Preparative Biochemistry and Biotechnology

Optimization of the Culture Conditions for the Production of a Bacteriocin from Halophilic Archaeon Sech7a

L. Pašić,*, B. H. Velikonja,*, N. P. Ulrih

*Department of Biology, Biotechnical Faculty, University of Ljubljana, Ljubljana, Slovenia
bDepartment of Food Science and Technology, Biotechnical Faculty, University of Ljubljana, Ljubljana, Slovenia

Online Publication Date: 01 July 2008


To link to this article: DOI: 10.1080/10826060802164637
URL: http://dx.doi.org/10.1080/10826060802164637

PLEASE SCROLL DOWN FOR ARTICLE

Full terms and conditions of use: http://www.informaworld.com/terms-and-conditions-of-access.pdf

This article may be used for research, teaching and private study purposes. Any substantial or systematic reproduction, re-distribution, re-selling, loan or sub-licensing, systematic supply or distribution in any form to anyone is expressly forbidden.

The publisher does not give any warranty express or implied or make any representation that the contents will be complete or accurate or up to date. The accuracy of any instructions, formulae and drug doses should be independently verified with primary sources. The publisher shall not be liable for any loss, actions, claims, proceedings, demand or costs or damages whatsoever or howsoever caused arising directly or indirectly in connection with or arising out of the use of this material.
Optimization of the Culture Conditions for the Production of a Bacteriocin from Halophilic Archaeon Sech7a

L. Pašić,1 B. H. Velikonja,1 and N. P. Ulrih2

1Department of Biology, Biotechnical Faculty, University of Ljubljana, Ljubljana, Slovenia
2Department of Food Science and Technology, Biotechnical Faculty, University of Ljubljana, Ljubljana, Slovenia

Abstract: An extremely halophilic haloarchaeon Sech7a, isolated from a solar saltern, was found to excrete halcin, a bacteriocin like substance. Optimal antimicrobial activity was obtained at 45°C using 0.5% (w/v) glycerol and 0.5% (w/v) yeast extract as nutrients in SW media containing 3.4 M NaCl with pH value 7.5. Halcin Sech7a is a 10.7-kDa polypeptide, which is stable in a wide range of pH and is thermolabile at temperatures above 80°C. As many other halophilic proteins, halcin Sech7a loses part of its activity upon exposure to low salt conditions, yet its activity can be restored after dialysis against initial saline conditions. Microscopic inspection revealed swelling and lysis of sensitive cells upon exposure to halcin Sech7a. These results indicate that haloarchaeon Sech7a excretes a novel bacteriocin.

Keywords: Archaea, Bacteriocin, Haloarchaeon Sech7a, Halophile, Halocin, Optimization

Address correspondence to Lejla Pašić, Department of Biology, Biotechnical Faculty, University of Ljubljana, Večna pot 111, 1000 Ljubljana, Slovenia. E-mail: lejla.pasic@bf.uni-lj.si
INTRODUCTION

Halophilic archaea (haloarchaea) of the order *Halobacteriales* are members of the euryarchaea, which thrive in NaCl saturated environments. To cope with high osmotic pressure, halophilic archaea keep a very high concentration of salts internally, thus remaining iso-osmotic with the environment. This contrasts compatible solute accumulation commonly observed in their bacterial and fungal counterparts.[1] To remain soluble and functional in intimate contact with salts, haloarchaeal proteins have a high content of acidic aminoacid residues accompanied by an increase of negative charge on the protein surface.[2] As a result, the majority of such proteins perform their functions *in vitro* and *in vivo* at 4–5 M NaCl, yet lose activity when exposed to low salt concentrations.[3]

To date, several unique characteristics of halophilic archaea were found of considerable biotechnological interest. These include halophilic enzymes able to perform their function at saturated NaCl; bacteriorhodopsin, a light driven proton pump; production of biopolymers, carotenoid pigments, and gas vesicles. It should be kept in mind that the high salt tolerance of extreme halophiles enables their cultivation under non-sterile and, thus, cost reducing conditions.[4]

Haloarchaea were the first members of *Archaea* found to produce bacteriocins.[5] Termed as halocins, these proteinaceous antibiotics act against related species and are universally produced by halophilic archaea.[6] In contrast with their ubiquity, only a handful of halocins has been described in any detail.

