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Cloning, expression, and structural modeling of two alkaline serine protease genes from extremophilic *Bacillaceae*-related species: Application in valorization of invasive crustaceans

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Abstract

Two novel protease genes *sapA* and *sapN* from the thermophilic *Anoxybacillus kamchatkensis* M1V and *Melghiribacillus thermohalophilus* Nari2A^T strains respectively, encoding a polypeptide of 381 and 379 residues, were identified, cloned and successfully heterologously expressed in *Escherichia coli* BL21(DE3)pLysS. The deduced putative amino-acid residues of SAPA and SAPN enzymes evinced identity with proteases from *Bacillus* strains. The highest sequence identity value (95%) of SAPA was obtained with peptidase S8 from *Bacillus subtilis* WT 168, but with 16 amino-acids of difference. While, the highest sequence identity (97.10%) of SAPN was observed with *Bacillus licheniformis* MP1 protease, but with 10 difference residues. rSAPA and rSAPN enzymes were purified until homogeneity, characterized, and compared to wild-type proteases. The purified recombinant enzymes rSAPA and rSAPN were two monomers of about 28 and 30 kDa, correspondingly. rSAPA displayed the highest activity at pH 11 and 70°C. While, rSAPN displayed the highest activity at pH 10 and 75°C. To initiate structure-function relationships, a 3D-model of the Pro-SAPA and Pro-SAPN proteins were thereafter built based on the available structures of common proteases. The comparative molecular modeling studies with the less thermostable protease, revealed extra charged residues at the surface of SAPA and SAPN potentially participating in the formation of intermolecular hydrogen bonds with solvent molecules or generating salt bridges, therefore contributing to the higher thermal stability.

Keywords: Recombinant proteases; *Anoxybacillus kamchatkensis*; *Melghiribacillus thermohalophilus*; Comparative modeling.

1. Introduction

Nature has evolved microbial enzymes for millions of years towards the catalysis of fastidious reactions in a diversity of environments. Seeing as the introduction of biochemistry, microbial proteases have been the most extensively studied enzymes [1, 2]. In fact, they have gained significant attention due to their key role in metabolic activities and substantial use in industries [3], with an annual rate running somewhere about several hundred tons. The leading factor prompting attention to serine alkaline proteases (SAPs) is the trade utility of this category of extremozymes [4-6]. Both the scientific community and industrial specialists embraced the innovative protein engineering attempts, since having a subtilisin fulfilling all industrial requirements is therefore like looking for a needle in a haystack. Noticeably, SAPs are key biocatalysts enzymes, used in many sectors likewise laundry detergents, peptides synthesis, food production, leather processing, and

waste bio-conversion [4,6,7]. The preparation of a high value protein hydrolyste from marine by-products is one of main target of proteolytic enzymes engineering and technology.

These fields are focused on optimization and production approaches, purification until homogeneity, and characterization in order to select the appropriate proteases [4, 7]. For particular interest, the microorganisms called extremophiles, which are colonized the environments characterized by extreme levels of temperature, pressure, pH, and salinity secreted an extraordinary biomolecules that are functional and stable at cruel conditions which result in the precipitation or denaturation of the other ones [4].

Since extremophiles can provide these new biomolecules meeting many industrial needs, many researchers and industries are more and more focusing on these microorganisms [8]. That does not prevent the necessity of enzyme engineering and gene expressions are to improve native proprieties and potentially introduce innovative functionalities of proteolytic enzymes [9, 10]. Thereby, much attention has been paid towards the development and the characterization of efficient expression vectors. Recombinant DNA strategies are tools extensively applied for the protein expression in cells from Prokaryotic or Eukaryotic origin for fundamental and practical seeking purposes to obtain tailor-made subtilisins with desired functions and yield. Consequently, it is crucial to investigate the purification and secretion procedure to improve the expression effectiveness for the homologous/heterologous enzymes expression by optimizing vectors and host strains. Prokaryote and Eukaryote host strains are procurable for protein expression system. Bacteria (*Bacillus subtilis* and *Escherichia coli*), yeasts (*Pichia pastoris* and *Saccharomyces cerevisiae*), and filamentous fungi (*Aspergillus oryzae* and *Trichoderma reesei*) are considered as the most employed, by benefiting from the capacity of each organism for the amelioration of the expressed protein. In addition, structural studies are critically important for understanding the molecular mechanisms of these proteases. The folding and structure predictions can be used to deduce catalytic active sites and potential binding partners and interfaces. Besides, in silico screening can be performed on close enzyme homologues with known structures. Owing to the ease of expression and purification and 3D-structures availability, bacterial proteases befit the pattern of a bioengineering system.

As a branch of an ongoing project aiming at the study of novel attractive hydrolases from extremophilic *Bacillaceae*-related species, we have previously isolated two protease producing bacteria, identified as *Anoxybacillus kamchatkensis* strain M1V and *Melghiribacillus thermohalophilus* gen. nov., sp. nov., Nari2A^T. The Algerian M1V and Nari2A^T strains were isolated respectively from Hammam Righa[1] hot spring and from the soil of Chott Melghir[2]Salt Lake. This article focuses, on the cloning, and structural modeling of *sapA* and *sapN* genes encoding two extracellular proteases SAPA and SAPN expressed in *E. coli*, correspondingly from M1V and Nari2A^T strains. The 3D-structural model of these enzymes was built following the cloning and expression of the *sapA* and *sapN* genes, and used to understand the interactive features of these proteases in these structural forms. The ability of rSAPA and rSAPN to deproteinize shrimp and blue crab by-products was also investigated.

