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ORIGINAL PAPER

Effect of organic solvents on the activity and stability of an extracellular protease secreted by the haloalkaliphilic archaeon *Natrialba magadii*

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Abstract The effect of various organic solvents on the activity and stability of an extracellular protease produced by the haloalkaliphilic archaeon *Natrialba magadii* was tested. This protease was active and stable in aqueous-organic solvent mixtures containing 1.5 M NaCl and glycerol, dimethylsulfoxide (DMSO), *N,N*-dimethyl formamide, propylenglycol, and dioxane. Among the solvents tested, DMSO, propylenglycol, and glycerol were effective in preserving enzyme stability in suboptimal NaCl concentrations. The stabilizing effect of DMSO on this haloalkaliphilic protease was more efficient at pH 8 than at pH 10, suggesting that DMSO may not substitute for salt to allow halophilic proteins to withstand the effect of high pH values. These results show that *Nab. magadii* extracellular protease is a solvent tolerant enzyme and suggest a potential application of this haloalkaliphilic protease in aqueous-organic solvent biocatalysis.

Keywords Protease stability · Organic solvent · Haloarchaea · *Natrialba magadii*

Introduction

Haloarchaea grow in hypersaline environments (>2.5 M NaCl) in either neutral or alkaline pH values. As a result of adaptation to high salt conditions, halophilic enzymes require high salt concentrations for activity and stability and undergo denaturation at NaCl/KCl concentrations below 1 M. Since salt reduces water activity, a feature in common with organic solvent systems, haloarchaeal enzymes may be valuable tools as biocatalysts in aqueous-organic media. There are several advantages that apply to biocatalysis in organic media: higher solubility of hydrophobic species, reduced microbial contamination, and reduced water activity which alters the hydrolytic equilibrium [10]. This condition is often used for peptide synthesis using proteases as catalysts. However, one disadvantage of using organic solvents in biocatalysis is that enzymes are easily inactivated. As halophilic extremozymes are best suited to function under harsh conditions, they offer the possibility of extending the repertoire of biocatalysts already available. Several haloarchaeal strains can grow in the presence of organic solvents [12], meaning that their enzymes are tolerant to these compounds. So far, studies on the behavior of haloarchaeal enzymes in organic solvents are limited to an extracellular protease from *Halobacterium salinarum* [5, 9], the alkaline *p*-nitrophenylphosphate phosphatase and malate dehydrogenase from *Halobacterium halobium* encapsulated in reverse micelles [7] and an α-amylase from *Haloarcula* sp. strain S-1 [3].

Proteases play a fundamental role in any cell and have numerous applications in biotechnology and industry [8]. The proteolytic enzymes from Archaea have been studied comparatively less than those from the domains Bacteria and Eukarya. Therefore, due to their ability to tolerate conditions which inactivate their mesophilic counterparts, their study is of interest for both basic and applied research. Extracellular

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proteases have been purified and characterized at the biochemical and molecular levels from haloarchaea [1]. Scarce information is available on haloalkaliphilic archaea (optimum growth in 4–5 M NaCl, pH 8.5–12) and a few enzymes, including proteases, have been characterized from this group [1, 2, 6]. The haloalkaliphilic archaeon *Natrialba magadii* secretes a halolysin-like extracellular protease at the end of the exponential growth, denoted as NEP for *Nab. magadii* extracellular protease. This protease was purified and biochemically characterized [4] and the corresponding gene was recently cloned and sequenced. NEP has optimal activity in aqueous buffer containing 1.5 M NaCl, pH 8 at 45°C and is irreversibly inactivated at lower salt concentrations [4]. In order to evaluate the potential application of NEP in biotechnology, the effect of organic solvents on the activity and stability of this haloalkaliphilic protease was assessed.

Materials and methods

Microorganism and culture conditions

Natrialba magadii ATCC 43099 was grown aerobically to the stationary growth phase in the medium for haloalkaliphilic archaea [11] and the cell free culture medium was precipitated with 50% (v/v) cold ethanol as previously described [4].