According to their size, halocins can be classified into protein halocins and peptide microhalocins. Protein halocins were found to be salt dependant proteins, losing activity rapidly after exposure to low salt conditions.[5] Purification schemes were developed for halocins H4 and H1, produced by *Haloferax mediterranei* strains ATCC33500 and Xia3, respectively,[7,8,12] and for halocin H6 produced by *Haloferax gibonsii* Ma2.39.[9] These proteins were 31–35 kDa in size and induced swallowing and lysis of sensitive cells.[9,10] Onset of halocin activity was observed in the culture supernatans at the beginning of the transition to the stationary phase of growth, except for halocin H1, whose activity was first detected during the mid exponential phase.[9,11] The gene for halocin H4 (*halH4*) was cloned and its expression correlated with halocin activity.[13] Killing of the sensitive cells by halocins H4 and H6 followed ‘single hit’ kinetics.[9,10] Mechanism of action was established for halocin H6, which was found to inhibit the Na\(^{+}/\)H\(^{+}\) exchanger in sensitive haloarchaeal cells.[14] Halocin H7 and H6 activity on the Na\(^{+}/\)H\(^{+}\) exchanger in mammalian cells and its cardio protective efficacy on the
ischemic and reperfused myocardium demonstrated the pharmaceutical potential of halocins.\cite{15,16}

In contrast with salt depended protein halocins, peptide microhalocins are quite robust, as most of them are resistant to changes in salinity and temperature, as well as exposure to acid, base, and organic solvents.\cite{17,18,19} As observed in protein halocins, the microhalocin activity in culture supernatant reaches maximum at the transition to the stationary phase of growth. Up till today, the genes for halocin S8 (\textit{halS8}) and C8 (\textit{halC8}), produced by \textit{Halobacterium} sp. S8 and AS7092, were cloned.\cite{18,20} Gene \textit{halC8} was found to direct the production and immunity of halocin C8 in strain AS7092.\cite{20} Both halocins S8 and C8 are processed from a larger precursor protein and have a wide activity spectrum amongst archaea.\cite{18,20}

To further assess halocin diversity and determine their biotechnological potential, many other halocins need to be studied. Here, we report optimization of the growth conditions for maximum halocin production and properties of partially purified halocin of haloarchaeal strain Sech7a originating from an Adriatic solar saltern.

**EXPERIMENTAL**

**Organism**

Halophilic archaea, isolated from brine samples of Sečovlje solar salterns crystallizers in Slovenia, were screened for bacteriocin like activity against \textit{Halobacterium salinarum} NRC817. The isolate Sech7a, phylogenetically related to \textit{Haloferax mediterranei}, produced the maximum halocin activity. Its 16S rRNA sequence was deposited in GenBank under the accession number AY823953. The archaeon was deposited in the EX culture collection maintained at the University of Ljubljana, Slovenia with accession number EX-B L 194.\cite{21}

**Growth and Media Composition**

Microorganisms were grown in basal media containing the mixture of marine salts (SW) in which the NaCl concentration was 3.4 M.\cite{5} The SW mixture used was supplemented with 0.5% of yeast extract (Difco, ZDA), pH 7.5, and 2% (w/v) agar (Difco, USA) for solid media. The growth was determined by measuring absorbance at 600 nm in a spectrophotometer (Lambda Bio, Perkin Elmer, USA).
Halocin Activity Assay

To test the halocin activity 0.2 mL of the indicator strain culture (Halobacterium salinarum NRC817, OD_{600} = 0.3) was transferred to 20 mL of basal media containing 2% agar, which had been melted and kept at 50°C, then the plates were poured. Next, 0.05 mL culture supernatants were inoculated into lawns aseptically punched wells (0.5 cm in diameter). The plates were incubated at 42°C in sealed plastic bags and the result was considered as being positive when a clear zone of inhibition appeared around the well. Halocin activity of Haloferax mediterranei Sech7a culture supernatants was determined by using serial two-fold critical end point dilutions to extinction. Activity was reported in arbitrary units (AU), which are defined as the reciprocal of the first dilution at which all trace of inhibitory activity disappears.\textsuperscript{13}

