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RESEARCH ARTICLE

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Purification and biological characterization of halocin H1 from *Haloferax mediterranei* M2a

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Abstract The production of halocins, bacteriocin-like proteins of ecological significance, is a frequent characteristic of species from the family Halobacteriaceae. Halocin H1, produced by *Haloferax mediterranei* strain M2a, is a single 31-kDa polypeptide. Its purification was achieved by combining two chromatographic systems: Sepharose 4B linked to bacitracin followed by hydroxylapatite Bio-gel HTP. Halocin H1 required concentrations of NaCl higher than 1.5 M to maintain its activity. Haoarchaeal strains showed a differential degree of sensitivity to the action of this halocin.

Keywords Halocin · Bacteriocin · *Haloferax mediterranei* · Haloarchaea · Halophiles

Introduction

The term bacteriocin is applied to a wide range of biologically active proteins of bacterial origin that exhibit antimicrobial properties against other bacterial species, usually closely related to the producer organism [1]. The production of these proteins has been reported in microorganisms inhabiting a wide variety of ecosystems such as soil [20], marine environments [4], or even the gastrointestinal tract [1]. During the last decade, protein antibiotics have been studied widely due to their potential as preserving agents in the food industry, controlling agents for infectious bacteria, etc. [3].

These antimicrobial proteins have been described not only in Bacteria but also among Euryarchaeota (extreme halophiles) or Crenarchaeota (hyperthermophiles) [14, 17]. Haloarchaea are the dominant heterotrophic population in hypersaline environments with salt concentrations exceeding 250–300 g l^{-1} [12]. The production of antimicrobial proteins, named halocins, is a widely distributed feature among haloarchaea [10 ,23]. Four halocins, produced by different species have been studied [8, 15, 16, 21]. Of these, three have been characterized fully [2, 8, 15, 21] and were found to demonstrate great heterogeneity in size, salt dependence, and spectrum of activity. The mechanism of action of the halocins characterized thus far seems to be related to changes in the bioenergetic steady-state across the membrane [9, 22]; in one case (halocin H6), the specific target has been described [11].

The production of halocin H1 from *Haloferax mediterranei* M2a, previously known as *H. mediterranei* Xia3, was initially described by Rodríguez-Valera et al. [17]. This halocin was found to have a broad inhibitory spectrum among different haloarchaea. This work describes the purification, characterization, and some ecological features of halocin H1.

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Materials and methods

Bacterial and Halobacteriaceae strains

Halocin H1 was obtained from the supernatant of *H. mediterranei* M2a (former Xia3) culture as described by Platas et al. [13]. The Haloarchaea used for the inhibitory tests were: *Haloarcula hispanica* ATCC 33960, *Haloarcula vallismortis* ATCC 29715, *Halobacterium salinarum* NRC 817, CCM 2090 and NRC 34002, *Halococcus morrhuae* CCM 537, *Haloferax gibbonsii* ATCC 33959, *Haloferax volcanii* NCMB 2012. *Haloferax mediterranei* ATCC 33500, M2b (Xia3b), M4 (Xia 10), M6 (Ma 2.31) and M10 (Gla 2.2) were kindly provided by the culture collection of the Department of Producción Vegetal y Microbiología, Universidad Miguel Hernández, Elche, Spain.

Media and growth conditions

All microorganisms were grown in media containing a mixture of marine salts (referred to as SW), described by Rodríguez-Valera et al. [17], in which the variable salt content is expressed as a percentage of its concentration [i.e. SW 25% has 25% (w/v) of salts in the medium]. Halocin H1 was produced as reported in [13]. Haloarchaea were grown in SW 25% medium supplemented with 0.5% (w/v) yeast extract (Oxoid), pH 7, for liquid cultures and with addition of 2% (w/v) agar for solid cultures.

Activity assays

Halocin activity was determined using *Halobacterium salinarum* NRC 817 as the target strain [21]. The degree of inhibition was measured as the inhibition zone diameter. Concentrated samples were titrated to extinction, and activity was reported as arbitrary units (AU) [21].

