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Review

Molecular adaptation to high temperatures: pernisine from the archaeon *Aeropyrum pernix* K1

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Summary. Pernisine is a subtilisin-like protease from the hyperthermophilic archaeon *Aeropyrum pernix*. Due to its high thermal stability and its activity in the presence of denaturants, pernisine represents a promising enzyme for use in various industrial applications. Another potentially applicable characteristic of this protease is its ability to degrade infectious prion aggregates. Production of pernisine in *A. pernix* does not provide sufficient yield for its commercial use, and alternative production strategies are hence needed. This review summarizes the biochemical and biophysical characteristics of pernisine and progress that has been made toward production of recombinant pernisine using *Escherichia coli* and *Streptomyces rimosus* as expression systems.

Keywords: expression systems, molecular adaptation, pernisine, thermally stable enzymes.

INTRODUCTION

Pernisine, like many other industrially important enzymes, comes from hyperthermophilic Archaea. Before describing pernisine in detail, we provide an overview of Archaea and thermostable enzymes in general.

Based on their 16S rRNA phylogeny, Archaea have been classified as an independent domain of life since 1990 (Woese et al. 1990). According to their morphology, size and cellular organisation (i.e., absence of organelles), Archaea are similar to Bacteria. However, according to some of their other characteristics, such as their transcription and translation systems, Archaea are more closely related to eukaryotes (Forterre et al. 2002). Archaea often inhabit ecological niches with extreme conditions (e.g., high temperature, salinity, pressure, extreme pH).

Hyperthermophiles are microorganisms that optimally grow at temperatures above 80 °C (Atomi 2005). Representatives of hyperthermophiles are found only in the archaeal domain and among bacteria from the Thermotogales and Aquificales phyla (Egorova and Antranikian 2005). Survival of these organisms under extreme conditions is possible due to adaptation of their biological macromolecules to such environments. For example, hyperthermophiles have different mechanisms to maintain DNA stability at high temperatures. The most important of these are reverse gyrase enzymes that promote the formation of supercoiled DNA, which increases the melting temperature of the DNA (i.e., temperature at which transition from a double helix to a single chain occurs) (Manaka 2011). Also, archaeal cytoplasmic membranes are composed of isoprenoidal lipids that are bonded with glycerol, and this is more stable than the bacterial double-layer phospholipid membrane (de Miguel Bouzas et al. 2006).

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ARCHAEON *AEROPYRUM PERNIX*

Aeropyrum pernix is a hyperthermophilic archaeon that was isolated in 1993 from a hydrothermal vent near Kodakarajima Island in Japan (Sako et al. 1996). Based on the 16S rRNA phylogeny, it is classified in the phylum Crenarchaeota and order Desulfurococcales. *Aeropyrum pernix* cells are spherical in shape with sharp edges and sizes of 0.8 μm to 1.0 μm; and they are surrounded by a unique ~25-nm-thick cell envelope (Sako et al. 1996). The *A. pernix* cell membrane consists of 2,3-di-sesperpanil-*sn*-glycerol ether lipids and polar components of phosphoglycolipids and phospholipids (Ota et al. 2012). The *A. pernix* genome was sequenced and published in 1999, and it has a relatively small size (1,669 kilobase pairs), with 2,694 potential genes predicted (Kawarabayasi et al. 1999).

Aeropyrum pernix was the first known obligatory aerobic hyperthermophilic organism. The aerobic metabolism of this archaeon is especially interesting due to low oxygen solubility at high temperatures, and the presence of reducing gases in its habitat (Sako et al. 1996). *Aeropyrum pernix* grows at temperatures from 70 °C to 100 °C. In the laboratory (Fig. 1), the optimal conditions for *A. pernix* cultivation were established to be temperatures from 90 °C to 95 °C, pH 7.0, and 3.5% salinity. Its doubling time under optimal growth conditions and in optimised medium is about 200 min (Milek et al. 2005). This archaeon is a heterotrophic organism that successfully grows on complex substrates, such as tryptone, yeast extract and trypticase peptone (Sako et al. 1996).