2. Materials and Methods

2.1. Materials

All substrates and chemicals were regent grade unless specified otherwise. General reagents were obtained from commercial suppliers. The Hammerstein casein (Merck, Darmstadt, Germany)[3] was used as a substrate for proteolytic activity assay. DNA kits and materials were Invitrogen brand. The T100™ Thermal Cycler and DC protein analysis kit, for Bradford-based assay, were purchased from Bio-Rad Laboratories Inc. (Hercules, CA, USA)[4]. The DNA polymerase (*Pyrococcus furiosus*, pfu) was from Biotools (Madrid, Spain)[5]. The ABI DNA Genetic Analyzer Automated Sequencer 3100 was supplied by ABI (PRISM®, USA)[6]. The pH-STAT 907 Titrando was from Metrohm AG (Herisau, Switzerland)[7]. Ampicillin sodium salt USP

[1] Located in Ain Defla district in northwestern of Algeria
[2] Located in Biskra district in north-east of Algeria
[3] https://www.sigmaaldrich.com/
[4] https://www.bio-rad.com/
[5] https://www.bionity.com/
[6] https://www.thermofisher.com/dz/en/home.html
[7] https://www.metrohm.com/en/

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was purchased from Bio Basic (Markham, Canada Inc.)[8]. Isopropyl-thio-*β*-D-galactopyranoside (IPTG) and lysozyme were bought from Sigma-Aldrich Co, (St Louis, MO, USA). The culture media used were Luria-Bertani (LB) composed of yeast extract, 5 g; NaCl, 5 g; and peptone, 10 g per liter. The LB media was complemented with 18 $g.L^{-1}$ agar to make solid media.

2.2. Strains and vectors

Two *Escherichia coli* strains: DH5α, Shot®TOP10, and BL21(DE3)pLysS came from Thermo Fisher Scientific, Inc., (Waltham, MA, USA) were used for cloning and expression of enzymes. Two wild-type extracellular protease SAPA and SAPN isolated respectively from Nari2A^T and M1V strains were used as the reference proteases [9, 10]. The pCR-Blunt™ was purchased from Invitrogen (Carlsbad, CA, USA). The pUT57 derivative from pUC19 and pTrc99A were from CAYLA (Toulouse, France) and Addgene (LGC Standards, Teddington, UK), respectively. The wild-type extracellular proteases SAPA and SAPN isolated from M1V and Nari2A^T strains, correspondingly were used as the reference enzymes [9, 10].

2.3. Amplification of *sapA* and *sapN*genes

General molecular biology protocols were determined as described by Sambrook *et al.* [11]. The amplification of the *sapA* and *sapN* genes encoding the SAPA and SAPN enzymes was performed using the genomic DNA of the strains *Anoxybacillus kamchatkensis* M1V and *Melghiribacillus thermohalophilus* Nari2A^T, respectively as a template. DNA amplification was performed with the Techne-Prime G Gradient Thermal Cycler, 96×0.2 mL. The amplification reaction mixtures (100 µL) contained 30 pg of each primer, 300 ng of DNA template, amplification buffer, and 2U of DNA polymerase. The cycling parameters used were 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, primer annealing at 60°C for 45 s, and extension at 72°C for 90 s. The PCR products were then purified using an agarose gel extraction kit (Jena Bioscience, GmbH, Germany)[9].

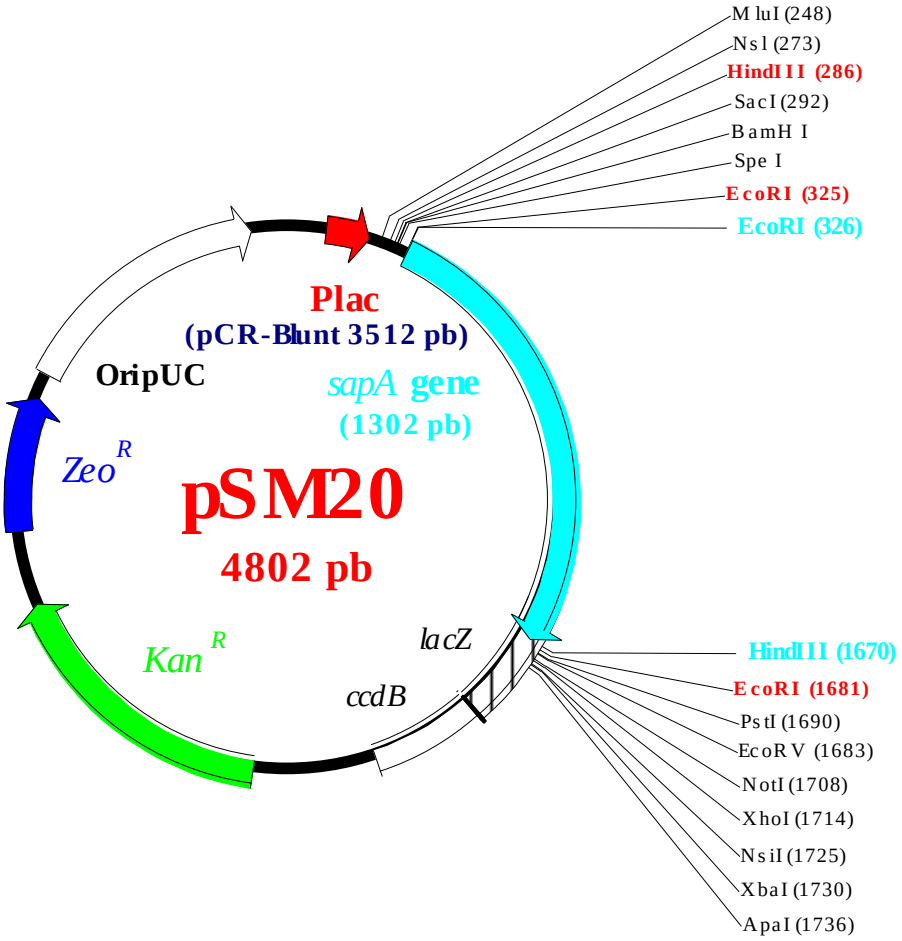
The amplification of the complete *sapA* gene and its flanking regions was performed using two primers: F-MS205′-CTGAGAAAGAAAAGGTTA-3′ and R-MS215′-GTTGCTGCTGTTTACGCA-3′, carrying flanking restriction site EcoRI. The purified ~1.3 kb PCR fragment was purified and cloned inpCR-Blunt™ into *E. coli* DH5α host strain, loading to a plasmid called pSM20 (Figure 1A).

