THE EFFECT OF Na^+/Mg^{2+} RATIO ON THE AMYLASE ACTIVITY OF HALOARCHAEA ISOLATED FROM TECHIRGHIOL LAKE, ROMANIA, A LOW SALT ENVIRONMENT

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A relatively large number of haloarchaea was isolated from a Techirghiol Lake, located in the proximity of the Black Sea coast, in Romania. Although this lake is characterized by salinity not higher than 60 g per liter, it harbors haloarchaea belonging to *Haloferax* and *Halorubrum* genera. The isolated strains were able to hydrolyze starch, a process that appeared to be influenced by Na^+/Mg^{2+} ratio. The increasing NaCl concentration conducted to decreasing or lost of amylase activity. The different requirements for Mg^{2+} for growth revealed the effect of this compound on the metabolism of halophilic archaea, and in particular on the process of starch hydrolysis. The registered data revealed that increasing salinity decreased the starch hydrolysis by the haloarchaeal strains isolated from salt lakes containing low salt concentrations.

Key words: Halophiles; Archaea; Amylase; Salt lakes; Ionic strength.

INTRODUCTION

A previous report[1] indicated that halophilic microorganisms were able to hydrolyze starch, which represents an important energy source for microorganisms. Starch is composed exclusively of D-glucose units that are linked by α-1,4- or α-1,6-glycosidic bonds. The two forms of starch are amylose (15–25%), a linear polymer consisting of α-1,4-linked glucopyranose residues, and amylopectin (75–85%), a branched polymer containing, in addition to α-1,4 glycosidic linkages, α-1,6-linked branching points occurring every 17–26 glucose units. Because of the complex structure of starch, cells require an appropriate combination of intracellular and extracellular enzymes for its conversion to oligosaccharides and smaller size sugars, such as glucose and maltose. Many starch-degrading proteins have been identified in various organisms[2], but relatively few studies focused on archaeal α-amylases. The evolutionary relatedness of this enzyme from archaea and plants has been also reviewed[2]. Recently, the haloarchaeal α-amylase from *Haloferax mediterranei* was purified and characterized[3]. The functional properties of this enzyme are similar to those reported for the amylases from other haloarchaea, *Halobacterium salinarum*[4,5], and *Natronococcus amylolyticus*[6], and the moderately halophilic bacterium *Halomonas meridiana*[7]. It is known that enzymes of both halophilic bacteria and archaea are active under high salt concentrations, and most of them are inactive in the absence of salt. In the case of *Haloarcula* sp. S-1, α-amylase can also tolerate relatively high concentrations of organic solvents present in high polluting environments[8].

A great deal of information on eukaryotic and bacterial amylases is currently available, therefore studies on archaeal amylases may contribute to elucidating properties of extracellular archaeal enzymes. The present study highlighted the effect of ionic strength on the amylase activity due to

variable concentrations between monovalent and divalent cations. These variable ions concentrations could be found in natural environments as a consequence of change in different ecological parameters of the environment.

Techirghiol Lake, located near the Black Sea coast, Romania, is characterized by variable concentrations of salt, ranging from negligible salinity to 60 g/L[9]. The lake is well known from biological community and saline regime and has been well investigated over time either from importance of therapeutic properties of the mud presented in the lake[10] or dynamic of moderately halophilic microbial community[11]. The present study revealed the effect of Na^+/Mg^{2+} ratio on the activity of the extracellular amylase from halophilic archaea isolated from Techirghiol Lake. According to our best knowledge, this is the first report on the influence of Na^+/Mg^{2+} ratio on the amylase activity of some archaeal strains isolated from such natural habitats.

MATERIALS AND METHODS

Cultures and media – Ten strains of extremely halophilic *Archaea* isolated from Techirghiol Lake were investigated. The strains were isolated and cultivated on the medium containing per liter 5 g K_2SO_4, 0.1 g $CaCl_2x2H_2O$, 1 g peptone, 2 g soluble starch, and 1 g yeast extract. The pH of medium was 7.0 – 7.2 before autoclaving. The medium contained 2.1 M and 3.4 M NaCl and various concentrations of $MgCl_2x6H_2O$ as follows (g/L): 0 (V1), 32 (V2), 64 (V3), 96 (V4), 128 (V5), 160 (V6) and 192 (V7). The strains were cultivated at 32^0C, in the absence of light, with moderate agitation at 250 rpm, in 100 ml medium containing 10 ml inoculum.