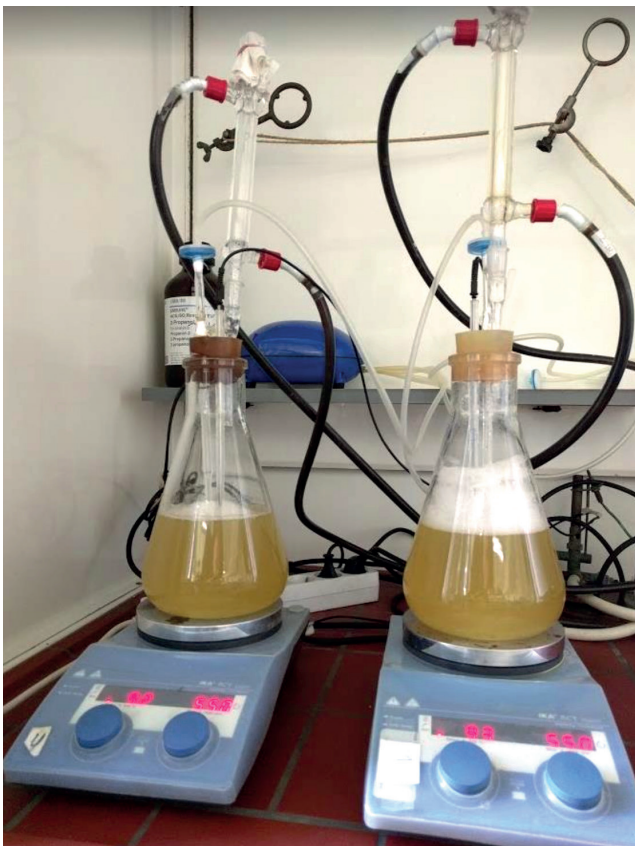

Fig 1. Cultivation of *Aeropyrum pernix* in the laboratory scale. The temperature of *A. pernix* liquid cultures are regulated using a hot plate magnetic stirrer. Air is provided by an air pump connected to a gas disperser submerged into the flask. Liquid evaporation is prevented using a chiller with circulating water.

MOLECULAR MECHANISMS OF ENZYME THERMOSTABILITY

The extreme environmental niches inhabited by archaeal species necessitated evolutionary adaptations of their enzymes to such extreme conditions, in particular to high temperatures. An enzyme is defined as thermostable when its denaturing temperature is >55 °C (Turner et al. 2007). Enzyme thermostability encompasses its thermodynamic and kinetic stability. Thermodynamic stability is defined by a stabilising free enthalpy (ΔG_{stab}), while thermal stability is defined by the temperature of denaturation of the protein (i.e., unfolding; T_m). The kinetic stability of an enzyme is often expressed as its half-life ($t_{1/2}$) at a defined temperature (Vieille and Zeikus 2001).

Comparisons of homologous enzymes from hyperthermophiles and mesophiles have revealed high amino-acid sequence similarities (i.e., up to 85% homology), high agreement in their three-dimensional structures, and identical catalytic mechanisms. In general, thermostability results from the intrinsic properties of an enzyme. Other factors, such as presence of salts, coenzymes, activators and stabilisers, and high protein concentrations, contribute to the stability of certain intracellular enzymes (Vieille and Zeikus 2001).

Given the complexity of protein structures, it is not surprising that there is no known universal protein stabilisation mechanism that is responsible for thermostability. The thermostability of proteins originates from a combination of many structural modifications through substitutions of some amino acids and modulation of stabilising intra- and intermolecular forces (Li et al. 2005). The factors that contribute to thermostability are additional inter- and/or intramolecular interactions (e.g., hydrogen bonds, disulphide bonds, hydrophobic interactions, electrostatic interactions, aromatic interactions) and a more robust conformational structure (i.e., greater rigidity, compact folding, reduced conformational entropy) (Li et al. 2005). Noncovalent intramolecular interactions represent the most important stabilising forces in proteins (Perl and Schmid 2002), whereby an increased number of these stabilising interactions increases the thermostability of an enzyme. In addition, more compact folding of enzymes is a conformational characteristic that generally

increases their thermostability. The compactness of an enzyme increases with shorter loops, a higher proportion of side chains directed toward the interior of the protein, and a reduction in the size and number of voids in the structure of the enzyme (Li et al. 2005).