Optimization of the Cultivation Conditions

To determine optimal conditions for halocin production, 50 mL of media were taken in 250 mL Erlenmeyer flasks and inoculated with 1 mL of a week old culture of H. mediterranei Sech7a. The effect of temperature, pH, and NaCl concentrations on the growth and halocin production was studied by cultivating the organism in basal medium at different temperatures (20°C–45°C), different initial pH values of the medium (pH 4–9), and different concentrations of NaCl (0.5 M–5.2 M) in an orbital shaker at 100 rpm for 96 h. Halocin activity was measured along the growth curve. The effect of different nutrient sources on halocin production was studied in basal media in which yeast extract was replaced with 0.5% of sucrose, glycerol, gelatin, casein, Na-acetate, glucose, pyruvate, mannose, fructose, lactose, L-arginine, starch, chitin, or peptone. Antimicrobial activity in these studies was determined at 96 h of cultivation.

Halocin Production in Shake Flasks

The media used for halocin production was SW media described above, supplemented with 0.5% of glycerol and yeast extract. The medium, of 500 mL, in 2 L Erlenmeyer flasks, was inoculated with 10 mL of a week old culture of haloarchaeon Sech7a and incubated at 45°C in an orbital shaker at 100 rpm for 96 h. The cell free supernatant was obtained by centrifugation (5,000 rpm, 20 min; 14,000 rpm, 20 min) followed by filtration through 0.22 μm pore size filters (MF\textsuperscript{TM}, Millipore, USA).
Assays of Stability of Halocin Activity

In order to evaluate the influence of different NaCl concentrations and buffer pH on the stability of halocin activity, aliquots of concentrated culture supernatants were subjected to dialysis/buffer exchange in a Microcon centrifugal concentrator with a 3-kDa MWCO filter (Millipore, USA). In order to assess the heat stability, the samples were subjected to heat treatment in temperature range of 20°C–100°C for 10 minutes. The halocin activities before and after each treatment were assayed.

Partial Purification of Halocin

All steps were done at room temperature as follows:

Ultrafiltration

A fresh culture supernatant (12L) was subjected to ultrafiltration in a Labscale TFF System unit (Millipore, USA), using a Pellicon XL Biomax 100 ultrafilter (Millipore, USA) to separate high molecular weight substances. The filtrate was then concentrated (1/100 of the original volume) using Pellicon XL Biomax 5 membrane (Millipore, USA). This concentrated material was designated H100.

Ion Exchange Chromatography

A 20 mL sample of H100 was dialyzed 48 h at 4°C against 0.02 M NaCl in 0.02 M Tris-HCl, pH 8, using 3.5 kDa MWCO SnakeSkin™ dialysis tubing (Pierce, USA). The sample was then applied to a 1 x 5 cm DEAE-Sephacel column (Amersham Bioscences, USA) equilibrated in 0.02 M Tris-HCl, pH 8. The column was washed with 150 mL of the starting buffer and eluted in a stepwise manner by a 0.1 M–1.0 M NaCl gradient in the same buffer (1.5 mL per fraction). The fractions containing high levels of halocin activity were pooled and concentrated to 1 mL using Microcon YM3 concentrator units (Millipore, USA). Protein concentration was measured using BCA™ Protein Assay Kit (Pierce, USA).

Electrophoresis

The degree of purification of halocin was monitored by SDS-PAGE using 12% acrylamide gels and applying a voltage of 200 V in a Mini-Protean II minigel system (Bio-Rad laboratories, Richmond, Ca, USA). The samples were desalted by adding trichloroacetic acid (final
concentration 10%) and incubating 10 minutes on ice. After centrifuging the samples for 10 minutes at 14,000 rpm in a microfuge, the precipitated proteins were washed with cold acetone and air dried. Samples were resuspended in SDS-PAGE loading buffer and heated at 65°C for 10 minutes. The halocin molecular mass was determined by MALDI-TOF mass spectrometry.

