HYDROLYTIC ENZYMES OF HALOPHILIC MICROORGANISMS AND THEIR ECONOMIC VALUES

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(Received April 1, 2010)

Halophilic enzymes, known as extremozymes produced by halophilic microorganisms, have identical enzymatic features like their non-halophilic counterpart, but they exhibit different properties mainly in structure. Among these, two main points could be mentioned, (i) a high content in acidic amino-acids located predominantly at the protein surface and (ii) requirement for high salt concentration for better biological functions. The latter could be understood as a consequence of the abundance of acidic amino-acids but these two fundamental characteristics of halophilic proteins are highly cross-linking each other, making it difficult to explain unambiguously which promotes stability and function at high salinity. The following communication reviews various extracellularly produced hydrolytic enzymes from halophilic microorganisms and their economical values together with basic characteristics of protein chemistry in media with high concentration of sodium chloride and other salts.

Key words: halophilic enzyme, extremozyme, halophilic microorganisms, salt, archaea.

INTRODUCTION

In recent years, investigations to search for biocatalysts that can cope with the conditions of industrial process have been continuously increasing. Organic chemical syntheses are characterized by high precision and purity of products, but they sometimes impose a hazardous effect on the environment. Enzymes, biocatalysts, on the other hand, often have better chemical precision, which can lead to a more efficient production of single stereoisomer, decreasing the number of secondary reactions, and with a lower environmental impact. Despite large numbers of enzymes that have been applied into various biotechnological and industrial processes, the demand for more efficient biocatalysts is high (1). The majority of important industrial processes are conducted under specific parameters,

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pH, ionic strength, temperature etc., but some of these enzymes cannot exhibit all the times their optimal activities (1). In respect of these requirements, extremozymes (2), the extracellular enzymes produced by extremophiles, microorganisms that are able to flourish in extreme environments, could help to develop new biotechnology.

Extracellular degrading enzymes, hydrolases, play an important role for microorganisms to utilize organic compounds in different ecosystems. For example, amylases, proteases, lipases have been used in various fields of chemical industry and in the domestic environment (3-5). Halophilic microorganisms (see below) that require high salt concentrations for growth are sources for both thermotolerant and salt adapted enzymes. Early investigations of halophilic amylases, nucleases, proteases, etc. have been described in previously published papers (6-15).

This work reviews various extracellularly produced hydrolytic enzymes from halophilic microorganisms and their economic values in different fields ranging from industry (food and pharmaceutical) to agriculture.

1. HALOPHILIC MICROORGANISMS

Saline and hypersaline environments are widely distributed throughout the world either salt lakes (The Dead Sea, The Great Salt Lakes etc.) or salt mines [Salzbad-Salzetnan (Austria), Slanic, Turda, Praid (Romania), Wieliczka (Poland), Nakhlichevan (Azerbaidjan), Chon-Tous (Kirghizstan), Cave Berezniki in Perm (Russia), Solotvino (Ukraine) etc.]. These hypersaline environments are too harsh for normal life to exist, but a variety of microbes, both Bacteria and Archaea, survive. These organisms have evolved to exist in these extreme environments and fall into a number of different categories, including halotolerant, moderately, borderline and extremely halophilic. They have adapted to thrive in ecological niches in saline and hypersaline environments described above. As a result, these microorganisms produce unique enzymes and metabolites able to develop biological activity in conditions in which their counterparts could not be functional. These properties could be exploited for the development of additional bio-industrial processes designed basically on the optimal conditions of these bio-molecules.

Two fundamentally different approaches are involved in order to cope with osmotic challenges associated with life in saline environments (16). In the case of halophilic Bacteria, the cytoplasm contains low concentrations of salt compared with surrounding environment, but it has high organic solute levels. The halophilic Archaea (halobacteria), on the other hand, have developed an entire biochemistry that functions at saturating salt concentrations. For more details of halophilic Archaea and Bacteria, please consult previous reviews (17-19).