The evidence that thermostability is often an inherent feature of an enzyme is the successful conservation of enzyme thermostability upon recombinant expression in mesophilic hosts (Vieille and Zeikus 2001; Li et al. 2005; Šnajder et al. 2015).

PRODUCTION OF THERMOSTABLE ENZYMES

Most commercial enzymes are produced in recombinant forms using mesophilic production organisms, such as different *Bacillus* species, *Escherichia coli* strains, the yeast *Saccharomyces cerevisiae* and *Pichia pastoris*, and the fungi *Aspergillus oryzae* and *Trichoderma reesei* (Turner et al. 2007). Many industrially relevant proteinases have been isolated from *Bacillus* species, which are also often used as enzyme production organisms, mainly because of their efficient protein secretory system (Singh et al. 2016).

Escherichia coli is a Gram-negative, facultative anaerobic bacterium that colonizes the digestive tract of warm-blooded animals (Terpe 2006). *Escherichia coli* is one of the most studied model organisms and the most frequently used host organism for production of recombinant proteins in the laboratory and at the industrial level. As there is good availability of genetic tools for *E. coli*, its genetic manipulation is generally simple, rapid and affordable. A large number of expression vectors and several strains of *E. coli* that are specialised for protein production are available, providing a number of potential expression systems for the optimal production of any specific protein (Wyre 2014). The most commonly used *E. coli* strains for synthesis of recombinant proteins are the BL21 and K12 strains, and their derivatives (Terpe 2006). The main advantages of *E. coli* as a host for the production of recombinant proteins are rapid cell growth, growth to high cell density, and cost-effective cultivation media.

Naturally occurring enzymes are often not suitable for commercial applications, where catalytic activity and high specificity under harsh non-physiological conditions are needed. Optimisation of enzyme stability and activity by protein engineering, and development of effective and inexpensive production on an industrial scale, are key for the commercial use of enzymes. Proteases and other enzymes can be improved by protein engineering, in terms of their thermostability, temperature and pH optimum of activity, tolerance to organic solvents, substrate specificity, stereoselectivity, co-factor dependence, and other properties that are important for commercial applications of the enzymes (Donlon 2007).

APPLICATIONS OF THERMOSTABLE PROTEINASES

The use of thermostable proteinases and other thermostable enzymes offers many advantages over mesophilic enzymes. Conducting industrial processes at higher temperatures enables greater substrate solubility and use of higher substrate concentrations, along with lower viscosity, lower risk of contamination, and higher reaction rates (Vieille and Zeikus 2001). Thermostable enzymes generally also show greater resistance to chemical denaturants, such as organic solvents and detergents. Thermostable enzymes are generally active across a wide pH range (Turner et al. 2007).

The thermostability of proteinases and their resistance to detergents and alkaline conditions have been exploited for their use in laundry and dishwasher formulations, which is the most common application of industrial enzymes (Li et al. 2013). Proteinases, oxidases, amylases, lipases, cellulases and peroxidases have been added to detergents to catalyse the breakdown of chemical bonds in the presence of water, thus removing stains of various types from clothing. Among the proteinases, bacterial subtilisins are the most suitable for use in detergent powders, where in addition to thermostability, they are characterised by broad substrate specificity (Smith et al. 1999). Enzymatic processes with thermostable proteinases have reduced the use of hazardous chemicals in leather production, where proteinases are used to selectively hydrolyze noncollagen skin parts and to remove non-fibrillary proteins (e.g., albumin, globulin), to improve leather quality (Rao et al. 1998).