**Effect of Haloarchaeon Sech7a Halocin Activity on Cells of* Halobacterium salinarum *NRC817**

Samples of 1.5 mL of fresh cultures of* Halobacterium salinarum *NRC817 at early stationary phase were collected by spinning in an Eppendorf tube, and washed twice with 1 mL of basal salt solution. The cells were incubated, with or without, halocin Sech7a (1024 AU) in the basal SW salt solution and treated for 4, 12, 24, and 48 h at 37°C. Morphological changes were observed with a phase contrast microscope Zetopan Binolux (Reichert, Germany).

**RESULTS**

**Haloarchaeon Sech7a Growth and Halocin Activity at Various Salt Concentrations**

Halocin activity in culture supernatants was observed as the haloarchaeon Sech7, a cell culture entered exponential phase of growth (~30 h), and the activity reached the maximum level at the entrance to the stationary phase (~40 h). Salt concentration in the media affected the time required to reach the stationary phase and to observe maximum halocin activity (Figure 1). Optimal growth and maximum halocin activity was obtained in SW media with 3.4 M NaCl. Media with saturated concentration of NaCl affected cell growth (maximum optical density OD$_{600}$ 2.5) and halocin production, which peaked later in the stationary phase. Cells of haloarchaeon Sech7 a required minimum 1 M NaCl for growth and below for which no growth was observed.

**The Effect of Temperature and pH on Haloarchaeon Sech7a Growth and Halocin Activity**

The effect of temperature on the growth of haloarchaeon Sech7a cells was observed in the temperature range 20°C–45°C. In these experiments,
Figure 1. Haloarchaeon Sech7a cell growth and halocin production at 37°C in media with (a) 2 M NaCl; (b) 3.4 M NaCl and (c) 5.2 M NaCl.
the thermophilic nature of haloarchaeon Sech7a cells became evident as the growth rate and halocin activity peaked at 45°C (Figure 2). Next, the growth rate of haloarchaeon Sech7a cells and halocin production was studied in the media with the broad range of pH 4–9. Media with a range of pH 5.5–8.5 supported the growth of haloarchaeon Sech7a cells with an optimum at pH 8 (Figure 3). However, the maximum halocin activity was observed in the media with slightly lower pH (7.0–7.5).

Figure 2. Haloarchaeon Sech7a cell growth (a) and halocin production (b) as a function of growth temperature.
The Effect of Different Nutrient Sources on Haloarchaeon Sech7a Growth and Halocin Activity

As shown in Table 1, there was a noticeable effect on the growth and antimicrobial activity depending on the nutrient source in the culture media. The growth of haloarchaeon Sech7a cells was not observed when casein, lactose, or L-arginine was used as a sole source of nutrients. Haloarchaeon Sech7a utilized carbohydrates, yet these induced poor growth and halocin production. Among the nutrients used, chitin and gelatin had a significant effect on growth. However, this was not correlated with halocin activity. The highest levels of halocin activity were observed in supernatants of cultures grown either in a media containing glycerol or yeast extract (2130 and 1194 AU OD\textsubscript{600}\textsuperscript{-1}, respectively, Table 1).

Figure 3. Haloarchaeon Sech7a cell growth (a) and maximum halocin activity obtained (b) as a function of culture media pH.
The time required for growing the culture of haloarchaeon Sech7a cells to reach stationary phase was shortened by using a combined glycerol/yeast extract media, while not affecting the halocin activity.

**Biological Properties of Haloarchaeon Sech7a Halocin Activity**

Biological properties of haloarchaeon Sech7a halocin activity are presented in Figure 4. In order to evaluate the salt dependence of haloarchaeon Sech7a halocin, aliquots of supernatant were dialyzed in Microcon YM3 centrifugal concentrators against 0.05 M Tris-HCl, pH 8 buffers at various NaCl concentrations. The antimicrobial activity was unaffected in buffers with ionic concentrations higher than 1 M NaCl. Dialysis of the supernatants against lower salt concentrations produced a decrease in halocin activity. Halocin activity remained detectable up to 0.02 M NaCl and was finally lost upon dialysis against water. However, we were able to restore approximately 40% of initial halocin activity by sample dialysis against the initial saline conditions.