Determination of protease activity

Protease activity was measured in a reaction mix (0.5 ml) containing 50 mM Tris–HCl (pH 8), 1.5 or 0.5 M NaCl, 0.5% (w/v) azocasein (Sigma) with or without organic solvent (15 and 30%, v/v) and 10–100 µl enzyme solution at 45°C, withdrawing samples at various time periods. The reaction was terminated by addition of one volume of cold 10% (v/v) trichloro acetic acid (TCA) and incubation on ice for 15 min. The samples were centrifuged at 3,000*g* for 15 min and then, acid soluble products were detected by measuring the absorbance at 335 nm (A_{335}). The amount of product (A_{335}) accumulated in 30 min was used as an estimation of azocaseinolytic activity.

Determination of protease stability

Natrialba magadii extracellular protease was preincubated at 30°C for 24 h in solutions containing 50 mM Tris–HCl, 1.5 or 0.5 M NaCl in the absence or presence of organic solvent (15 and 30%, v/v). The residual activity was measured under standard conditions (1.5 M NaCl, final solvent concentration below 6%, v/v) and expressed as the product (A_{335}) accumulated in 1 h.

Results and discussion

Activity and stability of NEP in aqueous-organic solvent mixtures

A partially purified fraction enriched in NEP protease was used as the source of enzyme, as identical biochemical properties had been determined for this fraction and the purified protease [4]. All determinations were performed in duplicates.

Under optimum salt concentrations (1.5 M NaCl) NEP activity was >75% in the presence of 15% (v/v) of high polarity solvents such as glycerol, dimethylsulfoxide (DMSO) and *N,N*-dimethyl formamide (DMF) and it was partially active in propylenglycol and dioxane (Table 1). Higher concentrations of organic solvent (30%, v/v) further decreased the azocaseinolytic activity. On the other hand, because of denaturation in aqueous solutions containing a suboptimal NaCl concentration (0.5 M), NEP activity decreased to 26% relative to the control (1.5 M NaCl). Under this condition, DMSO (and glycerol to a lesser extent) exerted a protective effect on NEP activity, which was more pronounced at 30% (v/v) concentration (activity >70%, Table 1). The low activity values determined in the presence of several organic solvents may be assigned to a negative effect of the solvent on the enzyme itself or on the substrate, as organic solvents may influence the conformation of azocasein, hence, affecting its hydrolysis by proteases. This may be the case of propylenglycol, which was very effective in maintaining NEP stability under optimal and suboptimal salt concentrations; however, it showed a negative effect on the enzyme activity (Table 1). To further examine the effect of organic solvents, NEP activity was measured in solutions containing 30% (v/v) DMSO with decreasing NaCl concentrations (1.5–0.15 M). NEP activity was undetectable in 0.3 M NaCl but 30% activity relative to the control (1.5 M NaCl) was measured in the presence of DMSO (data not shown). The effect DMSO on NEP protease was noted in the time course of azocasein hydrolysis. In aqueous buffer with 1.5 M NaCl, substrate hydrolysis was linear for at least 45 min while in 0.5 M NaCl the enzyme was rapidly inactivated. However, the reaction proceeded for at least 45 min in the presence of DMSO (data not shown).

The effect of organic solvents on NEP stability was analyzed by pre-incubation at 30°C for 24 h in aqueous-organic solvent mixtures containing 1.5 M or 0.5 M

Table 1 *Natrialba magadii* extracellular protease (NEP) activity and stability in aqueous/organic solvent mixtures

Solvent (15%, v/v)	log $P^{a}_{o/w}$	Activity (%)[b]		Residual activity (%)[c]	
		1.5 M NaCl	0.5 M NaCl	1.5 M NaCl	0.5 M NaCl
None		100	26	100	0
Glycerol	−1.76	76	33	107	14
Dimethylsulfoxide (DMSO)	−1.35	83	50	99	48
N,N-dimethyl formamide (DMF)	−1.0	76	5	73	0
Propylenglycol	−0.49	44	26	130	14
Dioxane	−0.27	30	5	93	0
Acetone	−0.24	22	1	59	0
Acetonitrile	−0.34	8	0	2	0
Ethanol	−0.31	6	1	52	0
2-Propanol	0.05	2	0	23	0
Solvent (30%, v/v)					
Glycerol	−1.76	54	38	108	89
DMSO	−1.35	48	72	111	56
DMF	−1.0	22	2	57	0
Propylenglycol	−0.49	20	13	102	55