Another important industrial application of thermostable proteinases is the digestion of substrates rich in keratin. By proteolytic degradation of waste products rich in keratin (e.g., feathers), soluble protein fractions can be obtained for animal feed, aquaculture food and biogas production plants, or as a source of nitrogen for plants (Deivasigamani and Alagappan 2008).

Proteinases, such as thermolysin, are also used to synthesise dipeptides in the pharmaceutical (e.g., somatostatin, vasopressin) and food (e.g., aspartame) industries. Under the appropriate kinetically controlled conditions (e.g., absence of water for hydrolysis), the high specificity and stereospecificity of optimised thermolysin can be exploited for the synthesis of peptides, which has many advantages over chemical synthesis (Mótyan et al. 2013).

Another area where thermostable proteinases may get widespread applicability is in degradation of infectious prion proteins (Poklar and Vilfan 2013). Prion diseases and transmissible spongiform encephalopathies are a group of neurodegenerative diseases that can occur in humans and animals. The cause of prion disease is structural transformation of the normal cellular prion protein PrP^{C} into the infective form

PrP^{Sc}, which can then convert other PrP^{C} proteins into PrP^{Sc} upon contact; this leads to exponential spread of the infective form (Prusiner 1998). Due to their structural properties, pathological prion proteins such as PrP^{Sc} are extremely resistant to decontamination by temperature, detergents, UV and ionisation, as well as via proteolytic degradation. Interestingly, pernisine isolated from the medium of *A. pernix* can successfully degrade pathological prion proteins PrP^{Sc} of various origins (e.g. human, cattle, mice) (Šnajder et al. 2012). This suggests that this protease may be developed for use in decontamination agents for contaminated surfaces and surgical tools.

GENERAL CHARACTERISTICS OF PERNISINE

The pernisine gene (EMBL: BAA79718.2) is 1293 base pairs long. Its product (UniProtKB: Q9YFI3) is a precursor that is composed of 430 amino-acid residues (Fig. 2). Based on the amino acid sequence, pernisine belongs to the subtilisin-like superfamily of proteases. The most closely related protease to pernisine is the well-studied subtilisin from *Thermococcus kodakarensis* (*Tk*-subtilisin). Comparisons of the amino-acid sequences of pernisine and *Tk*-subtilisin reveal 51.7% identity (Šnajder 2013). In general, subtilisin-like proteases are synthesised as inactive precursors, with a signal sequence and a pro-region at the N-terminus. The signal sequence directs the secretion of pernisine through the cell membrane into the extracellular space. In some proteases, the pro-region acts as an intramolecular chaperone, to facilitate folding of the catalytic domain (Chen and Inouye 2008; Tanaka et al. 2008). In general, the pro-region also inhibits the activity of subtilisin-like proteases, apparently by limiting the availability of the active site for substrate binding (Kannan et al. 2001; Sacco et al. 2013). Autocatalytic cleavage and degradation of the pro-region are needed for activation of subtilisins. Autocatalytic maturation is also obligatory to produce the active form of pernisine (Fig. 3) (Šnajder et al. 2015), while the chaperone function of the pernisine proregion remains to be confirmed.

The first 26 amino acids of pernisine correspond to its signal sequence, as predicted using the SignalP 3.0 tool. Based on the *Tk*-subtilisin primary sequence, the proregion has been predicted to comprise the region between amino acids residues Ala27 and Met94 (Fig. 2) (Šnajder et al. 2012). However, subsequent analysis of the N-terminal and C-terminal ends of processed and unprocessed pernisine using mass spectrometry have suggested that the site of pro-region cleavage is between residues Gln92 and Ala93 (Šnajder et al. 2015). This prediction of pernisine composition is in agreement with its molecular weight of ~45 kDa with the pro-region, and ~34 kDa without the pro-region, as estimated by SDS-PAGE (Catara et al. 2003; Šnajder et al. 2012). Using zymography, only the band corresponding to the processed pernisine (~34 kDa) was shown to be proteolytically active. Furthermore, an additional smaller active form of pernisine at ~23 kDa was detected by zymography, which appears to be a result of further autocatalytic processing that occurs with high concentrations of pernisine in enzyme reaction mixtures (Catara et al. 2003; Šnajder et al. 2012). It is not clear whether this truncated active form occurs only *in vitro*, or if it also occurs in nature by further post-translational processing into the final functional form of pernisine. Knowledge of the exact mechanism of the post-translational activation process is important for further development of pernisine, and for its production strategy for commercial use.