2. INTERACTION OF PROTEINS WITH SALTS

Interactions of proteins with salts are correlated with the position of salt in Hofmeister (20) or electroselectivity series, which are ordered according to increasing surface tension for anions: $SO_4^{2-}>CH_3COO^->Cl^->I^->NO^{3-}>SCN^-$. The kosmotropic (antichaotropic) salts located in the superior part of the series will promote salting-out processes. To the contrary, the chaotropic salts located in inferior part of series, as well as 8 M urea and 6 M guanidinium chloride, will promote the non-equilibrium state of solvent composition surrounding protein leading to salting-in processes. The effect could be due to the size of ions and the slow interactions between these ions and water (solvent) molecules (21). The effect of salt on protein solubility is characterized by a salting-in region at low concentration of salt and a salting-out region at high content of salt. The salting-in region could be considered as favorable electrostatic interactions between salt ions and protein charge (22). At high salt content, the unfavorable interactions between salt ions and protein conducted to a salting-out behavior (23). Proteins with negative charges could be dissolved at high salt concentrations in the presence of divalent cations due to specific binding of these cations to the protein surface (24).

The folding, stability and properties of proteins are strictly dependent on the interaction of these macromolecules with the solvent in which they are dissolved. Proteins are composed of various combinations of hydrophilic and hydrophobic amino acids, and interactions of these residues with water or other solvents determine the folding of the proteins. It has long been known that activities of enzymes of animal and plant origin are strongly inhibited by salts (25). Proteins, particularly enzymes, of halophilic microorganisms, however, are extraordinary in that they require high concentration of salts for their stabilities and activities.

3. HALOPHILIC PROTEINS

It has long been known that the amino acids compositions of halophilic proteins generally show an increased presence of glutamic and aspartic acids compared with their non-halophilic counterparts (26). An early work on comparison of amino acid composition of ribosomal proteins has also revealed a lowered frequency of hydrophobic amino acids in the halophilic system and a higher occurrence of borderline hydrophobic amino acids like serine and threonine (26). Some general mechanisms which contribute to stabilization of halophilic proteins are: (a) a charge screening of the protein molecules determined by abundance of acidic residues is effective at 0.5–1 M salt; (b) the mandatory close packing of non-polar marginally hydrophobic groups is determined by higher salt concentration; and (c) a hydrated protein surface is maintained through utilization of the carboxyl groups in glutamate and aspartate (26-28).

Examinations of three-dimensional structures of proteins from halophilic organisms have revealed that an abundance of acidic residues distributed at the surface of proteins, in opposition to the non-halophilic counterparts, contribute essentially to the stability and adaptation of proteins to media with high salt content (29). In these environments, acidic residues are favored due to their high capacity of binding water molecules, but also repulsive electrostatic interactions are present in halophilic proteins at physiologic pH values. The halophilic Archaea have evolved to function with intracellular ionic strength similar with those that surrounded the cell at external level. The halophilic adaptation is achieved by the abundance of acidic amino acids on protein surface in co-operation with electrostatic interactions and the presence of an increased number of salt-bridges. The proteins most well investigated in order to reveal the stability at high salt concentrations are malate dehydrogenase (30) and ferredoxin 2Fe-2S from a halophilic archaeon *Haloarcula marismortui* (31).

The malate dehydrogenase exists as a tetramer in high salt concentrations and dissociates into monomers when salt levels are reduced below 2.5 M, without dimeric forms being observed (32). This enzyme is stable in a wide range of pH values from 5 to 10 at 4 M NaCl, but it is dissociated at pH 4.8. If pH is maintained at 8.5, the enzyme keeps its stability until down to 1.8 M NaCl. The major point is pH values, which most probably promote some favorable electrostatic interactions and enzyme keeps stability at low salt content even if it is a halophilic protein (31). The tetrameric forms of the halophilic malate dehydrogenase are contrasting with the usual dimeric forms of non-halophilic counterparts, and this tetrameric form argues for similarity of halophilic malate dehydrogenase with non-halophilic lactate dehydrogenase. In fact, a single amino acid mutation, Arg100Gln, converts substrate specificity to pyruvate, which is similar with non-halophilic lactate dehydrogenase. The presence of a high number of acidic amino acid residues on the surface leads to a net charge to -156e, supposing that all ionizable residues are in protonated state, although lactate dehydrogenase from dogfish have a net charge of +16e (29; 33).