The amplification of the complete *sapN* gene and its neighboring regions was performed using two primers: F-MS155′-CTGAGAAAGAAAAGGTTA-3′ and R-MS16 5′-TCAAGATTTTTAAATAC-3′, carrying flanking restriction site EcoRI. The purified ~1.4 kb PCR fragment was purified and cloned inpCR-Blunt™ into *E. coli* TOP10 host strain, loading to a plasmid called pSM30 (Figure 1B).

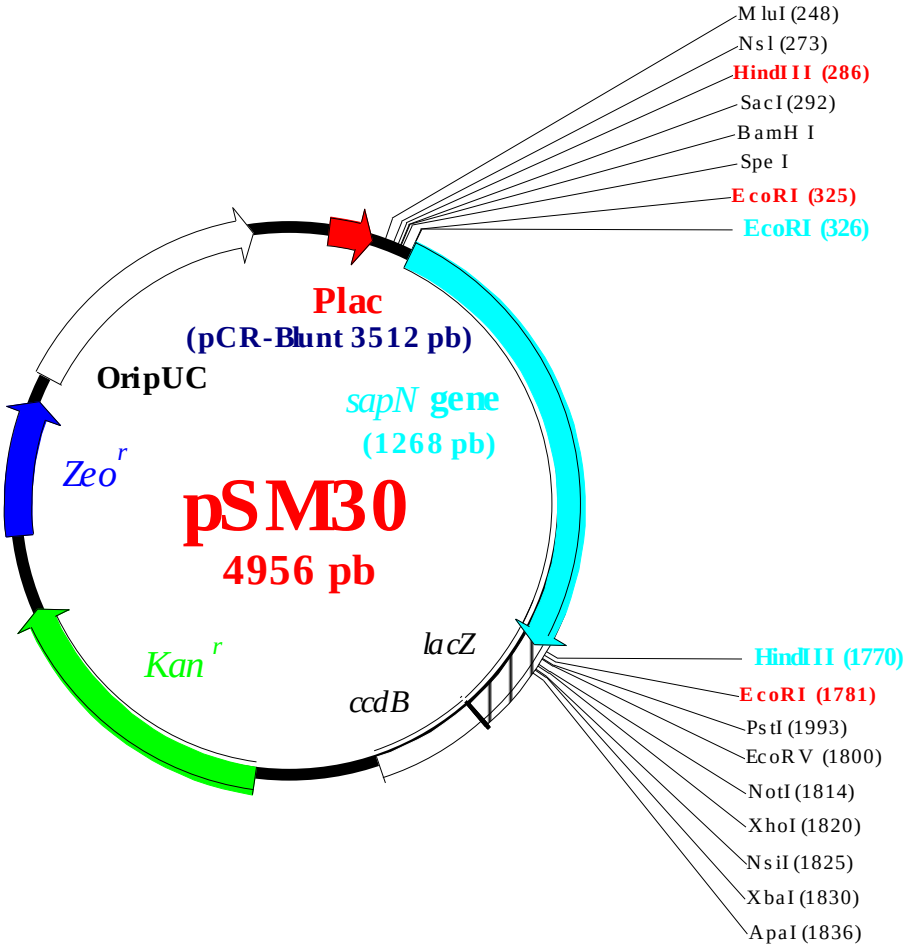
The upstream F-MS15 andF-MS20 primers and downstream R-MS16 and R-MS21 primers were elaborated on the basis of the high sequence similarity found in *Bacillus* and homologous genes. Recombinant clones were grown in LB agar medium supplemented with 100 $µg.mL^{-1}$ kanamycin sulfate, 160 $µg.mL^{-1}$ X-Gal, and 360 $µg.mL^{-1}$ IPTG. The colonies were screened by restriction digestion and by performing PCR amplification

The plasmids harboring DNA-inserts of predictable lengths were isolated using the DNA GeneJET Plasmid Miniprep Kit. The recombinant plasmids, termed pSM20 and pSM30, containing the open-reading-frame (ORF) encoding the pre-pro-enzyme SAPA and pre-pro-enzyme SAPN, correspondingly was used for extensive sequencing.

