A serine proteinase of an archaebacterium, *Halobacterium mediterranei*

A homologue of eubacterial subtilisins

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A homogeneous serine proteinase secreted by the extreme halophilic bacterium *Halobacterium mediterranei* 1538 was isolated by affinity chromatography on bacitracin–Sephacrose with a yield of 48 % (260-fold purification). The enzyme reveals an optimum for pyroglutamyl-Ala-Ala-Leu *p*-nitroanilide hydrolysis at pH 8.0–8.5 (K_m 0.14 mM; k_{cat} 36.9 s⁻¹). Its activity increases linearly with NaCl concentration over the range 2–5 M. The substrate specificity of the enzyme is comparable with that of secretory subtilisins, the extent of protein degradation approaching that attained with proteinase K. The enzyme has a molecular mass of 41 kDa and a pI of 7.5. The *N*-terminal sequence of *H. mediterranei* serine proteinase reveals a 50 % identity with that of *Thermoactinomyces vulgaris* serine proteinases, indicating that the enzyme belongs to the subtilisin family. Hence the serine proteinase secreted by the halophilic bacterium should be considered as a functional analogue, and a structural homologue, of eubacterial serine proteinases (subtilisins).

INTRODUCTION

Archaeobacteria, including halophilic bacteria, differ from Eubacteria in numerous biochemical traits [1,2]. The proteins produced by these micro-organisms possess specific features not found in eubacterial ones. This difference might be ascribed to different pathways pursued in evolution by the organisms of these two Kingdoms. On the other hand, the adaptation towards extreme conditions, e.g. to exceptionally high ionic strength for halobacteria, might act as an important factor causing very strong divergence of the enzymes evolving from an ancestor common to both Kingdoms.

Data comparing archaeobacterial and eubacterial enzymes are still rather scarce. We isolated a secretory proteinase produced by one species of halobacterium (*Halobacterium mediterranei*) and compared this enzyme with the thoroughly studied eubacterial serine proteinases (subtilisins). *H. mediterranei* cells, being strictly halophilic, grow in 25 % NaCl solution. The proteinase secreted by these cells is adapted to very high salt concentration; it therefore retains its activity in 0.7–5 M-NaCl and suffers fast autolytic inactivation when desalted. This phenomenon excludes the application of ion-exchange chromatography, electrofocusing and electrophoresis for the purification or analysis of any halobacterial proteinase. These difficulties explain why, until recently, the isolation of the only one halophilic proteinase, namely that from *H. halobium*, has been described [3], although no structural characteristics of this enzyme were given. In 1990, Kakemura & Seno described a halophilic extracellular proteinase from a strain of unidentified halophilic archaebacterium [4], an enzyme that shares common traits with that produced by *H. mediterranei*.

MATERIALS AND METHODS

Micro-organism cultivation

H. mediterranei, strain 1538, was grown as described [5] on the

growth medium that contained 5 g of peptone, 2.5 ml of yeast hydrolysate, 250 g of NaCl, 2 g of KCl, 20 g of MgSO₄ and 2 g of sodium citrate per litre of distilled water (pH 7.1–7.2) by shaking 250 ml flasks at 250 rev./min and 40 °C. The medium was inoculated with 51–10 % of the culture grown for 24 h under the same set of conditions. The maximal level of the proteinase was attained after 60 h growth.

Isolation of the enzyme (Table 1)

A 135 ml portion of the culture filtrate was applied to a bacitracin–Sephacrose 4B [6] column (1.4 cm × 10.5 cm) equilibrated with 4.5 M-NaCl in 0.05 M-Tris/HCl buffer, pH 8.0. The column was washed with 500 ml of 5 M-NaCl in the same buffer, which eluted the bulk of non-active proteins, pigments and other admixtures. The proteinase was then eluted with 100 ml of 10 % (v/v) ethanol in 4.5 M-NaCl, pH 8.0. The *A*₂₈₀, as well as the proteolytic activity against pyroglutamyl-Ala-Ala-Leu *p*-nitroanilide (Glp-Ala-Ala-Leu-NH-Np), were measured in the effluent.