Salt dependence of halocin H1

The salt dependence of halocin H1 was studied by recursive concentration and replacement with SW solutions at different ionic concentrations, of 1-ml aliquots of a filtered clarified supernatant of a culture (1,280 AU/ml) grown under the conditions reported in [13]. These samples were assayed just after the ionic concentration was changed, and again after 7 days of storage at 4 °C. In a separate experiment, the original ionic concentration of the samples was restored, using identical procedures, and assays were the carried out to observe changes in the degree of inhibition. The samples were concentrated and filtered through a PLGC NMWL 10000 ultrafilter (Millipore) connected to a vacuum system.

Purification of halocin H1

Two hundred milliliters of the clarified supernatant of a culture of *H. mediterranei* M2a grown under the conditions reported in [13] were filtered through a GVWP filter (13-mm diameter; 0.22 μm) to remove the remaining cells. This supernatant was concentrated by vacuum ultrafiltration [PLGC NMWL 10000 CX-10 ultrafilter (Millipore)] and dialyzed against 4.5 M NaCl, 50 mM Tris-HCl (pH 8) buffer. The final volume of the sample was 3.5 ml.

The first purification step was done using a 17×1.5-cm column of CNBr-Sepharose 4B (Pharmacia) linked to bacitracin (Sigma), as described by Stepanov and Rudenskaya [18,19], and equilibrated with buffer 4.5 M NaCl, 50 mM Tris-HCl (pH 8). The elution was carried out in the same buffer at a constant flow rate of 0.4 ml/min, and fractions (2 ml) were collected. Fractions showing the highest antimicrobial activity were pooled (60 ml), concentrated by ultrafiltration until a final volume of 3.5 ml was obtained, and dialyzed against 2 M NaCl, 10 mM potassium phosphate buffer (pH 8).

The second purification step was carried out in a 15×1-cm column of hydroxylapatite Bio-Gel HTP (Bio-Rad) equilibrated with 2 M NaCl, 10 mM potassium phosphate buffer (pH 8). The sample was eluted in the same buffer at a flow rate of 0.3 ml/min, and fractions (2 ml) were collected. The fractions showing the highest biological activity were pooled (40 ml), concentrated to 2 ml by ultrafiltration using the procedure described above, and stored at 4 °C.

Protein concentration was measured using the Bradford method, and the degree of purification of halocin was monitored by SDS-PAGE, using a 12% acrylamide gel and applying a voltage of 80 V for 4 h in a TYR-NR-G41 minigel system (Biometra). Prior to SDS-PAGE, the samples were concentrated and dialyzed against distilled water to avoid the interference produced by high salt concentrations in the electrophoresis. The halocin molecular mass was determined using protein standards of known mass [low-molecular-weight calibration kit (Pharmacia)].

Antimicrobial spectrum of halocin H1

Inhibition assays against haloarchaea were carried out in liquid media. The subject microorganisms were seeded in the presence of increasing concentrations of halocin (Table 3). The optical density (OD) of the cultures at 620 nm was determined after 48 h of incubation (late-exponential phase), and compared with the $OD_{620\ nm}$ of untreated control cultures The results were given as percentage of inhibition determined by (OD_{620nm} of control–OD_{620nm} of treated)/OD_{620nm} of control.

Inhibition of growth among strains of *H. mediterranei* was determined by a cross-sensitivity experiment: stationary cultures of the different strains were spread on agar plates, and 20 μl of a culture to be tested was spotted on the agar surface. The inhibitory capacity was determined by the observation of an antagonistic effect.

Results

Salt dependence of the halocin activity

The environments in which haloarchaea live are hypersaline. The stability of an extracellular protein at different ionic strengths is a variable of ecological interest, and critical for the design of a purification strategy. Halocins already characterized differ in their ionic stability: R1, H6, Ha1 and S8 are salt-independent [15, 16, 21], whereas H4 lost its antimicrobial activity when it was dialyzed below 15% SW [17].