The membrane lipid profile and biochemical tests of selected strains were analyzed according to the methods previously described[12]. In order to determine if the isolates were halophilic archaea, they were streaked on the solidified medium containing 0.25 g/L taurocholic acid sodium salt, or 20 mg/L chloramphenicol. It was previously shown[13] that halophilic archaeal cells lyse or do not grow in the presence of bile acids. The cultures were incubated for ten days at 37^0C, and strains that grew on the plates containing chloramphenicol but not on the plates with the bile acid, were considered halophilic archaea. Isolation and identification of membrane lipids by thin layer chromatography were performed using the method described by Kamekura[14]. Briefly, cells from 100 ml of cultures were harvested, mixed with water and chloroform-methanol (1/2 v/v). After centrifugation at 10 000 rpm, the supernatant was separated and the step was repeated. The lower phase resulted from the centrifugation at 10 000 rpm was isolated, dried and dissolved in the chloroform-methanol solvent mixture.

The amylase activity was measured as follow: 0.5 ml of culture supernatant were mixed with 3.5 ml of soluble starch solution (1% in 0.7 mM phosphate buffer, pH 6.1) and incubated at 37^0C for 15 minutes. After addition of 1 ml of 1N HCl and 5 ml water, samples of 0.25 ml of this mixture were added to 10 ml of 33×10^{-5} M I-KI. The optical density was measured spectrophotometrically at 595 nm, after 15 minutes of incubation at dark. One unit of the amylase activity was defined as the amount that hydrolyzed one gram of starch in one hour at 37^0C in one mL of bacterial culture.

The effect of pH of media on the amylase activity was investigated in the above medium containing 160g/L $MgCl_2\times6H_2O$ and 2.1 M NaCl.

RESULTS AND DISCUSSIONS

All tested strains were able to grow on medium supplemented with chloramphenicol, but showed no growth in the presence of sodium taurocholate. The strains showed growth on medium containing NaCl, ranging from 1 M to 5.2 M, with an optimum growth at 2.5–3.5 M NaCl. These results, together with the membrane lipids profile and biochemical tests of some investigated strains (Table 1), suggested that the microorganisms isolated from Techirghiol Lake were halophilic archaea and some of them belonged to *Haloferax* and *Halorubrum* genera.

Table 1

Lipid and biochemical profile of some investigated haloarchaeal strains

	5/3 95	2/3 95	1/2 95
PGP-Me	+	-	+
PG	+	+	+
PGS	-	-	+
S-DGA-1	+	+	-
DGA-1	+	+	-
Other glycolipid	-	-	+
Lysis in distil water	+	+	+
Chloramphenicol	+	+	+
Na taurocholate	+	+	+
NaCl range for growth (M)	1-5.2	1-5.2	1-5.2
Genera	*Haloferax*	*Haloferax*	*Halorubrum*

PGP-Me = phosphatidyl glycerophosphate methyl ester; PG = phosphatidyl glycerol; PGS = phosphatidyl glycerol sulphate; S-DGA-1 = sulphated diglycosyl archaeol; DGA-1 = diglycosyl archaeol.

The strains showed optimum growth with $MgCl_2$ varying between 64 and 160 g per litter on the medium with 2.1M NaCl (Table 2). Two strains showed optimum growth on the medium containing 64 g/L $MgCl_2$. Other five strains grew optimally in the presence of 96 g/L $MgCl_2$, one at 128 g/L $MgCl_2$ and two at 160 g/L $MgCl_2$. When the NaCl concentration was increased to 3.4 M, the optimum growth of the strains occurred at lower Mg concentrations: six strains in the absence of $MgCl_2$, two in the presence of 32 g/L $MgCl_2$, one at 64 g/L $MgCl_2$, and one at 96 g/L $MgCl_2$ (Table 2).

Table 2

The influence of $MgCl_2$ concentration on the growth of the haloarchaeal strains tested in the presence of 2.1 M or 3.4 M NaCl.