The fraction that contained the active enzyme was concentrated by ultrafiltration through a YM-10 Amicon membrane at 4 °C; 2 ml of the concentrated solution was applied to a Sephadex G-75 column (76 cm × 1.6 cm) and equilibrated with 4.5 M-NaCl in 0.05 M-Tris/HCl buffer, pH 8.0. Fraction II, which contained essentially homogeneous enzyme, was used to study its functional properties. To obtain dry material, the solution was treated with 5 µl of di-isopropyl fluorophosphate to inactivate the enzyme; it was then dialysed to remove the salts and freeze-dried.

Activity measurements

The activity against the chromogenic substrate Glp-Ala-Ala-Leu-NH-Np [7] was measured as follows. A 25 µl portion of the substrate solution in dimethylformamide (20 mg/ml) was added to 2.5 ml of 4.5 M-NaCl in 50 mM-Tris/HCl, pH 8.0. The mixture was incubated for 10 min at 37 °C, then the reaction was stopped

Abbreviations used: Glp, pyroglutamyl; NH-Np, *p*-nitroanilide; Ant, antraniloyl (*o*-aminobenzoyl); Z, benzyloxycarbonyl; Pth, phenylthiohydantoin derivatives; all amino acids are of the L configuration.

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Table 1. Isolation of *H. mediterranei* proteinase

Activity was measured against Glp-Ala-Ala-Leu-NH-Np

Purification stage	Volume (ml)	Total protein (A_{280})	Activity		Purification (fold)	Yield (%)
			Total (units)	Specific (units/ A_{280})		
Culture filtrate	138	1350	135	0.1	1	100
Affinity chromatography on bacitracin-Sepharose	46	12	105	9.3	93	80
Ultrafiltration (YM-1 membrane)	3.5	7.5	94.5	12.5	125	70
Gel-filtration on Sephadex G-75	25	2.5	65	26	260	48

with 0.5 ml of 50 % acetic acid and the A_{410} was measured. The incubation time might be extended when low concentrations of the enzyme were to be assayed, e.g. to assess its content in the culture filtrate. Under such circumstances, a yellow colouration of the mixtures serves as a convenient indicator of substrate hydrolysis. A unit of enzyme activity is defined as the amount that produces 1 μ mol of *p*-nitroaniline/min under the above conditions.

To determine the catalytic constants, to 2.5 ml of 4.5 M-NaCl in 50 mM-Tris/HCl buffer, pH 8.0, 50 μ l of the substrate solution in dimethylformamide was added to achieve a final concentration of 0.039–2.32 mM. Then the enzyme was added and the initial velocity of substrate hydrolysis was measured at 37 °C by monitoring the absorbance at 410 nm (corresponding to *p*-nitroaniline liberation). K_m and k_{cat} were calculated from $1/v$ versus $1/s$ plots.

The optimum pH for activity was measured against the same substrate in 4.5 M-NaCl, but the pH was varied from 5.35 to 6.15 (0.1 M acetate buffer) and from 7.3 to 10.3 (0.1 M-Tris/HCl buffer). The same set of solutions was prepared to monitor pH-dependence of enzyme stability.

Enzyme specificity

The proteinase specificity was tested against a series of synthetic peptide substrates and bovine insulin B-chain. The activity against peptide *p*-nitroanilides [7] was assessed as described for Glp-Ala-Ala-Leu-NH-Np. The activity towards the fluorogenic substrates antraniloyl(Ant)-Ala-Ala-Val-NH-Np and Ant-Ala-Ala-Phe-NH-Np was measured in 4.5 M-NaCl/0.1 M-Tris/HCl, pH 8, and 30 °C as described in [8].

To 0.3 mg of oxidized B-chain in 0.5 ml of 4.5 M-NaCl/0.05 M-Tris/HCl buffer, pH 8.0, 10 μ l of the enzyme solution was added. Aliquots of this mixture incubated for 3 and 17 h at 37 °C were chromatographed on an Ultrasphere Octyl column (15 cm $\times$ 0.46 cm) using a Beckman 344 h.p.l.c. system and a water/propan-2-ol gradient. The fractions containing peptides were collected and their amino acid compositions were determined.