The main characteristic of the subtilisin-like superfamily of proteases is the conserved catalytic triad that is composed of Asp, Ser and His (Fig. 3) (Uehara et al. 2017). Classification of pernisine according to catalytic type has also been confirmed by activity assays using metal-chelating agents (e.g., EDTA, EGTA), and specific inhibitors of serine proteases (e.g., phenylmethylsulphonyl fluoride) (Catara et al. 2003; Šnajder et al. 2012). Subtilisin-like proteases can be modulated by Ca^{2+} ions, whereby its binding increases protein stability, and consequently affects enzymatic activity (Pulido et al. 2006). The binding of Ca^{2+} appears to reduce the flexibility of protein polypeptide chains, thereby preventing protein unfolding (Smith et al. 1999). The effects of Ca^{2+} on protein stability and activity have also been demonstrated for pernisine (Catara et al. 2003; Šnajder et al. 2012, 2015).

Using circular dichroism and the CONTIN algorithm, the secondary structure of unprocessed recombinant perni-

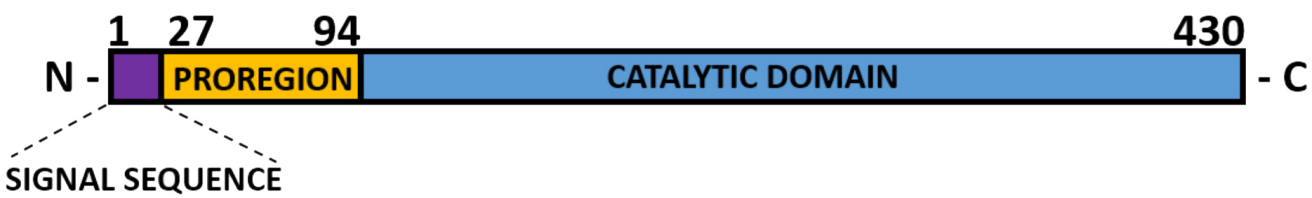

Fig. 2. Schematic representation of the primary structure of pernisine, showing the signal sequence (in violet; residues 1-26), the pro-region (in orange; residues 27-94), and the catalytic domain (in blue; residues 95-430).