[8] https://www.biobasic.com/
[9] https://www.jenabioscience.com/


M luI (248)
Nsl (273)
HindI I I (286)
SacI (292)
BamH I
Spe I
EcoRI (325)
EcoRI (326)
Plac
(pCR-Blunt 3512 pb)
OripUC
sapA gene
(1302 pb)
Zeo R
pSM20
4802 pb
Kan R
lacZ
ccdB
HindI I I (1670)
EcoRI (1681)
PstI (1690)
EcoR V (1683)
NotI (1708)
XhoI (1714)
NsiI (1725)
XbaI (1730)
ApaI (1736)

(A)



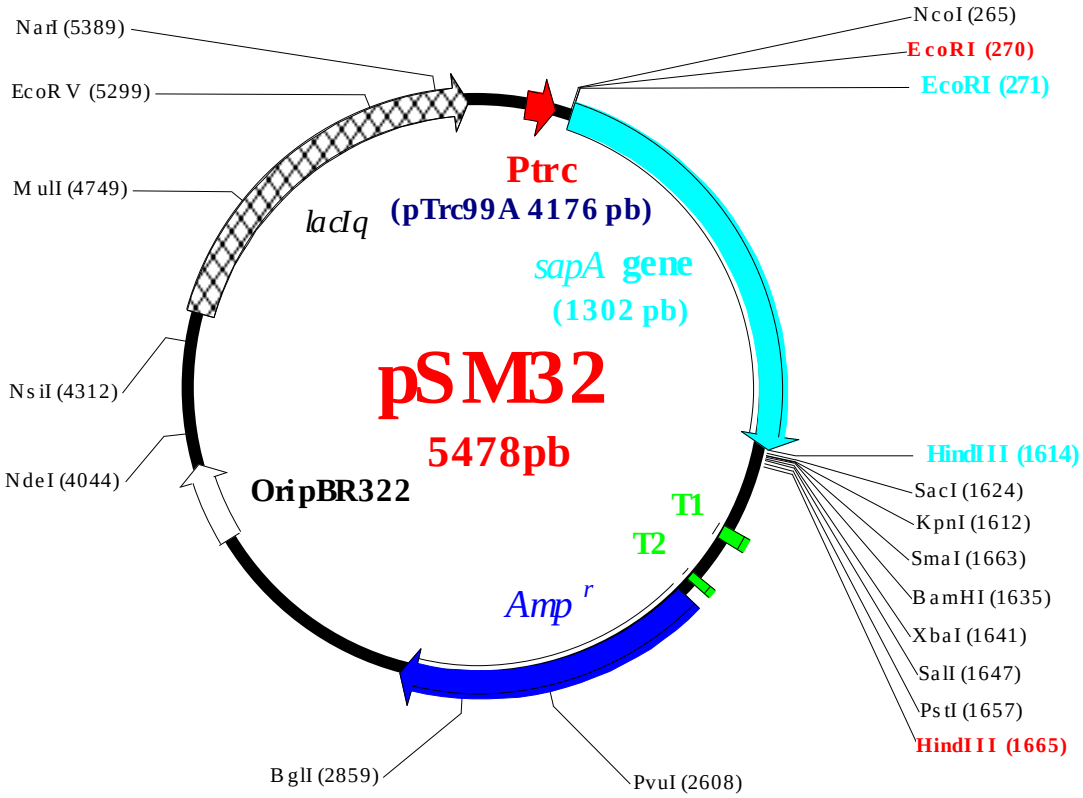
(B)

Figure 1: Maps of pSM20 (A) and pSM30 (B) plasmids

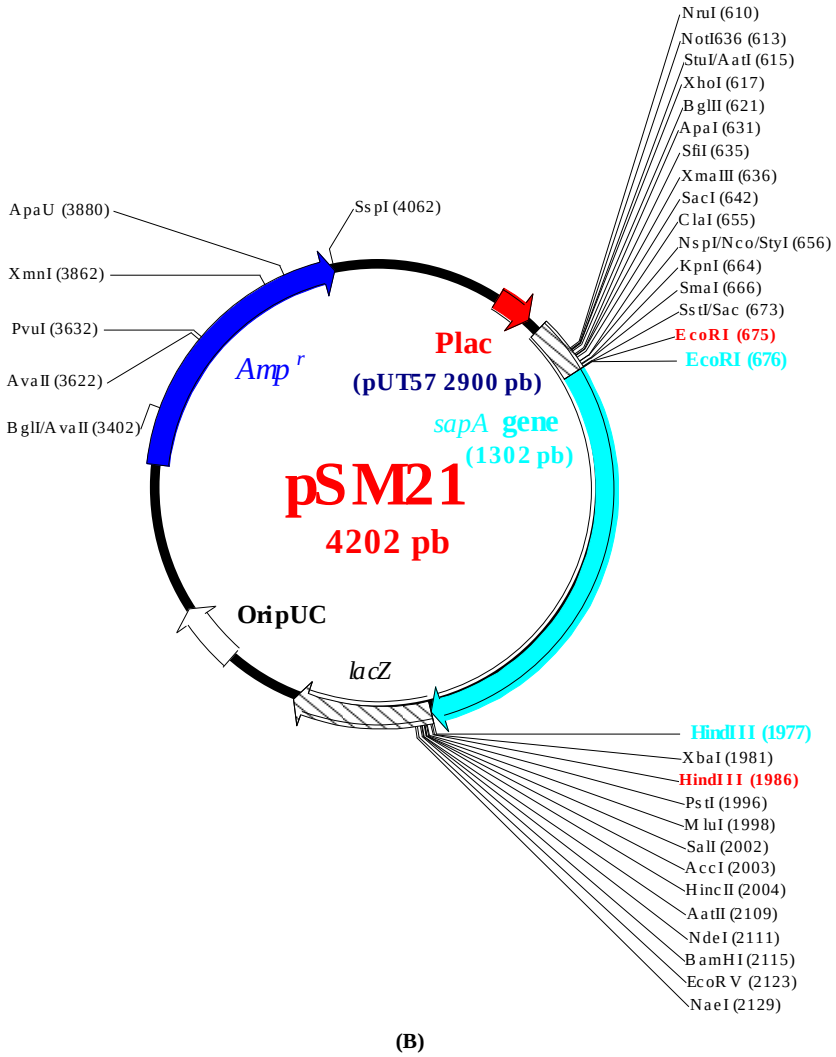
2.4. Cloning and expression of *sapA* and *sapN* genes