Solutions of several proteins in 4.5 M-NaCl, pH 8, were prepared and digested with the serine proteinase at 37 °C for 10 min. After precipitation of the digest with trichloroacetic acid, added to a final concentration of 4 % (w/v), the filtrate A_{280} was measured (A_{440} in the case if the azocasein hydrolysate, to which equal volume of 0.5 M-NaOH was added).

Molecular properties

The molecular mass of the native proteinase was determined by gel filtration on a Sephadex G-75 column (76 cm $\times$ 1.6 cm) in 4.5 M-NaCl; the column was calibrated with proteins of known molecular mass [BSA (67 kDa), ovalbumin (45 kDa), chymo-

trypsinogen (25 kDa) and pancreatic ribonuclease (13.7 kDa)]. Its molecular mass was also determined, as described in [9], by ultracentrifugation of the enzyme, inhibited with 0.1 mM-diisopropyl fluorophosphate, in 26 % NaCl at pH 8.0 and 20 °C for 8 h at 32000 rev./min using a Beckman model E ultracentrifuge. The partial specific volume was calculated from the amino-acid-analysis data. The molecular mass was computed by the 'all-speed' autocalibration method for sedimentation equilibrium [9].

The molecular mass of the denatured proteinase was determined, after its inactivation with 0.15 mM-phenylmethanesulphonyl fluoride or 0.1 mM-diisopropyl fluorophosphate in 4.5 M-NaCl, dialysis and freeze-drying, by electrophoresis in 10 %-(w/v)-polyacrylamide gel in the presence of 0.1 % SDS.

The pI of the proteinase was measured by chromatofocusing of a 5 mg sample on a column (21 cm $\times$ 1 cm) of PBE 94 (Pharmacia) after preliminary inactivation of the enzyme with 0.1 mM-diisopropyl fluorophosphate. A pH 8.7–5.5 gradient was formed by 25 mM-Tris/HCl starting buffer, pH 8.7, and gradient Polybuffer 96 ('Pharmacia'), to which acetic acid was added to pH 5.5.

Amino acid composition was determined, after hydrolysis of a sample with 5.7 M-HCl for 24 and 72 h, with a Hitachi 835 amino acid analyser. Half-cysteine and methionine were assayed respectively as cysteic acid and methionine sulphone after protein oxidation with formic acid, and tryptophan after hydrolysis with 4 M-methanesulphonic acid in the presence of 0.2 % tryptamine.

N-Terminal sequence

Proteinase was treated with 0.1 mM-diisopropyl fluorophosphate, dialysed and freeze-dried, then dissolved in minimal volume of phenol containing 20 % (v/v) of water, and precipitated with an excess of acetone. A 0.4 mg portion of this preparation was attached to 60 mg of aminopropyl-glass (Pierce) in 120 μ l of 0.5 M-Quadrol buffer, pH 9.0, by the *p*-phenylene diisocyanate procedure described by Laursen *et al.* [10]. In another experiment, 0.5 mg of diisopropyl fluorophosphate-inactivated enzyme was dissolved in 0.5 ml of 4.5 M-NaCl adjusted to pH 8.0. To this solution 50 mg of aminopropyl-glass, pretreated with *p*-phenylene diisocyanate, was added and the mixture kept at 40 °C for 1 h, then washed with water and methanol to remove non-covalently bound material. Edman degradation of the protein, attached to aminopropyl-glass by the procedures described, was performed using solid-phase Sequenator (Rank-Hilger). Phenylthiazolinone derivatives of amino acids were converted into their respective phenylthiohydantoin by heating with 1 M-HCl at 80 °C for 10 min. Phenylthiohydantoin were analysed by h.p.l.c. on an Altex Ultrasphere ODS/PTH column [11]. The N-terminal aspartic acid residue was identified separately by a manual Edman procedure.