Strain	2.1 M NaCl							3.4 M NaCl						
	V1	V2	V3	V4	V5	V6	V7	V1	V2	V3	V4	V5	V6	V7
1/2 95	2.40	2.49	2.43	2.61	2.70	2.76	2.67	2.94	2.94	2.82	2.73	2.67	2.70	1.80
2/3 95	2.43	2.28	2.34	2.58	2.52	2.67	2.64	2.76	2.85	2.70	3.06	2.06	1.82	1.78
3/1 95	2.02	2.10	2.16	2.13	2.22	1.95	1.89	2.61	2.34	2.22	2.10	1.62	1.04	1.62
3/4 95	3.64	3.88	4.00	4.04	3.15	2.61	2.34	2.85	2.88	2.88	2.67	1.98	1.94	1.82
3/8 95	3.48	3.72	4.45	4.60	3.72	3.92	3.92	3.06	3.42	3.48	3.12	2.40	1.82	1.64
5/3 95	2.07	2.31	2.34	1.92	1.92	1.77	1.74	1.96	1.80	1.78	1.76	1.70	1.60	1.36
3/1 96	0.32	0.90	0.94	0.97	0.90	0.86	0.80	0.95	0.94	0.89	0.88	0.82	0.78	0.68
3/2 96	0.70	1.40	1.52	1.54	1.24	1.22	1.32	1.68	1.60	1.72	1.64	1.66	1.34	1.16
2/1 97	2.26	2.37	2.40	1.74	2.13	1.98	1.95	1.88	1.92	1.88	1.84	1.76	1.72	1.64
2/5 97	2.14	2.16	2.28	2.31	2.31	2.22	2.22	1.94	1.90	1.88	1.90	1.92	1.80	1.76

V1-V7 represents various concentrations of $MgCl_2 \times 6H_2O$ (g/L): 0 (V1), 32 (V2), 64 (V3), 96 (V4), 128 (V5), 160 (V6), 192 (V7). The growth is represented by optical density at 660 nm.

Table 3

The effect of pH values on amylase activity

pH / Strains	7.0		8.0		8.5	
	G	A	G	A	G	A
1/2 95	2.46	22	1.65	0	1.71	0
2/3 95	2.22	22	1.65	0	1.74	0
3/1 95	2.28	9.2	1.86	0	1.80	0
3/4 95	2.04	0	1.95	0	1.86	0
3/8 95	3.74	12.2	3.04	11.8	3.00	5.0
5/3 95	1.74	17.1	1.56	0	1.53	0
3/1 96	1.18	0	0.87	0	1.00	0
3/2 96	0.84	3.4	0.78	0	0.40	0
2/1 97	3.68	14	3.52	6.0	3.48	2.4
2/5 97	2.46	16.3	1.38	0	1.29	0

The growth (G) in medium containing 160g/L $MgCl_2 \times 6H_2O$ and 2.1 M NaCl is represented by optical density at 660 nm. The amylase activity (A) is noted as units. One unit is considering as one gram of starch hydrolyzed in one hour by one ml of bacterial culture.

Although the presence of members of *Haloferax* and *Halorubrum* genera in environments containing low concentrations of NaCl was previously reported[15], there is no information about the ability of the strains to hydrolyze the starch or other sugar based polymer. To our best knowledge, this paper reports for the first time the effect of Na^+/Mg^{2+} ratio on the amylase activity of the halophilic archaea isolated from environments of low NaCl content.

The amylase activity of these strains was dependant on the NaCl and $MgCl_2$ concentrations. In the presence of 2.1 M NaCl, the amylase activity was detected for the majority of the strains (Fig. 1), except 3/4 95. In the case of V7 experimental conditions (192 g per litter of Mg^{2+}), there was no amylase activity detected, and in the case of V1 conditions (absence of Mg^{2+}), the amylase activity was present only for strain 2/3 95. Strains 1/2 95 and 2/1 97 did no produce amylase activity under V2 conditions, and strain 3/1 96 did not under V5 and V6 conditions either (Fig. 1). Under 3.4M NaCl, the amylase was not produced in six strains at all, and activities in the remaining four strains were generally lower (an exception was at V2 of 5/3 95) compared to those in the presence of 2.1M NaCl (Fig. 2). These results confirmed that the amylase activity of the extremely halophilic archaea isolated from Techirghiol Lake was influenced by the NaCl concentration present in the growth medium. The requirements for Mg^{2+} varied as a function of NaCl concentrations. In the absence of Mg^{2+} the amylase was not active in most of the strains. The minimum requirement for Mg^{2+} was 32 grams per liter. However, in the case of 3/2 96 strain, at 2.1 M NaCl, increased concentration of this cation resulted in inhibition of the amylase activity.

