESULTS

Proteolytic activity in P. furiosus. Proteolytic activity in cell extracts of P. furiosus was found to increase with temperature from 37 to 105°C as measured by azocasein

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presence of three additional proteins with molecular masses of 200, 102, and 52 kDa (data not shown). By SDS-PAGE gelatin overlay analysis, it was possible to distinguish five discrete bands with proteolytic activity in the cell extract and two distinct bands with activity in the sample treated with SDS for 24 h. The approximate molecular masses of the proteases present in the untreated cell extract were 140, 125, 116, 102, and 66 kDa. The molecular masses of the two SDS-resistant proteases were 102 and 66 kDa, respectively, corresponding to the two lower-molecular mass bands present in the untreated extract (Fig. 2B). These proteases were designated as S102 and S66. When the 24-h SDS-treated sample and the purified S66 enzyme were analyzed by using substrate-PAGE (Fig. 1), it was clearly seen that protease S66 was active during electrophoresis in the presence of SDS and was responsible for the smearing observed in the gels. This analysis also indicated the presence of two faint bands with proteolytic activity corresponding to 47 and 43 kDa in the 24-h SDS-treated sample. These proteolytic active bands could not be detected in samples incubated for shorter periods of time or in extracts treated for 48 h, suggesting that these bands are intermediate degradation products of a higher-molecular-mass protease(s).

All of these proteins exhibited some variability in their mobility in SDS-PAGE. In particular, protease S66 was observed to migrate to positions corresponding to molecular masses ranging from 60 to 70 kDa. This difference in migration pattern can be attributed in part to the fact that this protein is not completely denatured, since it retains enzymatic activity during electrophoresis. Also, it appears that this protein undergoes carboxy or amino terminal cleavage resulting in a progressively smaller protein during SDS treatment at 98°C (see later discussion). When protease S66 is heated at 120°C for 20 min, the protein is presumably denatured since all proteolytic activity is lost. When analyzed by SDS-PAGE, the molecular mass of the denatured protein corresponded to 66 kDa and this was used to designate the protein.

Different attempts were made to chemically denature the SDS-resistant proteases prior to SDS-PAGE separation. Samples of the SDS-treated cell extract were incubated first at 100°C for 3 min and then at room temperature for 1 h in the presence of the following combinations of denaturing agents: (i) 1% SDS, 5% β-mercaptoethanol, and 8 M urea; (ii) 1% SDS, 80 mM dithiothreitol, and 8 M urea; (iii) 1% SDS and 5% β-mercaptoethanol; and (iv) 1% SDS and 80 mM dithiothreitol. After electrophoresis, remaining proteolytic activity was determined by gelatin-gel overlay analysis. In all cases there was no apparent loss of activity in either one of the SDS-resistant proteases as compared with activity present in untreated samples.

Analysis of *P. furiosus* culture supernatants indicated the presence of the same five proteases found in cell extracts. It is unclear at the present if these extracellular proteases are the result of cell lysis or if actual secretion takes place.

**Immunoblot analysis of the SDS-resistant proteases.** The results obtained with the SDS treatment of the cell extract raised the question of whether proteases S66 and S102 corresponded to active fragments produced by hydrolysis of larger proteins and whether these two proteases were related to each other. Time course samples of the incubation with SDS were screened by immunoblot analysis with polyclonal antibodies against protease S66. The results show that the antibodies recognized a number of different proteins other than the 66-kDa protease (Fig. 3; Table 1). A protein of an estimated size of 200 kDa was recognized in samples treated
for up to 2 h but not in samples treated for 24 h. Since this protein did not exhibit proteolytic activity, it is unlikely that it represents an aggregate of protease S66. Rather, it may correspond to a precursor of this protein. Table 1 summarizes the effect of SDS heat treatment on the mobility of protease S66 and an immunologically related, but not proteolytically active, lower-molecular-mass (49 kDa) protein. These are represented in Fig. 3 as bands B* and C, respectively. Over the course of the 24-h treatment, there was a progressive and simultaneous decrease in the apparent mass of these bands. Both bands decreased by 8 kDa in apparent molecular mass; the active protease (S66) with a molecular mass of 70 kDa in the untreated cell extract decreased to a protein of 62 kDa in the 24-h sample. Similarly, the smaller protein showed a decrease in molecular mass from 57 to 49 kDa (Table 1). It should be emphasized that these values cannot be taken as an absolute measure of the molecular mass of these proteins because of the variability in protein mobility previously discussed. Rather, the molecular mass values are a convenient way to illustrate the apparent changes in mass that took place during the SDS-treatment. Lower-molecular-mass bands also were recognized in the early time course samples but not in the 24-h sample. None of these bands exhibited proteolytic activity. These fragments probably correspond to hydrolysis products of the larger proteins. In the time-zero sample, faint bands also were recognized at positions corresponding to molecular masses of 160, 132, 112, 96, and 86 kDa.