RESULTS

Affinity chromatography on bacitracin–Sephacrose [6] permitted the isolation of *H. mediterranei* proteinase from the culture filtrate in high yield. Halobacterial proteinase, adapted to function in highly concentrated salt solutions, was rather tightly bound to the specific ligand (the cyclopeptide bacitracin) even in the presence of 4.5 M-NaCl. The enzyme was eluted with 10 % ethanol in 4.5 M-NaCl, which suppressed its hydrophobic interaction with the ligand. Further purification by ultrafiltration and gel filtration on Sephadex G-75 (Fig. 1) gave essentially pure enzyme with the yield of 48 % (Table 1). The enzyme purity was confirmed by sequencing of the inhibited sample and chromatofocusing, which gave one peak of the enzyme at pI 7.5. Its molecular mass, as determined by gel filtration, corresponded to 40 kDa (Fig. 2), whereas sedimentation experiments gave the value 41.9 ± 1 kDa. SDS/PAGE gave a major band that corresponded to 40 kDa (Fig. 3). We use a value of 41 kDa for the molecular mass of the enzyme devoid of its quaternary structure. Di-isopropyl fluorophosphate at 0.1 mM, as well as 0.15 mM-

Fig. 1. Gel filtration of *H. mediterranei* serine proteinase on a Sephadex G-75 column (76 cm $\times$ 1.6 cm)

The column was equilibrated and eluted with 4.5 M-NaCl at 15 ml/h; 2.5 ml fractions were collected. Peak II contained the proteinase active against Glp-Ala-Ala-Leu-NH-Np (open bars).

Fig. 2. Determination of the molecular mass of *H. mediterranei* serine proteinase by gel filtration on a Sephadex G-75 column

The points correspond to the elution volumes of the following proteins: 1, ribonuclease A; 2, chymotrypsinogen A; 3, *H. mediterranei* serine proteinase; 4, ovalbumin; 5, BSA.

phenylmethanesulphonyl fluoride, completely inactivated the enzyme, which should therefore be considered as a serine proteinase. Partial inhibition was found with thiol-group-reactive reagents; thus the enzyme, when treated with 1 mM-HgCl₂, retained only 18 % of its initial activity, whereas 0.1 mM-*p*-chloromercuribenzoate reduced the activity of 50 % of the initial level. On the other hand, the enzyme was resistant even to 0.3 M-EDTA, indicating the absence of functionally important metal ion in its active site. The enzyme was completely inhibited by 0.15 mM-ovomucoid, which is common for subtilisins, whereas 9 μ M soybean trypsin inhibitor lowered its activity to 42 % of the initial value. In Table 2 the molecular and enzymic characteristics of the *H. mediterranei* serine proteinase are compared with those of serine proteinase from *H. halobium* [3] and serine proteinases known to belong to the subtilisin family, namely with subtilisin BPN' [12] and the thiol-dependent serine proteinase from *Thermoactinomyces vulgaris* [13–15].

The enzyme is stable within the pH range 5.5–8.5 in 4.5 M-NaCl (Fig. 4). Its activity against a synthetic chromogenic substrate (Glp-Ala-Ala-Leu-NH-Np) is optimal at pH 8.0–8.5 and linearly increases with NaCl concentration over the range 2–4 M. The K_m (0.14 mM) and k_{cat} (36.9 s⁻¹) values were determined by hydrolysis of this peptide in 4.5 M-NaCl at pH 8.0. The enzyme revealed no activity against benzyloxycarbonyl

Fig. 3. Electrophoresis of *H. mediterranei* serine proteinase inhibited by phenylmethanesulphonyl fluoride (lane 1) in a 10 %-polyacrylamide gel in the presence of 0.1 % SDS

As molecular-mass markers the following proteins were applied (lane 2): human serum albumin (67 kDa); aldolase (40 kDa) (the band with practically the same mobility as that of the proteinase); chymotrypsin (25 kDa); ferritin subunit (19 kDa); and cytochrome c (12000 kDa).

Table 2. Comparative characteristics of serine proteinases from Halo- and Eu-bacteria (activity measured against Glp-Ala-Ala-Leu-NH-Np)

Proteinase	Mol- ecular mass (kDa)	pI	Specific activity (units/ <i>A</i> ₂₈₀)	<i>K</i> _m (mM)	<i>k</i> _{cat} (s ⁻¹)	Optimum activity		Stability range (pH)
						pH	Temp. (°C)	
<i>H. mediterranei</i>	41	7.5	26	0.14	36.9	8–8.5	55	5.5–8
<i>H. halobium</i> [3]	41	—	15	—	—	8–9.0	37	—
Halophilic archaeobacterium 172 P1 [4]	42–46	—	—	—	—	10.7	75	5–11 (30 °C)
<i>Bacillus amyloliquefaciens</i> [12,13]*	27.5	7.8	30	0.8	4.2	7.5–7.8	37	6–10
<i>T. vulgaris</i> [13,14]*	28	8–9	20	1.9	—	8.2	55	7–9

* Activity measured against Z-Ala-Ala-Leu-NH-Np.