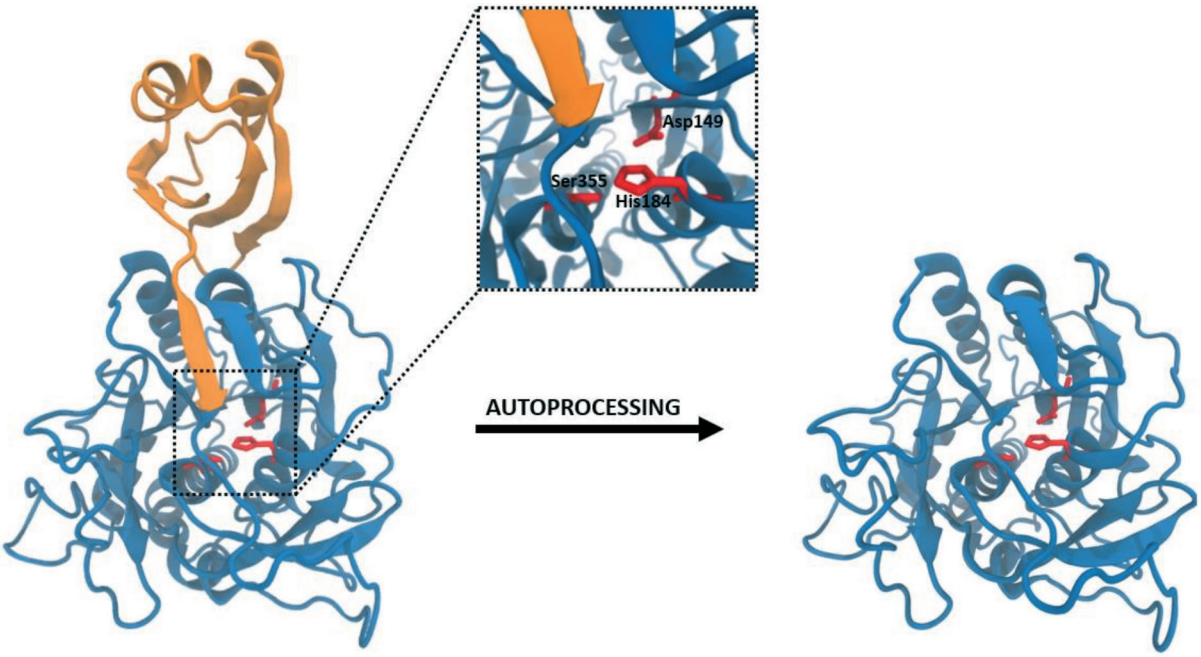

Fig. 3. Predicted three-dimensional structure of pernisine obtained through homology modelling using the SWISS-MODEL tool. The model was illustrated using VMD software (Humphrey et al. 1996). The pro-region is in orange and the catalytic domain is in blue. Unprocessed pernisine with the pro-region is shown on the left. Upon autoprocessing, the proregion is cleaved and degraded, leaving the activated catalytic domain (right). The catalytic triad of Asp149, His184 and Ser355 is shown in detail in the inset.

sine was estimated to be composed of 30% α-helix and 56% β-sheet. The secondary structure of additionally processed pernisine (~23 kDa) was estimated as 4% α-helix and 86% β-sheet (Šnajder 2013).

BIOCHEMICAL AND BIOPHYSICAL PROPERTIES OF PERNISINE

To date, pernisine has been biochemically and physiologically characterised in its native form (i.e., synthesised in *A. pernix*) as well as in its recombinant form (i.e., synthesised in *Escherichia coli*). The enzymatic activities of pernisine under various conditions was determined using azocasein as the substrate. Native pernisine is proteolytically active at temperatures ranging from 60 °C to 99 °C, at a pH from 3.5 to 8.0, and at NaCl concentrations from 0 mM to 500 mM. In the absence of Ca^{2+} ions, the highest enzymatic activity was achieved at ~85 °C and pH 7.0. Addition of 1 mM $CaCl_2$ significantly increased the proteolytic activity of pernisine, and shifted its temperature and pH optima to 105 °C and pH 6.5, respectively (Šnajder et al. 2012).

Recombinant pernisine from *E. coli* has comparable proteolytic activity to native pernisine based on azocasein assays. In the presence of 1 mM $CaCl_2$, recombinant pernisine reaches its optimal enzymatic activity at ~100 °C and pH 7.0 (Šnajder et al. 2015). The influence of protease inhibitors and various denaturants on the activity and stability of pernisine has also been studied. The proteolytic activity of pernisine was not significantly changed in the presence of denaturants (e.g. 4 M guanidine hydrochloride, 4 M urea) and detergents (e.g., SDS, sodium dodecyl sulfate, 0.1%). The chelating agents EDTA and EGTA and the protease inhibitor phenylmethylsulphonyl fluoride reduced pernisine activity, which confirms that pernisine is a serine proteinase that is stabilised by Ca^{2+} ions (Catara et al. 2003; Šnajder et al. 2015). Pernisine has broad substrate specificity. The possibility of modulating its specificity by regulation of reaction conditions has been demonstrated (e.g., temperature, presence of organic solvents, substrate:enzyme ratio), which is of interest for biotechnological applications (Palmieri et al. 2005).