Halocin activity appeared stable in a broad range of pH 2–10, as it remained unaffected by buffer exchange. These results indicate that halocin produced by haloarchaeon Sech7a is pH stable. In order to assess heat stability, the culture supernatants containing halocin activity were heat

---

**Table 1.** Effect of nutrient sources in the culture media on haloarchaeon Sech7a growth and halocin production.

<table>
<thead>
<tr>
<th>Nitrogen and carbon sources 0.5% (w/v)</th>
<th>Biomass (growth OD&lt;sub&gt;600&lt;/sub&gt;)</th>
<th>Halocin activity (AU)</th>
<th>Relative halocin activity (AU OD&lt;sub&gt;600&lt;/sub&gt;&lt;sup&gt;-1&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saccharose</td>
<td>0.62</td>
<td>32</td>
<td>51.6</td>
</tr>
<tr>
<td>Glycerol</td>
<td>0.48</td>
<td>1024</td>
<td>2129</td>
</tr>
<tr>
<td>Gelatin</td>
<td>0.63</td>
<td>256</td>
<td>406</td>
</tr>
<tr>
<td>Casein</td>
<td>0.00</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>Sodium acetate</td>
<td>0.48</td>
<td>32</td>
<td>65.4</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.62</td>
<td>512</td>
<td>821</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>0.43</td>
<td>512</td>
<td>1193</td>
</tr>
<tr>
<td>Manose</td>
<td>0.33</td>
<td>256</td>
<td>771</td>
</tr>
<tr>
<td>Fructose</td>
<td>0.41</td>
<td>256</td>
<td>631</td>
</tr>
<tr>
<td>Lactose</td>
<td>0.00</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>L-arginine</td>
<td>0.00</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>Starch</td>
<td>0.33</td>
<td>32</td>
<td>98.5</td>
</tr>
<tr>
<td>Chitin</td>
<td>1.04</td>
<td>1024</td>
<td>987</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>3.43</td>
<td>4096</td>
<td>1194</td>
</tr>
<tr>
<td>Peptone</td>
<td>0.92</td>
<td>512</td>
<td>557</td>
</tr>
</tbody>
</table>
Figure 4. Effects of NaCl concentration (a), temperature (b) and pH (c) on activity of halocin Sech7a.
treated and periodically sampled. Finally, halocin activity was found stable in temperature range of 20°C–80°C and was rapidly lost upon further heating.

Partial Purification and Molecular Weight Determination of Halocin Sech7a

Figure 5 shows the separation profile of different protein fractions contained in a H100 sample applied to a DEAE-Sephacel column and eluted with increasing NaCl concentration gradient. Crude proteins, concentrated by tangential flow filtration, were separated by DEAE-Sephacel chromatography into distinct peaks with halocin activity eluting in buffer with 0.3 M NaCl. Active fractions were pooled and concentrated. Following SDS-PAGE a single band was observed, indicating that halocin exists as a monomer. The molecular weight of partially purified halocin Sech7a was determined by MALDI-TOF mass spectrometry. The estimated molecular weight was 10765 Da. Molecular weight of haloarchaeon Sech7a halocin estimated by SDS-PAGE was of similar value (Figure 6). However, the SDS-PAGE procedures abolished enzymatic activity of halocin.
Effect of Haloarchaeon Sech7a Halocin Activity on Cells of Halobacterium salinarum NRC817

Morphological changes of the Halobacterium salinarum NRC817 cells that have been exposed to halocin (1024 AU) were monitored by a phase contrast microscope (data not shown). The morphology of untreated Halobacterium salinarum cells remained rod shaped and unchanged throughout the experiment. For the treated cells, there were no significant changes observed in the first 3 hours. Gradually the cells started to swell and showed spherical morphology. After 24 h of treatment the cells were completely lysed.

DISCUSSION

Halocin production by haloarchaeal strain Sech7a was found to be growth dependant. The onset of halocin activity was observed in the early
exponential phase of growth. This contrasts other halocins studied, most of which are first detectable when cultures of the producing cells enter the stationary phase of growth.\cite{17,11} However, the peak of halocin Sech7a activity was observed at the entrance of the stationary phase of growth. The Sech7a halocin activity levels were observed for a long time during the stationary phase. The correlation of this phenomenon with halocin gene expression remains to be elucidated, yet it allowed for an effortless production in shake flasks.