Fig. 4. Dependence of *H. mediterranei* serine proteinase stability on pH in 4.5 M-NaCl at 20 °C

×, 4 h incubation; O, 22 h incubation. Maximal activity was taken as 100 %.

Z-Glu-NH-Np, Glp-Phe-Nh-Np, Glp-Phe-Ala-NH-Np and Glp-Ala-Ala-Leu-Arg-NH-Np. If its relative activity against Glp-Ala-Ala-Leu-NH-Np was taken as 100 %, the activities against Ant-Ala-Ala-Xaa-NH-Np were equal to 115 % (Xaa = Leu), 50 % (Xaa = Phe), 5 % (Xaa = Val) and 0 % (Xaa = Ile). Hence the halophilic serine proteinase favours Leu and Phe in the P₁

position of a substrate. The enzyme is sensitive towards the presence of organic solvents. Thus, in 10 % (v/v) propan-2-ol in 4.5 M-NaCl, its activity decreases to 1 % of its initial level and in 10 % ethanol to 10 %, whereas in 10 % dimethylformamide approx. 30 % of the enzyme activity against Glp-Ala-Ala-Leu-NH-Np was observed.

H. mediterranei serine proteinase in 4.5 M-NaCl efficiently degraded oxidized insulin B-chain, cleaving 12–14 peptide bonds (Fig. 5). The pattern of insulin-B-chain hydrolysis is similar to those characteristic for subtilisins [16], including their homologues, serine proteinase from *T. vulgaris* [17] and aqualysin I [18]. In addition to the bonds formed by the carboxyl groups of Phe, Tyr and Leu, quite a few bonds formed by polar amino acid residues (i.e. those after Gln⁴, Ser⁹, His¹⁰, Glu¹³, Glu²¹, Arg²² and Lys²⁹) were cleaved. It appears that the enzyme specificity is especially similar to that of proteinase K [19]. When allowed to act on insulin B-chain in the absence of 4.5 M-NaCl the enzyme still cleaved the bonds Leu¹⁵-Tyr¹⁶ and Phe²⁵-Tyr²⁶.

The enzyme easily degraded several protein substrates in 4.5 M-NaCl, pH 8.0 at 37 °C and showed the following relative activities (substrate concentrations are given in parentheses): ovalbumin, 100 % (10 mg/ml); BSA, 30 % (5 mg/ml); bovine haemoglobin, 13 % (20 mg/ml); azocasein, 6.6 % (2.5 mg/ml).

The N-terminal sequence of the enzyme, determined by solid-phase Edman degradation is shown in Table 3 in comparison with that of the proteinase of the halophilic archaeobacterium strain 172 P 1 [4] and of *T. vulgaris* serine proteinase, an enzyme unambiguously homologous with the subtilisins [20,21].

The unique sequence (residues nos. 1–21 according to the *T.*

Fig. 5. Hydrolysis of oxidized insulin B-chain with *Halobacterium mediterranei* proteinase after 3 and 17 h of incubation and with other eubacterial serine proteinases (aqualysin I [18], proteinase K [19] *T. vulgaris* serine proteinase [17] and subtilisin BPN [16])

Arrows indicate the bonds hydrolysed by the enzymes.

Table 3. Amino acid sequences from the N-terminal part of *Halobacterium mediterranei* serine proteinase and related eubacterial proteinases

The sequence starting from residue no. 33 appears as a result of autolytic cleavage and contains an Asp (marked with asterisk) residue at position 35 identical with that belonging to the catalytic site of known subtilisins. The residue Xaa³³ escaped identification because of its amino-group attachment to the support.