The different requirements for Mg^{2+} revealed the effect of this compound on the metabolism of halophilic archaea, in particular on starch hydrolysis. For all the studied strains isolated from salt lakes of low salt concentrations, increasing salinity determined a decrease of starch hydrolysis. In the presence of a relatively low NaCl concentration (2.1 M), increasing concentrations of $MgCl_2$ determined an increase of the amylase activity (Fig. 1) for most of the strains. On the contrary, at a higher NaCl (3.4 M), increasing concentrations of $MgCl_2$ decreased the amylase activity (Fig. 2), with an exception of 3/1 96.

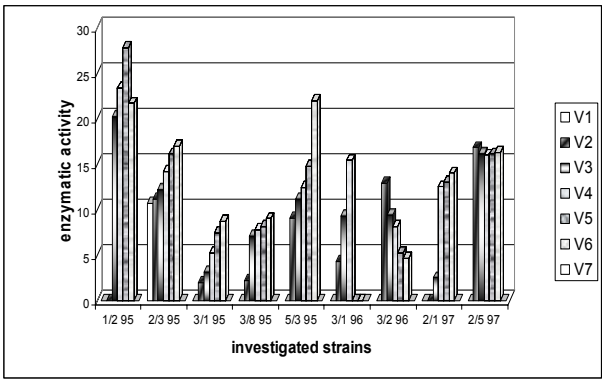

Fig. 1. The effect of Na^+/Mg^{2+} ratio on the amylase activity of haloarchaea in the presence of 2.1 M NaCl. V1 to V7 represents various concentrations of $MgCl_2 \times 6H_2O$ as follows (g/l): 0 (V1), 32 (V2), 64 (V3), 96 (V4), 128 (V5), 160 (V6) and 192 (V7). The enzymatic activity was noted as units and is considering as one gram of starch hydrolyzed in one hour by one ml of bacterial culture.

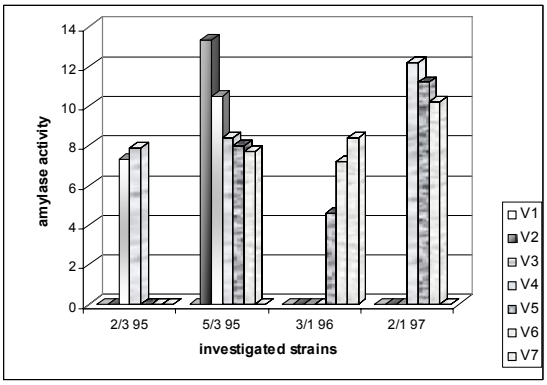

Fig. 2. The effect of Na^+/Mg^{2+} ratio on the amylase activity of haloarchaea in the presence of 3.4 M NaCl.

These data indicate that low concentrations of NaCl and high concentrations of $MgCl_2$ are required for the optimum amylase activity, revealing the requirement for divalent ions of amylase activity of these haloarchaea. Most probably, these ions contribute to binding the appropriate number of water molecules to the protein surface, for maintaining the active folding of the amylase under various ionic strength conditions.

The amylase activity was reduced or, in most cases, was abolished in media of pH higher than 7.0. Two strains presented amylase activities even at pH 8.0 and 8.5, but they were reduced to around half as compared to that at neutral pH (Table 3). These results indicate that the amylase activity of these archaea requires neutral or slightly acid pH values. Since pHs higher than 8.0 caused the precipitation of Mg^{2+}, the lose of amylase activity could be associated also with the absence of this cation in the medium. This is in accordance with the requirement of high concentrations of Mg^{2+} for the growth of all investigated strains, and in accordance with the hypothesis that divalent ions contribute to optimum conformation of amylases for preserving their active form in the case of haloarchaea thriving in moderate halophilic environments. The presence of amylase activity at pH higher than 8, as revealed in the case of 2/1 97 and 3/8 95 strains, could represent an advantage for biotechnological processes involving starch hydrolysis at moderately alkaline pH.

Archaea have developed a variety of molecular strategies to survive the often harsh environments in which they exist. However, relatively few haloarchaeal extremozymes are currently used in biotechnologies, due to the limited demand for salt-tolerant enzymes in current manufacturing or related processes, or limited knowledge about enzyme function and stability under high ionic strength, in the presence of salts mixtures. In this respect, the present study revealed the influence of various concentrations of sodium and magnesium ions, and their combined effect, on the activity of extracellular amylase of moderate halophilic archaea isolated from Techirghiol Lake.

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