[a] log $P_{o/w}$ is the logarithm of the partition coefficient, P, of the solvent between *n*-octanol and water and is used as a quantitative measure of the solvent polarity (http://www.syrres.com/eSc/est_kowdemo.htm)

[b] Protease activity was measured in 50 mM Tris–HCl (pH 8), 1.5 or 0.5 M NaCl and 0.5% (w/v) azocasein with or without organic solvent at 45°C for 30 min. Protease activity was expressed as the product (A_{335}) produced in 30 min of reaction

[c] *Natrialba magadii* extracellular protease was preincubated in the absence or presence of organic solvent at 30°C for 24 h in solutions containing 1.5 or 0.5 M NaCl. The residual activity was measured in standard conditions (1.5 M NaCl, final solvent concentration below 6%, v/v) and expressed as the product accumulated in 1 h. The percentage of remaining activity relative to the non-solvent, 1.5 M NaCl control is shown

NaCl. In high salt (1.5 M NaCl) NEP was stable in the presence of various kinds of organic solvents (>50% residual activity) except acetonitrile and 2-propanol. Particularly, NEP was very stable in glycerol, DMSO and propylenglycol, retaining >100% residual activity in 15 and 30% (v/v) organic solvent. In addition, these solvents were able to sustain high levels of protease activity when the enzyme was pre-incubated in suboptimal NaCl concentrations (0.5 M), DMSO being effective at a lower concentration (Table 1). The effect of these solvents on NEP activity and stability was also tested with a highly purified protease sample, which behaved similarly to the partially purified enzyme (data not shown).

Activity and stability of NEP in the presence of DMSO at different salt concentrations and pH values

Since NEP is a haloalkaliphilic protease, the effect of DMSO on enzyme stability was examined at various salt concentrations and at two pH values (pH 8 and 10). The enzyme was pre-incubated in solutions containing 30% (v/v) DMSO and decreasing NaCl concentrations at 30°C for different time periods. The residual activity was measured as indicated above in duplicate samples. Stability was expressed as the residual activity relative to the non-solvent containing 1.5 M NaCl control without pre-incubation. NEP was very stable in high salt buffers of pH 8–10 with or without DMSO (~80% residual activity after 7 days at 30°C). However, its stability was greatly improved by DMSO in solutions containing suboptimal concentrations of NaCl. The residual activity was 60% in 0.15 M NaCl and 50% in 0.5 M NaCl after 2 h and 7 days of incubation at pH 8, respectively (Fig. 1). Subtilisin Carlsberg was similarly stable in the presence of 30% (v/v) DMSO (90% residual activity after 7 days at 30°C, not shown). As expected for an alkaliphilic enzyme, NEP was slightly more stable at pH 10 in high salt. However, the protective/stabilizing effect of DMSO in diluted buffers was lower at pH 10 than at pH 8. This observation suggests that, in order to maintain activity at very alkaline pH values, haloalkaliphilic enzymes may need to preserve the structure gained in high salt media. DMSO may not completely substitute for salt for native folding of halophilic proteins and withstand the effect of high pH values.

Altogether, these results show that NEP protease is an organic solvent tolerant enzyme and that it is stable and active in aqueous solutions containing various kinds of organic solvents in the presence of high salt (1.5 M NaCl). In addition, this protease remains stable when salt is partially replaced by DMSO, propylenglycol or glycerol. This observation is of interest for the use of NEP protease in processes that are not compatible with high salt concentrations. A stabilizing effect of DMSO on the enzyme's structure in low salt media was previously reported for an extracellular protease produced