The pSM20 plasmid was digested with EcoRI restriction enzyme and used for expression studies. The resulting DNA fragment, harboring the *sapA* encoding gene, was sub-cloned in the pUT57 vector into an *E. coli* BL21(DE3)pLysS previously digested with *Eco*RI enzyme giving the pSM21 plasmid (Figure 2.A).

Based on this construction, the *sapA* gene was generated as an EcoRI fragment and sub-cloned in the pTrc99A vector linearized with the same restriction enzyme leading to pSM21 plasmid, noticed to harbor the *sapA* encoding gene, was sub-cloned in the pTrc99A vector into an *E. coli* BL21(DE3)pLysS under the control of the inducible P*tac* promoter that was previously digested with the *Eco*RI restriction enzyme leading to the pSM22 plasmid (Figure 2.B).


NcoI (265)
EcoRI (270)
EcoRI (271)
NarI (5389)
EcoR V (5299)
MulI (4749)
Ptrc
(pTrc99A 4176 pb)
lacIq
sapA gene
(1302 pb)
pSM32
5478pb
OripBR322
T1
T2
Amp r
NsiI (4312)
NdeI (4044)
HindIII (1614)
SacI (1624)
KpnI (1612)
SmaI (1663)
BamHI (1635)
XbaI (1641)
SalI (1647)
PstI (1657)
HindIII (1665)
BglI (2859)
PvuI (2608)

(A)