In order to evaluate the salt dependence of halocin H1, aliquots of the supernatant were dialyzed against buffers at various salt concentrations (Table 1). The

Table 1. Titration of the antimicrobial activity of 1,280 AU/ml of halocin H1 after its dialysis against different saline concentrations

Dialysis conditions	Titration (AU) of the antimicrobial activity of the sample after its dialysis	Titration (AU) of the antimicrobial activity of the sample when its initial ionic conditions were restored	Titration (AU) of the antimicrobial activity of the dialyzed sample after being stored at 4 °C for a week
SW 20%	1,280	1,280	1,280
H_2O	640	640	0
SW 1%	640	640	0
SW 2%	640	640	0
SW 3%	640	1,280	160
SW 4%	640	1,280	320
SW 5%	1,280	1,280	1,280
SW 10%	1,280	1,280	1,280
SW 15%	1,280	1,280	1,280

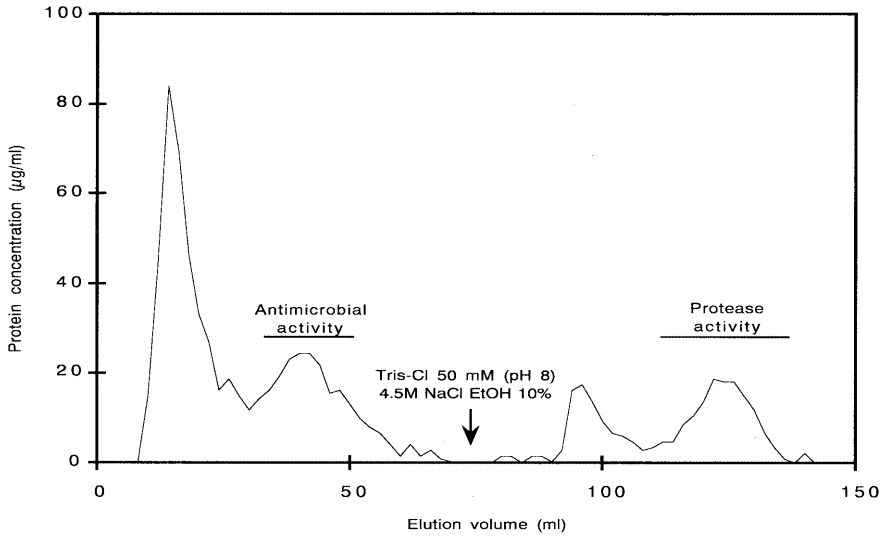

Fig. 1. Elution profile of the chromatography in Sepharose 4B linked to bacitracin of a concentrate supernatant of *Haloferax mediterranei* M2a. Chromatographic conditions are described in Materials and methods

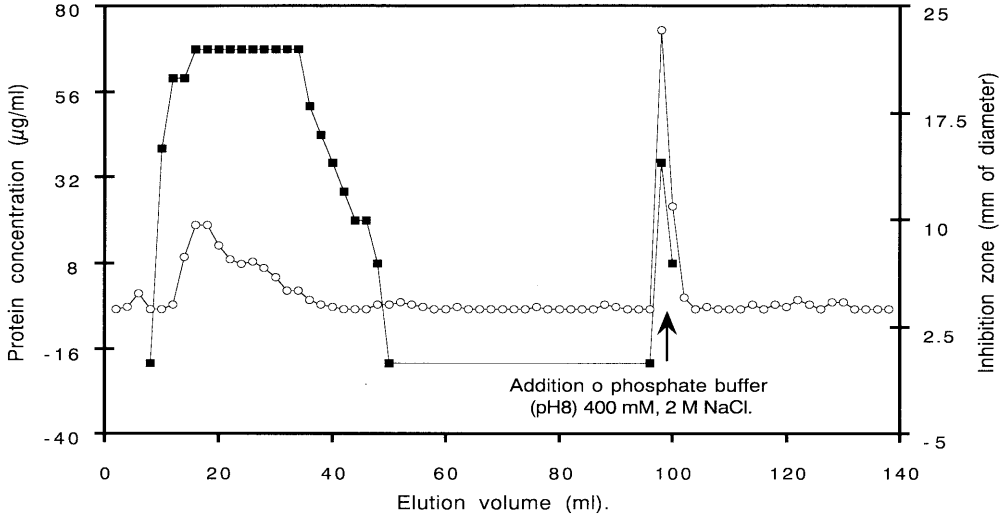

Fig. 2. Elution profile of the chromatography in hydroxylapatite of the active fractions purified in the Sepharose column. ○ Protein concentration, ■ antimicrobial activity against *Halobacterium salinarum* NRC 817. The chromatographic conditions are described in Materials and methods