The model of halophilicity derived from the observation on malate dehydrogenase is also supported by data obtained by dihydrofolate reductase of another halophilic archaeon *Haloferax volcanii* (34). Surface charge is asymmetrically distributed, with positively charged amino acids centered around the active site and negative charges on the opposite side of the protein. These negative charges due to abundance of negative amino acids form clusters at high concentrations of counterions (35).

The haloarchaeal 2Fe-2S ferredoxins are longer than plant type ferredoxins by 30 amino-acids sequence. A comparison of *Anabaena* ferredoxin with *Haloarcula marismortui* ferredoxin revealed that the N-terminal domain of the latter protein contains 34 residues, from which 15 are acidic (36). In addition of these acidic residues, large numbers of acidic residues appear throughout the rest of the sequence. Similarly with halophilic malate dehydrogenase, these residues are

located on the protein surface. These data confirmed modeling studies in which structures of halophilic proteins were aligned with the three-dimensional structures of their non-halophilic counterparts (29).

The structure of thermostable enzyme glucose dehydrogenase from the extreme halophile *Haloferax mediterranei* (37) has been solved during the last years (38) and revealed a surface with acidic residues which is only partially neutralized by bound potassium counterions, involved also in substrate binding. The structure reveals a unique highly ordered, multilayered solvation shell organized into one dominant network covering much of the exposed surface accessible area, supporting the requirement of enzyme to form a protective shell in a dehydrating environment.

Since the halophilic enzymes possess salt-enriched solvation shells (39), they are constructed to retain catalytic activity in environments with low water activity, such as in the presence of high levels of organic solvents. Such properties could be of interest in a variety of applications (40).

3.1. EXTRACELLULAR HALOPHILIC ENZYMES

According to literature, the major interest in products derived from halophilic microorganisms has focused on either stabilizing agents, such as betaine, ectoine or hydroxyl ectoine on exopolysaccharides for use as biodegradable plastics, or salt-tolerant lipids (41, 42). In some cases, the resistance to high salt concentrations of haloarchaeal compounds is exploited, whereas in other cases, the halophilic property is secondary to thermotolerance or pH values resistance. For example, retinal proteins, such as bacteriorhodopsin, have found uses in holographic films or in other light-sensitive or 'bioelectric' applications (43, 44). Although we have seen advances in understanding the biophysical properties and behavior of halophilic enzyme's stability, activity and solubility (39), potential uses of haloarchaeal extremozymes have received less interest. The haloarchaeal extremozymes are not only highly salt-tolerant, but also reveal the capacity of retaining stability at ambient temperatures for long periods of time and often appear to be thermotolerant. At present, a few haloarchaeal extremozymes have been adopted for applied purposes due to the relatively limited demand for salt-tolerant enzymes in current manufacturing or related processes. There are few examples of extremozymes from haloarchaea of commercial value. Obayashi *et al*. (45) patented the production of a novel restriction enzyme with unusual specificity from a strain of the genus *Halococcus*. There is a report that a chymotrypsinogen B-like protease was isolated from the haloalkaliphilic archaeon *Natronomonas pharaonis*, which has an optimal activity at 61^0C and pH 10 (46). Although previously reported haloarchaeal proteases lose their catalytic activity at low salt content (47), the alkaliphilic *N. pharaonis* protease is able to function in salt concentrations below 3 mM. This could be an advantage for this extremozyme as an additive to detergent, which is the current major role of alkaliphilic enzymes (48).

3.1.1. Starch-degrading enzymes

The starch, composed exclusively of D-glucose units, is one of the common polymers in nature. The two forms are amylose (15–25%), a linear polymer consisting of α-1,4-linked glucopyranose residues, and amylopectin (75–85%), a branched polymer containing α-1,6-linked branching points occurring every 17–26 α-1,4 glycosidic linkages. Many starch-degrading enzymes have been identified in various organisms (49), but more investigations are necessary to elucidate the processes due to the complex structure of starch, which requires an appropriate combination of intracellular and extracellular enzymes for its conversion to smaller size sugars, glucose and maltose.