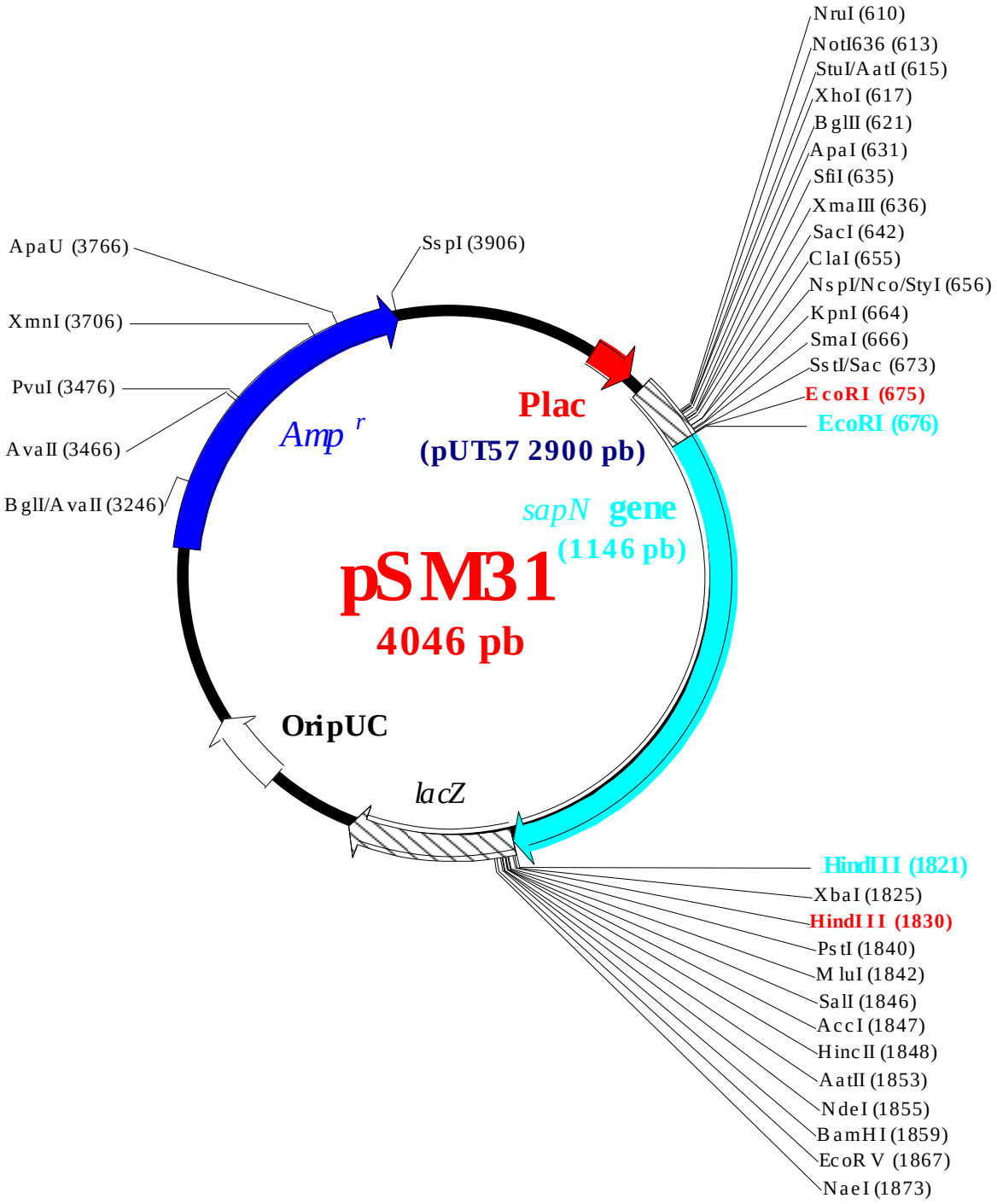


(B)

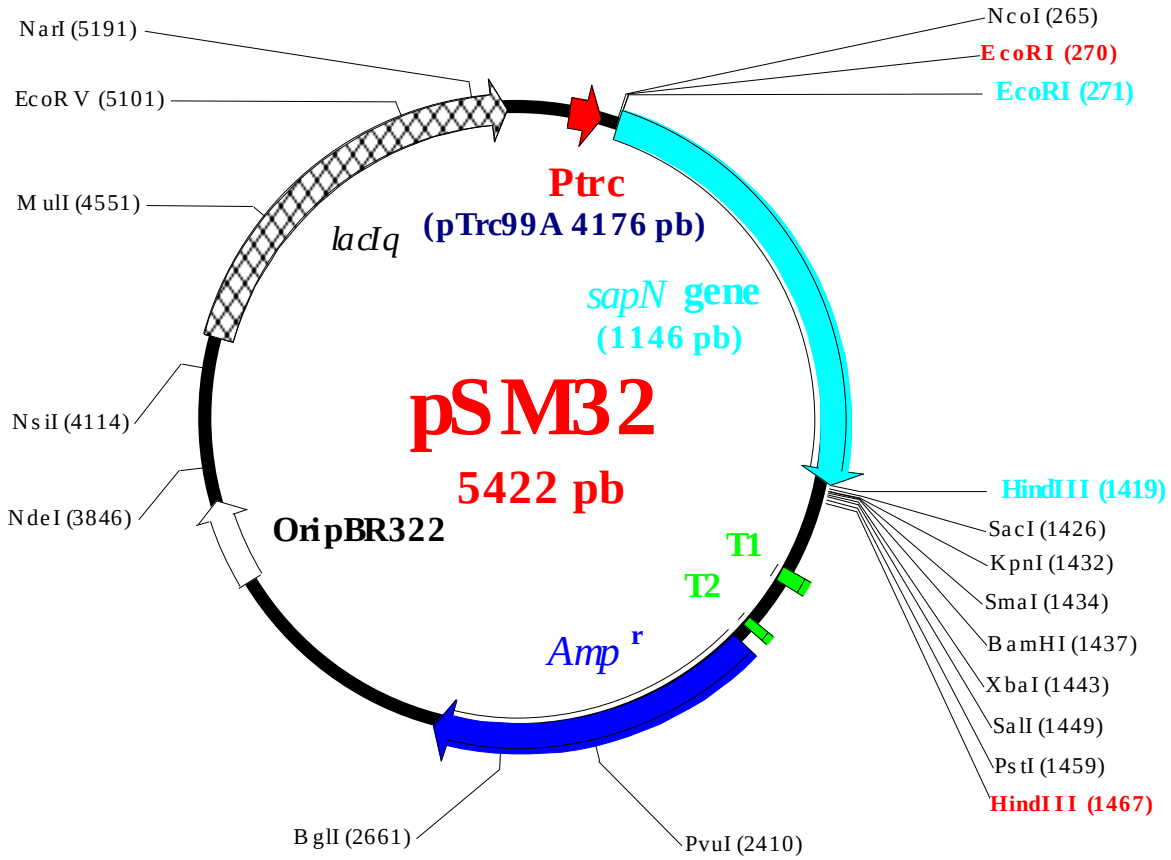
Figure 2: Maps of pSM21 (A) and pSM22 (B) plasmids.

The ORF region encoding SAPN was amplified with F-MS19 5′-GCGAATTCATGATGAGGAAAAA GAGTTTTTG-3′ and R-MS20 5′-CGAAGAAGCTTAT TGAGCGGCAGCTTC-3′ primers by using pSM30. The PCR fragment containing the *sapN* gene was then double restricted with EcoRI and HindIII and inserted into the pUT57 and pUC19 vectors leading to pSM31 (Figure 3.A) and pSM32 (Figure 3.B) plasmids, respectively, containing the pre-pro-enzyme SAPN (Figure 1). The pSM31 and pSM32 ligations served to transform *E. coli* strain BL21(DE3)pLysS.