Adaptations of the extremely halophilic haloarchaeon Sech7a to its NaCl saturated environment of a solar saltern crystallizer are well reflected in biological properties of its extracellular bacteriocin. Opposite to the marine waters, the crystallizer brine is not a stable environment as it consists of relatively small water bodies, which can undergo wide oscillations depending on the solar radiation and rainfall. Hence, the halocin remained active in wide NaCl concentration range (0.02 M–5.2 M) with highest production observed in high salt media (3.4 M NaCl). Next, haloarchaeon Sech7a was thermophilic in character with optimal growth occurring at $\geq 45^\circ$C, although the temperature in its native solar saltern crystallizer rarely exceeds $32^\circ$C.\cite{23} This finding is not unexpected as the thermophilic nature of halophilic archaea was demonstrated in a recent study.\cite{24} Consistent with the physicochemical properties of a crystallizer, the optimal growth of the culture was observed at pH 8, yet the halocin production reached maximum at neutral pH.

We have concluded that halocin production is a constitutive property of haloarchaeon Sech7a, as it remained unaffected when haloarchaeon Sech7a cells were cultured aerobically in media with different nutrient sources. The latter, however, did influence the growth rate and halocin activity observed, which was maximal in media with glycerol. This is not surprising, as in solar salterns glycerol produced by blooms of unicellular green alga, *Dunalliela* is considered the most important source of organic carbon for the heterotrophic prokaryotes.\cite{25} In line with previous observation is haloarchaeon Sech7a increased growth rate in media with chitin, produced at lower salinities by brine shrimp *Artemia salina*.

Bacteriocinogenic activity of many bacteria can be induced by the DNA crosslinking effect of UV light exposure or mitomycin C. Furthermore, induction of bacteriocinogenic activity by use of sublethal concentrations of quinolone antibiotics known to inhibit DNA replication and transcription has recently been described.\cite{26} In contrast, halocin synthesis appeared unaffected by DNA crosslinking agents.\cite{8} In addition to these observations, we report inability to induce synthesis of halocins by use of sublethal concentrations of quinolone ciprofloxacin (data not shown) indicating that halocin synthesis is not SOS dependent.

Morphological changes of sensitive cells upon exposure to halocin Sech7a were similar to those observed in previously studied halocins
H4, H6, and C8, and included swelling of the cells and cell lysis\(^{[8,9,19]}\). The primary target of halocin Sech7a might be located in the cell wall or in the cell membrane, yet more data are needed to support this hypothesis. It is worth to mention that hemolytic activity of halocin Sech7a against bovine and humane erythrocytes was not observed and our attempts to develop halocin activity assays based on turbidimetry failed.

Classical gel filtration technique was employed in early attempts to develop the halocin Sech7a purification scheme. High ionic strength of chromatography buffers (3.5 M NaCl) affected protein resolution if cross linked agarose or dextrose beds were used, but not if the column was filled with cross linked alyl-dextrose bed. Further attempts in this direction were omitted due to low protein concentration of the sample and, instead, the purification scheme described above was used. Molecular weight of 10.750 Da of halocin Sech7a was obtained by MALDI-TOF mass spectrometry and by ESI-MS (data not shown).

**CONCLUSIONS**

Biological properties of antibacterial activity in the supernatants of haloarchaeon Sech7a cultures did not correspond to any halocin described so far. Halocin Sech7a is active in wide range of pH and salinity and, therefore, appears more robust than its protein counterparts (e.g. H4). Like microhalocins S8 and C8, halocin Sech7a is thermally stable at temperatures up to 80°C, yet is not resistant to boiling. It therefore appears that extremely halophilic archaeon Sech7a produces a novel halocin differing in molecular size (10.7 kDa) and its biological properties.

**ACKNOWLEDGMENTS**

This work was supported by Ministry for School and Sports of the Republic of Slovenia research programs P1-0198 and J1-6487–0481–04. We are thankful to dr. Bojan Sedmak and dr. Tom Turk for their helpful suggestions during this work.

**REFERENCES**


Received January 22, 2008
Accepted February 18, 2008
Manuscript 7543