	1	10
<i>H. mediterranei</i>	Asp-Thr-Ala-Asn-Asp-Pro-Lys-Tyr-Gly-Ser-	- -
Archaeobacterium 172 P1 [4]	Ala-Thr-Pro-Asn-Asp-Pro-Gln-Tyr-Gly-Gln-	- -
<i>T. vulgaris</i> [21]	Tyr-Thr-Pro-Asn-Asp-Pro-Tyr-Phe-Ser-Ser-Arg-	
Subtilisin BPN' [12]	Ala-Gln-Ser-Val-	
	12	20
<i>H. mediterranei</i>	Gln-Tyr-Ala-Pro-Gln-Lys-Val-Asn-Ala-Asp-	
Archaeobacterium 172 P1	Gln-Tyr-Ala-Pro-Gln-Gln-Val-Asn-Xaa-Glu-	
<i>T. vulgaris</i>	Gln-Tyr-Gly-Pro-Gln-Lys-Ile-Gln-Ala-Pro-	
Subtilisin BPN'	Pro-Tyr-Gly-Val-Ser-Gln-Ile-Lys-Ala-Pro-	
	35	45
<i>H. mediterranei</i>	Xaa-Val-Asp*-Gln-Gly-Val-Xaa-Xaa-Xaa-His-Pro-Glu-Leu-	
<i>T. vulgaris</i>	Ile-Val-Asp*-Thr-Gly-Val-Gln-Ser-Asn-His-Pro-Asp-Leu-	
Subtilisin BPN'	Val-Ile-Asp*-Ser-Gly-Ile-Asp-Ser-Ser-His-Pro-Asp-Leu-	

Table 4. Amino acid composition of serine proteinases from halo- and eu-bacteria

For the *H. mediterranei* proteinase, (a) shows average values from three experiments; (b) shows the nearest integer.

Amino acid	Composition (mol/mol)			
	Proteinase ...		<i>H. halobium</i> (3)	<i>B. amylolique faciens</i> [12] (subtilisin BPN')
	<i>H. mediterranei</i>			
	(a)	(b)		<i>T. vulgaris</i> [21]
$\frac{1}{2}$ -Cys	5-7	6	4	0
Asx	60.3	60	69	28
Thr	30.6	31	32	13
Ser	54.0	54	43	35
Glx	34.3	34	34	15
Pro	14.6	15	18	14
Gly	53.5	53	56	33
Ala	37.5	37	47	37
Val	24.8	25	24	30
Met	2.0	2	2	5
Ile	15.3	15	16	13
Leu	27.6	28	28	15
Tyr	14.3	14	14	10
Phe	5.9	6	7	3
Trp	2.8	3	3	3
Lys	10.3	10	5	11
His	5.5	6	3	6
Arg	7.8	8	11	2
Sum...		407	416	275
				283

vulgaris enzyme numbering) was determined after the attachment of di-isopropyl fluorophosphate-inhibited enzyme to the support in the presence of 4.5 M-NaCl (Table 3). By contrast, the sequencing of the desalted enzyme revealed the presence of two peptide chains, although the enzyme was preinhibited with di-isopropyl fluorophosphate. One of these chains corresponded to the *N*-terminus of the enzyme, whereas another one, apparently formed as a result of the autolytic cleavage of the polypeptide chain after the residue no. 32, was traced from the residues 34-45.

The aspartic acid residue in the *H. mediterranei* proteinase sequence, coinciding as it does with Asp³⁶ in *T. vulgaris* serine proteinase (Asp³² in the subtilisin BPN' numbering) warrants special mention. The latter residue is known to belong to the subtilisin catalytic triad. Therefore one might assume that this Asp residue plays the same role in the *H. mediterranei* enzyme.

DISCUSSION

We have found that the halophilic bacterium *H. mediterranei* secretes a serine proteinase in the culture medium. Previously it has been shown [3] that another halobacterium, namely *H. halobium*, also produced a secretory serine proteinase. The functional properties, as well as the structural characteristics, of these two enzymes reveal several common traits (Table 2). Their molecular masses and pH-activity profiles are the same, whereas the amino acid composition indicates certain differences in primary structures. Unfortunately, the absence of even limited data on the primary structure of *H. halobium* serine proteinases prevents a more extended comparison of both enzymes. Nevertheless, one can assume that these two proteinases are closely related.