The transformants were selected on LB agar with 100 µg.mL^{-1} ampicillin, 160 µg.mL^{-1} X-Gal, and 360 µg.mL^{-1} IPTG by the means of the blue white screening method. The constructions were verified by sequencing.


NruI (610)
NotI636 (613)
StuI/AatI (615)
XhoI (617)
BglII (621)
ApaI (631)
SfiI (635)
XmaIII (636)
SacI (642)
ClaI (655)
NspI/Nco/StyI (656)
KpnI (664)
SmaI (666)
SstI/Sac (673)
EcoRI (675)
EcoRI (676)
ApaU (3766)
SspI (3906)
XmnI (3706)
PvuI (3476)
AvaII (3466)
BglI/AvaII (3246)
Amp r
Plac
(pUT57 2900 pb)
sapN gene
(1146 pb)
pSM31
4046 pb
OripUC
lacZ
HindIII (1821)
XbaI (1825)
HindIII (1830)
PstI (1840)
MluI (1842)
SalI (1846)
AccI (1847)
HincII (1848)
AatII (1853)
NdeI (1855)
BamHI (1859)
EcoRV (1867)
NaeI (1873)

(A)



(B)

Figure 3: Maps of pSM31 (A) and pSM32 (B) plasmids.

2.5. Production and purification of the rSAPA and rSAPN

The transformants were grown in LB broth containing ampicillin and IPTG. After reaching an absorbance ($A_{600\ nm}$) of 0.8 units, the expression of each recombinant was induced by the addition of IPTG (0.5 and 0.4 mM for SAPA and SAPN, respectively). Next, the *E. coli* BL21(DE3)pLysS/pSM22 and *E. coli* BL21(DE3)pLysS/pSM32 were cultivated during 14 h (200 rpm and 37°C) and centrifuged. The culture supernatant was used for rSAPA and rSAPN purification, using the same protocol as for the native enzymes [9, 10].

After a centrifugation for 30 min at 10,000 ×g of a 500 mL of each culture of *E. coli* BL21(DE3) pLysS/pSM22 and *E. coli* BL21(DE3) pLysS/pSM32, the both recombinant enzymes were purified to homogeneity by ammonium sulfate fractionation, heat treatment, followed by sequential column chromatographies. For the rSAPA enzyme, the proteins were subjected to a precipitation with $(NH_4)_2SO_4$ at 35% and then centrifuged for 20 min at 10,000 ×g. The obtained supernatant was saturated up to 70% with $(NH_4)_2SO_4$, re-centrifuged, re-suspended in a minimal volume of 25 mM PIPES buffer at pH 6 supplemented with 2 mM $CaCl_2$, and dialyzed overnight against the repeated changes of the same buffer. Hence, the obtained sample was re-centrifuged and loaded onto a UNO Q-6 column (12 mm × 53 mm) (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using FPLC system. The column was washed extensively with buffer B. The elution of proteins was executed by buffer B, containing a linear of NaCl gradient 0 to 500 mM at a rate of 30 mL/h. The collected of fractions was carried manually, and estimated by measuring absorbance at

280 nm. Fractions containing rSAPA enzyme activity, were concentrated and applied to HPLC system using a ZORBAX PSM 300 HPSEC (6.2 mm × 250 mm), Agilent Laboratories, pre-equilibrated with 25 mM HEPES buffer at pH 8 supplemented with 2 mM $CaCl_2$. Fractions of each peak were collected at a flow rate of 30 mL/h and analyzed for caseinolytic activity and protein concentration. While, for rSAPN enzyme, the supernatant was precipitated to 35% with solid $(NH_4)_2SO_4$ and then re-centrifuged. Next, the clear supernatant was saturated to 60% $(NH_4)_2SO_4$ and centrifuged; the pellet was dissolved in a minimal volume of 50 mM Tris–HCl buffer at pH 8 supplemented with 2 mM $CaCl_2$. The clear supernatant was heated for 30 min at 80°C. The insoluble denatured proteins were removed by centrifugation. The supernatant was loaded on a Sephacryl S-200 HR column (2.5 cm × 150 cm) pre-equilibrated with 500 mM Tris–HCl buffer (pH 8). The pooled active fractions were then injected onto a UNO Q-6 column pre-equilibrated with 20 mM MOPS buffer at pH 7 supplemented with 2 mM $CaCl_2$. The column was washed extensively with the same buffer. The proteins were eluted with 20 mM MOPS buffer (pH 7) containing a linear gradient of NaCl from 0 to 500 mM at a rate of 30 $mL.h^{-1}$. The pooled fractions containing rSAPA and rSAPN activity were concentrated in centrifugal micro-concentrators (Amicon Inc., Beverly, MA, USA) with 10 kDa cut-off membranes and were stored at -20°C in a 20% glycerol (v/v) solution and then used for the determination of the biochemical properties.

2.6. Assay of proteolytic activity

Protease activity was investigated through the accumulation of free tyrosine resulting from casein degradation using the Folin-Ciocalteu reagent [12]. The rSAPA activity, was assessed at 70°C and bicarbonate-NaOH buffer (100 mM + 2 mM $CaCl_2$) at pH 11. While for rSAPN protease reaction, the activity was assayed at 75°C and glycine-NaOH buffer (100 mM + 2 mM $CaCl_2$) at pH 10. Then, 2.5 mL of TCA (200 $g.L^{-1}$) were added to arrest the reaction. 30 min later, the cooled mixture was spin down (15,000 rpm, 20 min). The supernatant (0.5 mL) was merged with 500 mM Na_2CO_3 (2.5 mL) and Folin-Ciocalteu's phenol (0.5 mL), accompanied by incubation during 30 min at 23±2°C. One unit of SAPN activity corresponds to protease quantity necessary to generate one µg of tyrosine/min.

