INFLUENCE OF TEMPERATURE ON THE GROWTH, PROTEASE PRODUCTION, AND HEAT RESISTANCE OF AEROMONAS HYDROPHILA (HG-1), A. BESTIARUM (HG-2), AND A. SALMONICIDA (HG-3)

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Abstract

The aim of the study was to evaluate the effect of different temperatures on caseinase and elastase production and growth of Aeromonas hydrophila K-101, A. bestiarum 15s, and A. salmonicida A-11 strains, isolated from diseased carp. In order to study the influence of the temperature on proteolytic yield and growth of the strains, standard spectrophotometric methods were used. For the determination of caseinase and elastase activity, the azocasein and elastin-Congo red as substrates were used. It was shown that K-101, 15s, and A-11 strains isolated from motile Aeromonas septicaemia (MAS) affected carp grew better at 28ºC than at 18ºC and 38ºC. The highest proteolytic activity of all studied strains was obtained when the bacteria were grown at 28ºC. At 60ºC the D-value for K-101, 15s, and A-11 were 7, 4, and 3, respectively. In summary, the temperature influenced the growth of the strains isolated from MAS affected carp. The adaptation of these strains to environmental factors imply their possible long survival in the water, which is a potential threat to public and animal health. From these results, it can be concluded that the potential virulent ability of the ECP might vary due to culturing at different incubation temperatures.

Key words: fish, Aeromonas, temperature.

Aeromonas species are ubiquitous inhabitants of the aquatic environment and are also an opportunistic and primary pathogen of fish, predisposing to the infection as a result of stress (4, 30, 31). As part of the normal microbiota, aeromonads usually do not cause disease in healthy fish. Some of the aeromonads produce a number of toxic extracellular products such as haemolysins, cytotoxins, enterotoxins, and different proteolytic enzymes (11, 24, 28). These properties have been suggested to be associated with the virulence of these pathogens (2). Widely distributed in aqueous environments, aeromonads have been isolated from rivers, drinking water, swimming pools, estuaries, and lakes (4, 16). Aeromonas strains could be of public health significance in food products that have an extended shelf-life at refrigeration temperatures (3, 6).

The classification of the genus Aeromonas has been dogged by confusion and controversy. According to Joseph and Carnahan (13), this genus is now classified within the family Aeromonadaceae and consists of 14 different confirmed species. Species Aeromonas hydrophila (HG-1), A. bestiarum (HG-2), and A. salmonicida (HG-3) are included in the so-called “A. hydrophila” complex (12).

The effect of temperature on the growth kinetics of strains of A. hydrophila was evaluated by Knochel (18), Stecchini et al. (37), Santos et al. (32), Sautour et al. (33), and Wang and Gu (39). Many studies are done to assess the influence of different factors on the survival of A. hydrophila (15, 22, 39). Palumbo et al. (26) studied the combined effects of temperature, NaCl, pH, and NaNO2 on the aerobic growth of A. hydrophila. It is well known that the temperature is an important factor controlling the rate of development of microbial populations. A modulation of enzyme synthesis by the growth temperature has been observed in several microorganisms (8, 22). There are no data concerning the effect of temperature, protease yields and growth of bacteria belonging to the different hybridisation groups (HG) of A. hydrophila “complex” species.

The aim of the study was to evaluate the effect of different temperatures on caseinase and elastase yields and growth of A. hydrophila K-101, A. bestiarum 15s and A. salmonicida A-11 strains, isolated from diseased carp.

Material and Methods

Bacteria and growth conditions. A. hydrophila K-101 (HG-1), A. bestiarum 15s (HG-2), and A. salmonicida A-11 (HG-3) strains, isolated from motile aeromonas septicaemia (MAS) affected carp (Cyprinus carpio L.), were kindly provided by Dr.
Kozierska (Department of Fish Diseases, National Veterinary Research Institute, Poland). The bacteria were cultured in tryptic soy agar (TSA). The agar plates were incubated for 24 h at 28°C. For the production of extracellular proteases, the bacteria were grown in tryptic soy broth (TSB) at 28°C for 24 h. The culture from slants was inoculated into 250 ml of TSB in 500 ml Erlenmeyer flasks, and then incubated at three different temperatures (18°C, 28°C, and 38°C). The samples were removed from the incubator at specified time intervals (0, 6, 12, 24, 48, 72 and 96 h) and examined for bacterial growth by determining in the spectrophotometer their optical density at 620 nm.