The conformational stability of native and recombinant pernisine has been studied using spectrometric methods (e.g. UV, fluorescence emission, circular dichroism spectrometry). The secondary and tertiary structures of the mature form of native pernisine are thermally stable. Conformational changes that suggest the onset of temperature denaturation have been observed at temperatures above 90 °C. The recombinant form of pernisine is less thermally stable than the native form, as denaturation of its tertiary structure has been detected at temperatures >55 °C (Šnajder, 2013).

PRODUCTION OF PERNISINE IN *ESCHERICHIA COLI*

Production of native pernisine in its original host organism has proven to be demanding, as cultivation of *A. pernix* yields only low quantities of pernisine (<0.1 mg per 1 L of culture; Catara et al. 2003; Šnajder et al. 2012). Therefore, the production of pernisine in *E. coli* appears to be a more reasonable option to prepare sufficient quantities of pernisine for biochemical characterisation and the development of commercial applications. Catara et al. (2003) first attempted to produce recombinant pernisine in *E. coli*. They cloned the pernisine gene (with omission of the coding region for the pernisine native signal sequence) from the *A. pernix* genome into the expression vector pGEX, to express pernisine fused with the glutathione-S-transferase protein in *E. coli* BL21. However, successful recombinant pernisine production was not demonstrated in that study, although Catara et al. (2003) claimed they observed proteolytic activity and putative pernisine degradation products in the crude *E. coli* extracts. The first successful production of pernisine in *E. coli* was reported by Šnajder et al. (2015), where the codons in the nucleotide sequence that encode full-length pernisine were optimised for translation in *E. coli*. Using a combination of the pMCSG7 expression vector and the *E. coli* BL21(DE3) strain, His_6-tagged pernisine was synthesised and isolated from cell extracts using immobilised metal affinity chromatography followed by gel filtration. With this approach, ~10 mg recombinant pernisine was isolated from 1 L of *E. coli* culture. The identity of the recombinant pernisine was confirmed by tandem mass spectrometry. Also, the expression of pernisine fused with glutathione-S-transferase or the maltose binding protein at the N-terminus was attempted to improve the synthesis and solubility of the protein produced, but no improvement in the final yield was obtained (Šnajder et al. 2015).

PRODUCTION OF PERNISINE IN *STREPTOMYCES RIMOSUS*

An expression system for biosynthesis of pernisine was also created for the less-established host organism *Streptomyces rimosus* (unpublished results). This bacterium is of interest for recombinant protein production due to the availability of industrial-scale bioprocesses for its cultivation and efficient secretion of recombinant proteins into the extracellular medium, thus simplifying subsequent purification. For its expression in *S. rimosus*, the pernisine gene was modified such that only the catalytic domain was synthesised, with a srT signal sequence from *S. rimosus* attached to the N-terminus of pernisine for secretion, and a His_6-tag attached to the C-terminus to facilitate protein purification. As for *E. coli*, the nucleotide sequence of pernisine was 'codon-optimised' for translation in *S. rimosus*. The pernisine produced that lacked the proregion was purified from clarified medium after *S. rimosus* cultivation, using only immobilised metal affinity chromatography. This resulted in yields of up to 12 mg purified pernisine per 1 L culture. No activation step was needed to obtain proteolytic activity in this case. This pernisine synthesised in *S. rimosus* had similar biochemical characteristics to the native pernisine, and was also proteolytically active against infectious prion aggregates from bovine brain.

CONCLUDING REMARKS

Remarkable stability and proteolytic activity towards infectious prion protein aggregates make pernisine suitable for use in industrial applications, where enzyme robustness is often desired. The properties of pernisine that have been reviewed in this article support the use of hyperthermophilic archaea as a promising source of exceptionally stable and industrially relevant enzymes. However, feasible industrial-scale bioprocesses for pernisine production need to be developed to enable purification of sufficient amounts of this enzyme for commercial use. The production of recombinant pernisine was already developed in *Escherichia coli* and is in progress in *Streptomyces rimosus*. Further understanding of the mechanisms of pernisine thermostability and its auto-activation is needed in order to simplify downstream processing for specific industrial applications of pernisine.

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