It is clear from this immunoblot analysis that the two SDS-resistant proteases (S66 and S102) are not immunologically related, since the antibodies against protease S66 do not recognize a protein of 102 kDa in the SDS-treated samples. These results also show that protease S66 is not related to the other proteases present in *P. furiosus* cell extracts. Control experiments showed that guinea pig sera prior to immunization did not recognize any of *P. furiosus* proteins.

**Characterization of the SDS-resistant proteases.** The effect of temperature on the proteolytic activity of the SDS-resistant proteases partially purified through treatment with 1% SDS at 98°C for 24 h is shown in Fig. 4. The activity increased with temperature from 40 to 100°C. Because azocasein is increasingly less stable at and above 100°C, quantitative measurement of proteolytic activity at these temperatures is less certain. However, qualitatively, activity is apparent to at least up to 105°C. The $t_{1/2}$ of this protease mixture at 98°C in 50 mM phosphate buffer was found to be 33 h. The SDS-resistant proteases showed activity over a wide range of pH, from 5.8 to 8.3, with an optimum at pH 7.0 (Fig. 5).

Inhibitor studies were performed by using the 24-h SDS-treated extract and purified S66 protease (Table 2). Proteolytic activity of S66 was almost completely inhibited by the presence of phenylmethylsulfonyl fluoride and diisopropylfluorophosphosphate but was not significantly affected by iodoacetic acid, EDTA, or EGTA. These results indicate that
S66 is a serine protease. Essentially identical inhibition profiles were obtained with the SDS-treated extract containing both S66 and S102 proteases. Considering that protease S66 is more abundant in terms of mass, this result seems to indicate that this protease is the main contributor to proteolytic activity in the SDS-treated extract. However, the possibility that both enzymes are serine proteases cannot be ruled out. Attempts to recover protease S102 by electroelution have been so far unsuccessful. This is primarily a result of the low concentration of this protein in the SDS-treated extracts. Preliminary specificity studies with synthetic substrates showed that the 24-h SDS-treated extract was hydrolytically active towards N-benzoyl-L-arginine ethyl ester and L-lysine-p-nitroanilide but not N-benzoyl-L-tyrosine ethyl ester or N-α-benzoyl-DL-arginine-4-nitroanilide. Hydrolytic activity could be detected towards only N-benzoyl-L-arginine ethyl ester when purified S66 protease was used. It is not known at present if this difference is due to the presence of S102 in the extract sample or to the fact that higher enzyme concentrations are required to hydrolyze the amide substrates compared to the ester analogs (19).

**DISCUSSION**

The results reported here show that *P. furiosus* produces five intracellular proteases, two of which are resistant to SDS denaturation and have unusually high thermostability. When cell extracts were incubated in the presence of 1% SDS at 98°C for 24 h, the majority of the cellular proteins were hydrolyzed so that only few proteins could be detected.

**TABLE 1. Estimated molecular mass of fragments**

<table>
<thead>
<tr>
<th>Estimated molecular mass (kDa) of fragments found in cell extract samples incubated for:</th>
<th>0 h</th>
<th>0.25 h</th>
<th>0.5 h</th>
<th>1 h</th>
<th>2 h</th>
<th>24 h</th>
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<td>200 (A)</td>
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<tr>
<td>70 (B*)</td>
<td>68 (B*)</td>
<td>66 (B*)</td>
<td>64 (B*)</td>
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<tr>
<td>57 (C)</td>
<td>55 (C)</td>
<td>53 (C)</td>
<td>51 (C)</td>
<td>51 (C)</td>
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* a Recognized by immunoblot analysis with antibodies against protease S66 in cell extract samples incubated at 98°C with 1% SDS for various times.

* Letters in parentheses correspond to bands shown in Fig. 3.

**FIG. 4.** Effect of temperature on proteolytic activity of cell extracts treated with 1% SDS at 98°C for 24 h. Activity is based on azocasein hydrolysis.