The samples for the measurement of proteolytic activity were centrifuged for 30 min at 10,000 g at 4°C, filtered through 0.22 µm membrane (Millipore), and frozen at -80°C for later analysis.

**Measurement of proteolytic activity of ECPs.** Protein levels of ECP solutions were determined using the Sigma protein assay kit with bovine albumin as a standard.

**Caseinase activity.** The caseinase activity was determined by the azocasein procedure described by Leung and Stevenson (20), with slight Mateos et al. (22) and own modifications. Briefly, the reaction mixture consisting of 0.1 ml of a 10% (w/v) azocasein solution (Sigma), 0.1 ml of supernatant fluid sample, and 2.3 ml consisting of 0.1 ml of a 10% (w/v) azocasein solution and own modifications. Briefly, the reaction mixture Leung and Stevenson (20), with slight Mateos determined by the azocasein procedure described by

**Elastase activity.** The elastase activity was determined by the elastin-Congo red procedure described by Bjorn et al. (1), with slight Mateos et al. (22) and own modifications. Briefly, 1 ml of culture supernatant fluids was added to 2 ml of Tris-maleate buffer (0.1 mol l⁻¹, pH 7.0) supplemented with CaCl₂ (0.001 mol l⁻¹) containing 10 µg of elastin-Congo red. The mixture was incubated at 28°C for 30 min and the reaction was stopped by the addition of 2 ml of sodium phosphate buffer (0.7 mol l⁻¹, pH 6.0). The precipitate was removed by centrifugation. The blank consisted of 3 ml of the buffer containing 10 µg of elastin-Congo red. Elastase activity was determined by reading absorbance of the supernatant fluid at 495 nm.

**Heat stability.** Heat stability of the bacteria was measured as described by Spinks et al. (35) with own modifications. Briefly, 10 ml portions of the final stationary phase cultures were centrifuged (35,000g, 10 min) at 4°C, and pellets were resuspended in sterile distilled water to give approximate concentrations of 10^9 cells ml⁻¹. The inocula were determined by serial dilutions and plated on TSA. A fixed volume of sterile distilled water was placed into an Erlenmeyer flask held in water bath at the appropriate lethal temperature (55°C, 60°C, and 65°C) prior to inoculation. After temperature stabilisation, 1 ml of resuspended culture was injected into the water medium and timing was immediately initiated. Surviving bacteria were enumerated by serial dilutions, plated on TSA, and then incubated at 28°C for 48 h. The “decimal reduction time” (D-value) was defined as the time required to reduce a bacterial population by 90% or 1 log reduction, and was derived from the formula:

\[
D_x = \frac{(T_x - T_1)/(\log C_1 - \log C_2)}
\]

where \(D_x\) is the D-value in seconds for temperature \(x\), \(T_x\) is the number of elapsed seconds at the final sample point since time zero, \(T_1\) is the number of elapsed seconds at the initial sample point since time zero, \(C_1\) is the concentration of bacteria at \(T_1\), and \(C_2\) is the concentration of bacteria at \(T_2\).

The stability of proteases was measured as described by Khalil and Mansour (15) by subjecting the samples to heat treatment ranging from 30 to 100°C for 15 min. After the heat treatment, the residual proteolytic activity was measured as described above.

**Results**

Three bacterial strains isolated from MAS affected carp for the current investigations were identified as hybridisation groups HG-1, HG-2, and HG-3 (19).

After 96 h cultivation, the optical density, expressing the growth rate at 3 different temperatures, for *A. hydrophila* K-101 (HG-1), *A. bestiarum* 15s (HG-2), and *A. salmonicida* A-11 (HG-3) strains were 3.2, 2.4, 1.6 at 18°C, 4.6, 3.7, 2.8 at 28°C, and 4.1, 2.8, 1.8 at 38°C, respectively (Fig. 1).

The highest proteolytic activity of ECPs from K-101, 15s, and A-11 cultures grown at 18°C, 28°C, and 38°C was obtained when the bacteria were grown at 28°C (Fig. 2). The lowest proteolytic activity was obtained when the strains were grown at 18°C, while the cultures grown at 38°C showed moderate proteolytic activity (Fig. 2).