A few α-amylases were purified and characterized from halophilic microorganisms, *Haloferax mediterranei* (50), *Halobacterium salinarum* (51, 52), *Natronococcus amylolyticus* (53), *Halomonas meridiana* (54) etc., having similar functional properties. The enzyme isolated from *Haloarcula* sp. S-1 showed a relatively high tolerance to various organic solvents (55), which differentiated it from other haloarchaeal α-amylases usually active under high salt concentrations and generally inactive in the absence of salt. The effect of ionic strength on the amylase activity, at various ratios of Na^+ and Mg^{2+} concentrations, has also been reported (56).

3.1.2. Cellulose-degrading enzymes

A cellulose-utilizing, extremely halophilic bacterium was first reported by Bolobova *et al*. (1992). The obligate anaerobic organism named *Halocella cellulolytica* is able to utilize cellulose as a sole carbon source. Another work has shown that many cellulose-utilizing extremely halophilic Archaea are present in subsurface salt formation (57).

A preliminary work on extracellular hydrolytic enzymes of halophilic microorganisms from subterranean rock salt revealed the presence of cellulase (58). The strains shown to exhibit this enzyme activity are Gram-negative rods unable to grow in the presence of various antibiotics, neomicin, penicillin, anysomicin and erythromycin, and tolerate salt concentrations of up to 3M NaCl. They are able to grow at 12^0C. The cellulase activities were associated with starch, casein and tween 80 hydrolysis (58).

3.1.3. Proteases

The proteases play a key role in many metabolic pathways and could be used in different applications in various areas of industry, such as subtilisin BPN' and Carlsberg (from Novo) used widely in detergents. Halophilic microorganisms produce proteases with high stability at saturated salt concentrations or organic solvent tolerance (59) which can have novel applications (60).

Fish sauces (nam-pla, pla-ra etc.) are condiments produced in South-East Asia, similar to *Garum* or *Liquamen* of ancient Roman society. They are prepared from raw fish themselves in concentrated and sometimes saturated brine, and it has been shown that proteases of haloarchaea present in solar salt used in brine play an important role in degradation of fish protein into amino acids (61). An extracellular serine protease from *Halobacterium salinarum* requires 4 M NaCl for optimal catalytic activity and stability in aqueous solutions (62). This enzyme has potential to be used for peptide synthesis, particularly those containing glycine. Another protease produced by *Natrialba asiatica* (63) and *Haloferax mediterranei* (64, 65) was purified and characterized. Amino acid sequences deduced from gene sequences showed that they were subtilisin-type serine proteases, but they possessed long-stretch of C-terminal extension (115-125 amino acids) not observed in non-halophilic counterparts. The calcium ions increased the stability of the enzyme from *Hfx. mediterranei,* which is differentiated by a different calcium binding site from other members of the same enzyme type family (65). The other serine proteases were isolated from *Natronomonas pharaonis* (46), *Natrialba magadii* (66), *Natronococcus occultus* (67). The biochemical characterization and modification of these enzymes based on their cloned genes (63, 65, 68) will help to improve the understanding halophilic protease and contribute to elucidating mechanisms for their activation and extracellular secretion (69). The tolerances of halophilic microorganisms to various organic solvents contribute also to stabilization of halophilic proteases by these solvents and lowering the required salt concentration, which in some biotechnological process could have a corrosive nature.

3.1.4. Lipases

Esterases represent a family of enzymes with high potential in various industrial processes, such as stereospecific hydrolysis, trans-esterification, ester synthesis and other organic biosynthesis reactions. Indeed, the range of reactions in which esterases are employed has been extended due to the discovery of extremophilic versions of these enzymes. Lipase is one of the most important hydrolytic enzymes with potential in various fields of pharmaceutical industry and agriculture. Various moderately or extremely halophilic microorganisms, *Salinivibrio* sp. (70), *Natronococcus* sp. (71), haloarchaeal strains (72) etc., have been shown to produce lipases stable at relatively high temperatures.

3.1.5. Xylanases and chitinases

Xylanases are enzymes that degrade xylan in the hemicellulose complex, which represents a huge reserve of utilisable biomass. Since hemicelluloses are, after cellulose, the most abundant renewable resource, the importance and commercial values of xylanases are very high. The purification and properties of two new extremely halotolerant xylanases with stability and activity in NaCl

concentrations from 0 to 5 M have been reported (73, 74). Recently, xylanase and cellulase producing halophilic microorganisms were isolated from Romanian subterranean rock salt (58).