titration of the antimicrobial activity of the sample was not affected in solutions with ionic concentrations higher than 5% (SW 5%). Dialysis of the supernatant against lower salt concentrations produced an immediate decrease in its activity. This effect was more evident when the samples were stored at lower salt concentrations for longer periods of time. These observations suggested that halocin is inactivated at low ionic concentrations, as has been observed for halocin H4, produced by a different strain of *H. mediterranei*. Activity could not be restored by dialysis against the initial saline conditions in samples that had lost their activity when dialyzed against water and stored for 7 days. This observation suggests that low ionic concentrations irreversibly inactivate halocin H1.

Purification of halocin H1

Due to the lack of stability of halocin H1 at low ionic conditions, most of the classical techniques reported for protein purification would have failed to maintain its inhibitory activity. Only a few procedures are available for the purification of halophilic proteins in the presence of high ionic strength to maintain activity [5, 19].

The first step of the purification of halocin H1 was carried out by affinity chromatography using bacitracin linked to Sepharose 4B. This step removes the extracellular halophilic protease produced by *H. mediterranei* [19] including strain Ma2. The usual methods for protease inactivation, (e.g. the addition of protease inhibitors such as phenyl methyl sulfonyl fluoride), could not be used because *Halobacterium salinarum* NRC 817, the test strain, was also sensitive to these agents (data not shown).

Figure 1 shows the elution profile of a concentrated supernatant of 3.5 ml obtained from chromatography on bacitracin-linked Sepharose 4B. The elution profile shows a protein peak that is retarded by the column and which coincides with the maximum halocin inhibitory activity. Sepharose 4B linked to bacitracin retarded the elution of the halocin, probably due to hydrophobic

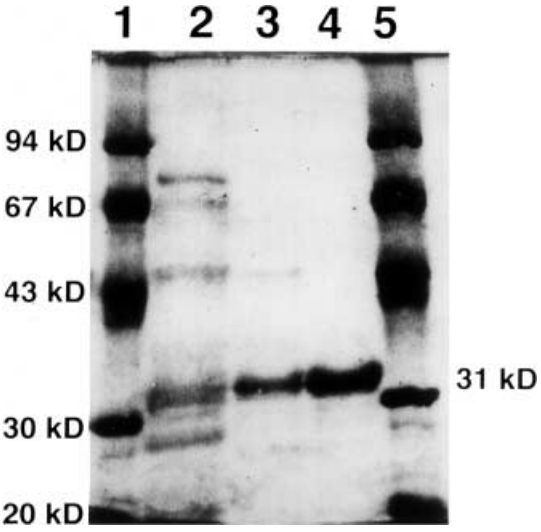

Fig. 3. SDS-PAGE of the pooled fractions obtained in the different purification steps of halocin H1. *Lanes*: *1 and 5* Low-molecular-weight calibration proteins (Pharmacia), *2* culture supernatant, *3* pooled active concentrated fraction after chromatography in Sepharose 4B-bacitracin, *4* pooled fraction containing activity after hydroxylapatite chromatography

interactions. This fact allows the recovery of a protein fraction enriched in halocin activity.

While different strategies were followed for the next purification step, the most successful result was obtained by using chromatography on hydroxylapatite Biogel-HTP column. This chromatographic system initially retains proteins, which are released gradually by increasing the ionic strength of the elution buffer. Figure 2 shows the elution profile obtained for this column. Most of the inhibitory activity was released when the sample was eluted with 2 M NaCl, 10 mM potassium phosphate buffer (pH 8). Fractions showing the highest antimicrobial activity were pooled and concentrated to 3.5 ml as described in Materials and methods.

The degree of purification obtained in the successive steps was monitored by gel electrophoresis. Figure 3 shows that complete purification is achieved after the second chromatography step. The use of hydroxylapatite as the only chromatographic system did not result in the purification of halocin H1 in a single step (data not shown). The low recoveries obtained for the purification of halocin H1 (Table 2) could be explained by the general tendency observed for halophilic proteins to be retained in the membranes used. The molecular mass of the protein that produces the inhibitory activity was 31 kDa by SDS-PAGE.