More intriguing appears the comparison of the serine proteinase from *H. mediterranei* with subtilisins, the serine proteinases produced by bacilli (e.g., subtilisins Carlsberg and BPN'), thermoactinomycetes (e.g. 'thermitase' synthesized by

T. vulgaris) and certain fungi (proteinase K). It is well known that serine proteinases form several families of enzymes that are not necessarily evolutionarily related, although their active sites are strikingly similar, an observation which might be explained by convergence. It may be that halobacteria, as well as other archaeobacteria, have also developed their own family of serine proteinases.

The results presented here indicate, however, that the serine proteinase secreted by *H. mediterranei* not only reveals a number of functional traits similar to those of subtilisins (pH-activity profile, substrate-specificity pattern etc.), but also (which is of crucial importance) showed a rather convincing percentage of identical residues (~50%) when its *N*-terminal sequence was aligned with that of *T. vulgaris* serine proteinase. It has to be stressed that, at this level of primary structure analysis, clear-cut sequence identity was found only with *Thermoactinomyces* serine-proteinase sequence. The similarity of the latter sequence with those of subtilisins has been well proven previously. Essentially the same conclusion was arrived at by Kakemura & Seno [4] after sequencing of another halophilic serine proteinase produced by a halophilic archaeobacterium.

This observation, as well as only a marginal similarity between the studied stretch of *Halobacterium* enzyme and subtilisins, has to be explained by the fact that *Thermoactinomyces* enzyme and *H. mediterranei* serine proteinase both have the *N*-terminal extension not present in well-known subtilisins produced by bacilli. The conservation of the Asp residue that belongs to subtilisin active sites implies that the comparison of substantially longer sequences would directly confirm the homology between halobacterial proteinase and the 'typical' subtilisins such as subtilisins BPN' or Carlsberg.

Nevertheless, the data available at the moment are sufficient to conclude that *H. mediterranei* produces a serine proteinase evolutionarily related to eubacterial subtilisins.

It is clear that halobacterial serine proteinases, being adapted to their existence and function in highly concentrated salt solutions, had diverged rather strongly from subtilisins. Thus a

remarkably high proportion of aspartic and glutamic acid residues and/or their amides (Table 4) (their sum corresponds to a quarter of all residues as compared with one-sixth in eubacterial subtilisins) appears to be typical for halobacterial proteins [22]. A comparatively high pI value of 7.5 indicates that the protein contains a rather high proportion of asparagine and glutamine residues.

Presumably strongly hydrated side chains of these residues help to retain hydration water under conditions of high ionic strength, a property of critical importance for the stability of protein molecules under such circumstances. A high content of leucine and glycine residues may also be considered as a specific property of the secretory halophilic proteinase, whereas the presence of half-cysteine residues, usually absent in subtilisins produced by bacilli, is also characteristic for several subtilisin-like enzymes from thermoactinomycetes and fungi.

It seems that the enzyme contains at least one free SH group, like the so-called 'thiol-dependent serine proteinases' [19]. Its activity is markedly suppressed, although not abolished, by treatment with HgCl₂ or *p*-chloromercuribenzoate. It should be taken in account that both these thiol-group-reactive reagents act in the presence of high concentration of Cl⁻ ions, which are capable of forming complexes with Hg²⁺, thus lowering the effective concentration of the inhibitor. Unfortunately, no data on the amino acid composition of the halophilic proteinase from the archaeobacterium strain 172 P1 were presented [4].

The difference in the molecular masses, which are substantially higher in the case of halobacterial serine proteinases (41 kDa instead of 29 kDa typical for eubacterial subtilisins) might indicate the presence of additional loops or even domains within the halobacterial enzyme structure. A molecular mass of 42–46 kDa has been estimated for another halophilic archaeobacterium serine proteinase [4].

It appears that halobacterial and eubacterial serine proteinases are descended from a common precursor. This indicates that the subtilisin family of serine proteinases is an ancient one and its members already existed in a common precursor of eu- and archaeobacteria. It is tempting to presume that subtilisin-like enzymes existed even in the Progenota, a hypothetical group of eukaryotic organisms thought to be precursors of contemporary Eu- and Pro-karyota [23,24]. Indeed, there are indications that subtilisin-like serine proteinases might be present in certain plants [25,26] and even in animals [27].

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