**FIG. 5.** Effect of pH on proteolytic activity of cell extracts treated with 1% SDS at 98°C for 24 h. Activity was measured by the hydrolysis of azocasein in 100 mM sodium phosphate buffer adjusted to the desired pH value.
TABLE 2. Effect of enzyme inhibitors on the proteolytic activity of purified S66 protease and P. furiosus SDS-treated cell extract

<table>
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<tr>
<th>Inhibitor</th>
<th>% Inhibition</th>
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<tr>
<td>PMSF</td>
<td>78.9</td>
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<tr>
<td>DFP</td>
<td>83.8</td>
</tr>
<tr>
<td>EDTA</td>
<td>11.3</td>
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<tr>
<td>EGTA</td>
<td>10.6</td>
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<tr>
<td>iodoacetic acid</td>
<td>1.4</td>
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* PMSF, Phenylmethylsulfonyl fluoride; DFP, diisopropylfluorophosphate.

by SDS-PAGE. The SDS-treated extract retained 19% of the initial proteolytic activity and contained two proteases (S66 and S102). These enzymes correspond to the lower-molecular-weight proteases present in the untreated cell extracts and are presumably responsible for protein hydrolysis during the SDS treatment. The highest proteolytic activity of the SDS-resistant proteases was measured at 105°C. However, it is possible that the optimum temperature might be even higher. To determine this, suitable thermostable substrates must be identified. The measured $t_{1/2}$ of the proteolytic activity in the SDS-treated sample was 33 h at 98°C, corresponding to the most thermostable proteolytic activity reported thus far. Optimization of conditions for protease stability should lead to enhanced resistance to thermal denaturation. In addition to being resistant to SDS, proteases S66 and S102 were not denatured by treatment with 8 M urea together with 5% β-mercaptoethanol or 80 mM dithiothreitol. This result suggests that either disulfide bonding is not involved in preserving proteolytic activity or that existing disulfide bonds were not reduced under the test conditions. These observations are not unique to P. furiosus proteases. Cowan et al. (7) reported that disulfide bridges seem to play an insignificant role in stabilizing archaealysin, and it is well established that subtilisins do not have disulfide bonds in their polypeptide chains (16). Clearly, more information is needed to establish the importance of sulfur bridges, if present, in the preservation of activity in P. furiosus proteases. Also, data on the contribution of hydrophobic interactions in hyperthermophilic enzyme stability will provide a better understanding of protein chemistry at high temperatures.

Immunoblot analysis of time course samples of the SDS treatment showed that proteases S66 and S102 are not related and correspond to different enzymes. In addition, the results indicate that protease S66 is distinct from the other three proteases present in P. furiosus cell extracts. Also, the results suggest that protease S66 is the hydrolysis product of a 200-kDa precursor which does not exhibit proteolytic activity. This precursor disappears upon prolonged treatment at 98°C, producing the active protease and other smaller fragments. The immunoblot analysis also indicated that the 66-kDa protease and a smaller immunologically related but inactive protein (molecular mass, 49 kDa) progressively decrease in size upon SDS treatment. These results and the fact that these two proteins undergo simultaneous changes in apparent molecular mass suggest that the lower-molecular-mass protein is the hydrolysis product of protease S66 and that the missing fragment is essential for enzyme activity. This fragment, of approximately 13 kDa, should correspond to either the amino or carboxy terminus of the 66-kDa protein. If this analysis is correct, it would also help to explain the simultaneous decrease in size of both proteins upon thermal SDS treatment. Since the proteins get smaller by a discrete fragment (2 kDa) and no smearing is evident, it is likely that a precise cleavage site is being recognized at the amino or carboxy terminus (whichever one both proteins share). The sequential nature of these cleavages suggests that all sites are not accessible and that the more terminal fragment has to be cleaved off to expose the next cleavage site. Amino acid sequencing of the amino and carboxy termini of these proteins will provide information on the actual mechanism of this hydrolysis process. It would also be interesting to determine if this process is autocatalytic or if other proteases are involved. These issues are currently under investigation. Characterization of purified protease S66 has determined that this enzyme is a serine protease and that it can hydrolyze N-benzoyl-l-arginine ethyl ester, a trypsin substrate. Additional biochemical characterization of this novel collection of proteases will result most likely in important information about protein stability.

The physiological significance of the proteases found in P. furiosus is not clear, but they may play an important role for heterotrophic bacteria inhabiting high-temperature niches. It has been established that P. furiosus can grow in a defined medium with amino acids as the only carbon source (I. I. Blumentals, S. H. Brown, R. N. Schicho, A. K. Skaja, H. R. Costantino, and R. M. Kelly, Ann. N.Y. Acad. Sci., in press) and in complex media formulations including Casamino Acids or trypitone (9). These observations suggest that these proteases might provide nutritional diversity for P. furiosus, allowing it to degrade proteinaceous material in its environment. However, considering that the proteases described here are intracellular, it is likely that these enzymes play an important role in intracellular protein turnover so that the bacterium can break down and reassemble peptides depending upon metabolic requirements. The capability of recycling amino acids might prove to be an important adaptation of hyperthermophilic bacteria to cope with the extreme conditions of their habitats. The capability of P. furiosus to recycle amino acids, together with its capacity to degrade and utilize polysaccharides (3), suggest that this organism is well adapted to utilize available substrates in its environment.

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LITERATURE CITED