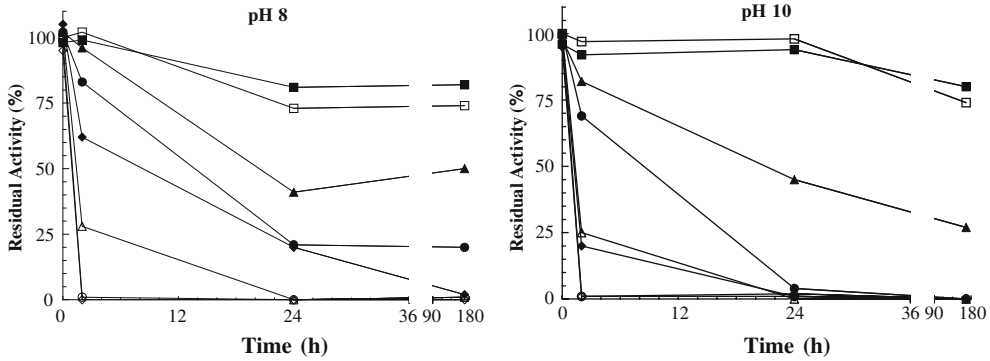

Fig. 1 Time course of residual *Natrialba magadii* extracellular protease (NEP) activity in the presence of dimethylsulfoxide (DMSO) at different salt concentrations and pH values. NEP was pre-incubated in solutions containing 30% (v/v) DMSO and various concentration of NaCl at 30°C for different time periods. The residual azocaseinolytic activity was measured under standard conditions (1.5 M NaCl, pH 8, 45°C). Stability was expressed as the residual activity relative to the non-solvent 1.5 M NaCl control without preincubation (100%). Aqueous buffer containing 1.5 M NaCl (*open square*, *filled square*), 0.5 M NaCl (*open triangle*, *filled triangle*), 0.3 M NaCl (*open circle*, *filled circle*), 0.15 M NaCl (*open diamond*, *filled diamond*). *Open symbols* without DMSO; *filled symbols* with 30% (v/v) DMSO

by *Hbt. salinarum* by using fluorescence spectroscopy [5]. This protease was destabilized by DMF, dioxane, and tetrahydrofuran. However, DMF increased the ratio of esterase/amidase activity which allowed the use of this enzyme for peptide synthesis [9]. To our knowledge, that was the only report on the application of haloarchaeal enzymes in biocatalysis in aqueous-organic media. Another recently reported organic solvent tolerant enzyme is the α-amylase from *Haloarcula* sp. strain S-1 [3]. This protein is stable and active in various non-polar organic solvents (benzene, toluene, chloroform, styrene, xylene, *n*-octane, *n*-nonane, and *n*-decane); however, it is inhibited by hydrophilic organic solvents (1-butyl alcohol, ethyl alcohol, 2-propyl alcohol, DMSO, methyl alcohol, and acetone). Interestingly, α-amylase activity is higher at low salt concentrations in the presence of chloroform, as observed for NEP protease in aqueous mixtures containing DMSO or glycerol. The effect of organic solvents on the activity and stability of halophilic enzymes is not universal and seems to depend on the properties of the solvent and/or the particular enzyme. Solvents with similar polarity such as DMSO (log $P_{o/w}$ − 1.35) and DMF (log $P_{o/w}$ − 1.0) showed an opposite effect on the stability of the halophilic protease from *Hbt. halobium* [5, 9] and NEP (this work). Besides, while DMSO was effective in stabilizing these proteases, it had a negative effect on the activity and stability of *Haloarcula* α-amylase [3].

Conclusion

Haloarchaeal enzymes display optimal activity and stability in solutions containing high salt concentrations, meaning low water activity. For this reason, they are excellent candidates as biocalalyst in aqueous-organic and organic solvent reactions. Particularly, halophilic proteases may have application in protease-catalyzed peptide synthesis. In this report, the activity and stability of the haloalkaliphilic protease NEP in the presence of various organic solvents was evaluated. It was shown that NEP is a solvent tolerant protease. It was active and stable in aqueous-organic solvent mixtures containing 1.5 M NaCl and glycerol, DMSO, DMF, propylenglycol, and dioxane. In addition, DMSO, propylenglycol, and glycerol were effective in preserving enzyme stability in suboptimal NaCl concentrations. Future experiments will be conducted to examine the ability of NEP to catalyze peptide synthesis reactions. These results contribute to fundamental and applied aspects of extremozymes and show the potential of halophilic proteins as biocatalysts in aqueous-organic media.

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