Chitinases catalyze the hydrolysis of chitin, an insoluble linear biopolymer with β-1,4 linkage of N-acetyl-D-glucosamine. A gene encoding chitinase homologue (ChiN1) was found in the genome of an extremely halophilic archaeon, *Halobacterium salinarum* NRC-1, and the gene was expressed in another extremely halophilic archaeon *Haloarcula japonica* TR-1. The recombinant ChiN1 exhibited the optimal activity at 1 M NaCl and it was almost stable in 1-5 M NaCl. ChiN1 was also active in the presence of 30% (v/v) DMSO, suggesting that the enzyme could be applicable to synthetic reactions of oligosaccharides in organic solvents (75). Furthermore, preliminary data (Mizuki T, personal communication) suggested that another halophilic archaeon decomposes colloidal chitin. Genes coding for chitinase have been isolated from an alkaline hypersaline lake and an estuary, expressed and characterized (76). These halophilic chitinases are quite important in view of the use of a huge amount of chitin, the second most abundant renewable biomass beside cellulose, particularly as arthropod integuments in marine environments.

4. BIOTECHNOLOGICAL POTENTIAL OF HALOPHILIC EXTREMOZYMES

Enzymes have been used in detergents since the 1960s. The use of enzymes in laundry and automatic dish washing detergents provides consumers clear cleaning performance with lighter burden on environment. It is important that enzyme properties may be improved by the use of protein engineering technique, whereby specific amino acids are substituted. Examples are enzymes that replace surfactant, those with the improved cold washing efficiency, and the bleach-resistant starch degrading enzymes, etc.

The increasing numbers of available data on haloarchaeal genomes promise a major increase in the use of halophilic extremozymes in a variety of applied fields in the near future. These data will help to reveal novel halophilic enzymes with biotechnological potential in various economical and technological fields. For example, a gene encoding the extracellular serine protease halolysin (*hly*) is present in *Halobacterium salinarum* NRC-1, but the presence of this gene was unknown because this particular strain does not excrete this protease. The same is true for the chitinase gene of the same strain, suggesting that exploitation of halophilic enzymes from cultures of halophilic microorganisms should be done in parallel with search for expected genes in the genome library.

The use of 16S ribosomal RNA genes revealed a huge diversity of microbiota in hypersaline environments (77–79). These unexplored microbiotas are supposed to harbor genes of a great variety of extremozymes. Although cultivable number of

halophilic microorganisms is relatively low, the increasing numbers of described novel species support this optimism for exploitation of halophilic extremozymes (2). Given that the structure-function rules which confer the stability of enzymes under high salt milieu are becoming well understood (80), the potential of halophilic extremozymes for economic fields will be better exploited. The investigations on the mechanism of protein translocation and activation across the halophilic archaeal membranes will be also conducted at well-characterized protein with enzymatic activity and stable for biotechnological processes. Based on the results of such kind of research, novel biotechnological processes designed for current demands of modern society could be tailored.

5. CONCLUDING REMARKS

There is a general argument that less than 10% of the living organisms in a defined environment are cultivable and further improvement of gene expression technologies will help to increase the exploration of microbial diversity (81). It could be possible to construct gene expression libraries from the most diverse sources, which might lead to the discovery of many new extremozymes in the near future. These extremozymes will be used to design a wide range of novel biocatalytic processes that are faster, more accurate, specific and environmentally friendly. In order to increase the role of extremozymes in various areas of industries, it is necessary to develop concurrent protein engineering and convergence of bio/nanotechnologies (2).

The study of proteases and their regulation *in vivo* of halophilic microorganisms continues to contribute to our overall understanding of the physiology of these unusual organisms that have adapted (evolved) their entire repertory of proteins to function optimally in extreme environments.

The great economic potential of extremozymes will bear plenty of fruits in various industrial processes, agricultural, food, chemical and pharmaceutical from further exploration of extremozymes (82).

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