Antimicrobial spectrum

Another major feature in the characterization of a bacteriocin is its spectrum of activity. An important characteristic of bacteriocins is that they inhibit microorganisms closely related to the producer strain.

Halocin H1 does not inhibit the growth of halotolerant eubacteria (data not shown). Haloarchaeal strains showed a differential degree of sensitivity (Table 3). This result strongly suggests that the range of action of halocin H1 is limited to haloarchaea. Halocin H1 was very active against two of four species of the genus *Haloferax*. The two strains of *Halobacterium salinarum* tested could, in general, be considered sensitive, even though major differences were found in their degree of susceptibility. This could be due to the ability of *H. salinarum* CCM 2090 to form aggregates during growth, which make OD measurements difficult. The two species of the genus *Haloarcula* showed only

Table 2. Purification of the halocin H1

	Volume (ml)	Total protein (μg)	Activity		Purification (fold)	Yield (%)
			Total (AU)	Specific (AU/protein)		
Supernatant	200	7	256,000	36	1	100
Concentrate	3.5	6.29	245,760	37.7	1	91
Sepharose 4B linked to bacitracin	3.5	0.5	81,920	146.2	4	32
Hidroxylapatite Bio-gel HTP	3.5	0.28	40,960	146.2	4	16

Table 3. Sensitivity of different haloarchaea to several concentrations of halocin H1, measured in percentage of inhibition with respect to non treated controls

	Inhibition (%)				
	64 UA/ml	32 UA/ml	16 UA/ml	8 UA/ml	4 UA/ml
Haloferax volcanii NCMB 2012	98	91	84	54	18
Haloferax gibbonsii ATCC 33959	100	100	75	50	9
Haloferax mediterranei M2a	0	0	0	0	0
Haloferax mediterranei ATCC 35500	0	0	0	0	0
Halobacterium salinarum CCM 2090	80	56	4	0	0
Halobacterium salinarum NRC 34002	100	100	97	83	51
Haloarcula hispanica ATCC 33960	59	42	30	20	10
Haloarcula vallismortis ATCC 24745	31	19	10	2	0
Halococcus morrhuae CCM 537	8	15	3	0	10

moderate sensitivity, whereas a strain from the taxonomically distant genus *Halococcus* was insensitive. Other strains of *H. mediterranei* were found to be resistant to halocin H1 using this test.

A cross-sensitivity assay was performed to check whether there were antagonistic effects between strains of *H. mediterranei*. The results showed that the H1 producer, *H. mediterranei* M2a, could inhibit the growth of other strains, while being insensitive to the halocins produced by the rest of the strains tested [17]. The only cases in which this halocin failed to inhibit the growth of other strains are M2b and M4, strains closely related to the producing strain M2a [7] and thus which probably produce the same halocin.

Discussion

The production of halocins is a common feature among halobacteriaceae, although a recent study [6] questioned the ecological, importance of these proteins in the competition between different halobacteria in hypersaline environments.

The aim of this work was to identify various properties of halocin H1 and compare them with the properties of halocins produced by other strains of the same genus. Halocin H1 differs from halocin H4 produced by *H. mediterranei* ATCC 35500 in its molecular mass (31 vs 34.9 kDa) [2], and its producer strain can inhibit the growth of *H. mediterranei* ATCC 35500, whereas the opposite antagonism does not occur. Halocin H1 differs from halocin H6, among other characteristics, in its sensitivity to low ionic strength.

Halocin H1 also shares some characteristics with other proteinaceous ecological agents of prokaryotic origin. For instance, this protein shows some degree of taxonomic specificity, being more effective against organisms classified within the same genus than the producer strain, a feature observed in gram-negative bacteria [3]. Also, other cultures of *H. mediterranei* studied were inhibited by this bacteriocin, with the exception of the producer and related strains, suggesting that these organisms have mechanisms of resistance to the action of the halocin, as has been reported for some bacteriocin-like proteins from gram-positive bacteria. The mechanism of action of this bacteriocin is still unclear, although preliminary experiments have shown that its effects in *H. salinarum* NRC 817, are similar to those observed for halocin H4.

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