The heat resistance of the strains was studied at 50°C, 55°C, and 60°C. D-values expressed as the time required to achieve 90% reduction in the concentration of bacteria from three replicate experiments were calculated (Table 1). The reductions in bacterial count were observed at all temperatures used (Fig. 3). The capacity for heat resistance was greatly diminished at 60°C with several log reductions occurring below 30 s. The caseinase activity of K-101 and 15s ECPs was relatively stable when heated for 15 min at 60°C (95% and 72.5%, respectively), although caseinases of A-11 were more labile (30.5% activity). Complete inactivation of the caseinolytic enzymes was observed after heating the ECPs at 100°C, 90°C and 80°C, respectively (Fig. 4). The elastase activity of K-101, 15s, and A-11 ECPs was stable when heated for 15 min at 50°C (98%, 92%, and 93%, respectively). Complete inactivation of the elastolytic enzymes was observed after heating the ECPs at 90°C, 90°C, and 80°C, respectively (Fig. 4).
**Fig. 1.** Effect of incubation time and temperature on *A. hydrophila*, *A. bestiarum*, and *A. salmonicida* growth at 18°C (A), 28°C (B), and 38°C (C).
Fig. 2. Effect of incubation time and temperature on *A. hydrophila*, *A. bestiarum*, and *A. salmonicida* caseinase (A) and elastase (B) activity. A unit of caseinolytic activity was defined as the enzyme activity in a 0.1 ml volume of sample that produced an increase in absorbance of 0.1 at 450 nm. Elastase activity unit is expressed as the activity contained in 1 ml of supernatant fluid that increased the absorbance by 0.1 at 495 nm.
Fig. 3. The reduction of cells following exposure to heat for *A. hydrophila* (A), *A. bestiarum* (B) and *A. salmonicida* (C).
Fig. 4. Heating stability of extracellular caseinase (A) and elastase (B) treated at different temperatures for 15 min.

Table 1

_D-values expressed as the time required to achieve 90% reduction in the concentration of bacteria._

_Means (± standard error) from three replicate experiments_

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Temperature (ºC)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>50</td>
</tr>
<tr>
<td><em>A. hydrophila K-101</em></td>
<td>27 (±2)</td>
</tr>
<tr>
<td><em>A. bestiarum 15s</em></td>
<td>33 (±3)</td>
</tr>
<tr>
<td><em>A. salmonicida A 11</em></td>
<td>10 (±0.8)</td>
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Discussion

Temperature is considered as the major controlling factor in the distribution of the bacteria in natural environment. Temperature dependent seasonal variations have been observed for Aeromonas sp. with the highest population in summer and the lowest one in winter (14). The growth temperature range for aeromonads is from 4 to 44°C, but individual strains typically have a restricted growth range according to their ecological niche, and growth of strains at both extremes of the range are rare (4, 17). Our investigations have shown that A. hydrophila K-101 (HG-1), A. bestiarum 15s (HG-2) and A. salmonicida A 11 (HG-3) strains, isolated from MAS diseased carp, grew better at 28°C than 18°C and 38°C. These results are consistent with the findings of Khalil and Mansour (15), who found that the optimum temperature for A. hydrophila growth in TSB medium was 30°C, but in contrast to our study, at this temperature the bacteria growth reached its maximum after 24 h of incubation time. Palumbo et al. (27) observed the same lag time at 28°C and 37°C with strains, do not grow at 35°C (7, 21). Merino

The capacity for heat resistance of A. hydrophila was greatly diminished at 60°C with several log reduction occurring within 1 min (35). Sheldon and Schuman (34) determined D-values (1.5, 0.10, and 0.03) at 51°C, 57°C, and 60°C, indicating that such thermal processes can provide a large safety factor with regard to the inactivation of A. hydrophila in liquid egg. Isonhood et al. (10) found that A. hydrophila is not heat or freeze/thaw resistant and does not appear to have a measurable phenotypic cross-protective stress response to starvation or cold storage that enhances heat or freeze thaw tolerance. In our study, at 60°C the D-value for K-101, 15s, and A-11 were, 7, 4, and 3, respectively. For all the strains studied, the inactivation curves were linear at 60°C, while survival curves at 50°C and 55°C were characterised by a slower initial phase of inactivation followed by a faster phase.

In summary, the temperature influenced the bacterial growth of three isolates from MAS diseased carp. Adaptations to environmental parameters by these strains imply their possible long survival in water, which is a potential threat to public and animal health. From these results, it can be concluded that the potential virulent ability of the ECP might vary due to cultivating at different incubation temperatures.

References