2.7. Protein determination and analytical methods

The protein contents were ascertained according to Bradford's method using the DC protein assay kit with bovine serum albumin as a standard [13]. The rSAPA and rSAPN molecular masses in denatured forms was resolute by the means of polyacrylamide gel electrophoresis (PAGE) under reducing conditions, using 12% separating gel (pH 8.8) and 5% stacking gel (pH 6.8) as described previously by Laemmli [14]. The protein bands were visualized with Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, Inc., Hercules, CA, USA)[10] staining. Substrate-PAGE (Zymography staining) was estimated by incorporating azocasein (1 $mg.mL^{-1}$) into the separating gel before polymerization as detailed [15]. To remove SDS, the gels were rinsed twice, after the electrophoresis, for 30 min in 2.5% Triton X-100. The rSAPA and rSAPN protease activities were elucidated by the occurrence of clearance zone around the protein band on the blue background. Bands of purified recombinant enzymes were separated on SDS gels, transferred to a ProBlott membrane, and then the NH_2-terminal sequence analysis was performed by automated Edman degradation using an Applied Bio system Model 473A gas-phase sequencer. The sequence was compared with those in the Swiss-Prot / TrEMBL data base with homology search[11].

2.8. Biochemical characterization of the purified rSAPA and rSAPN

2.8.1. Behaviour against pH

The activity of the purified recombinant enzymes was measured at a pH range of 3 to 13 at 70°C for rSAPA enzyme and at 75°C for rSAPN enzyme and at using casein as substrate. Its pH stability was determined by pre-incubation of each recombinant enzyme in buffer solutions with different pH values for 24 h at 40°C. Aliquots were withdrawn, and residual enzymatic activity was determined at pH 11 and 70°C

[10] http://www.bio-rad.com/
[11] www.ncbi.nlm.nih.gov/blast

for rSAPA and at pH 10 and 75°C for rSAPN. The following buffer systems, supplemented with 2 mM $CaCl_2$, were used at 100 mM: glycine-HCl (pH 3-5), MES (pH 5-6), PIPES (pH 6-7), HEPES (pH 7-8), Tris-HCl(pH 8-9), glycine-NaOH (pH 9-11), bicarbonate-NaOH (pH 11-11.5), sodium phosphate dibasic-NaOH (pH 11.5-12), and potassium chloride-NaOH (pH 12-13).

2.8.2. rSAPA and rSAPN thermoactivity and thermostability assessment

The effect of temperature on each recombinant enzyme was examined at 30°C to 90°C for 15 min at pH 11 for rSAPA and at pH10 for rSAPN using casein as substrate. Their thermostability was determined by incubation for 24 h at 50°C to 80°C in the appropriate buffer for each enzyme in the presence or in the absence of 2 mM $CaCl_2$. Aliquots were withdrawn at specific time intervals to test the residual activity under standard conditions. The non-heated enzymes, which were cooled on ice, were considered as a control (100%).

2.8.3. Substrate specificity

The substrate specificity of rSAPA and rSAPN was carried using a variety of natural and modified protein substrates as well as esters. The substrate specificity profile of rSAPA and rSAPN was also investigated using some synthetic peptides substrates. Enzymatic activity assays were determined on each substrate according to standard conditions aforementioned [4].

2.9. Hydrolysis assessment of shrimp and crab by-products

The degree of hydrolysis (DH) using crustaceans was determined at pH 11 and 70°C for rSAPA and at 75°C and pH 10 for rSAPN. An amount of 2 g of shrimp or crab by-product was dissolved in 100 mL of each appropriate assay buffer and then treated with 2,000 U of each enzyme. The pH was titrimetrically maintained constant by using the pH-Stat set at the desired pH value of each enzyme by continuous addition of 4 N NaOH [12] as given below:

$$\alpha = \frac{10^{pH-pK}}{1+10^{pH-pK}} \quad (1)$$

where *B* refers to the amount of the base consumed (mL) to keep the pH constant during the reaction, *Nb* to the normality of the base, *MP* to the mass (g) of protein (N×6.25), and α to the average dissociation degree of the α-NH_2 groups released during hydrolysis expressed as:

$$\text{D(H) (\%)} = \frac{h}{h_{tot}} \times 100 = \frac{B \times Nb}{MP} \times \frac{1}{\alpha} \times \frac{1}{h_{tot}} \times 100 \quad (2)$$

where *pH* and *pK* refer to the values at which the proteolysis was performed and h_{tot} refers to the total number of peptide bonds in protein substrate (meq.g^{-1} of protein for casein; h_{tot} = 8.6) [16].

2.10. Sanger sequencing, in silico tools, and comparative modeling

The DNA sequencing of the *sapA* and *sapN* genes cloned in *E. coli* strains was done at least three times on direct and reverse strands with the ABI DNA Genetic Analyzer Automated Sequencer 3100. The protein sequence alignments were accomplished through the Blast tools associated with Clustal Omega program and web portals (ExPASy, SWISS-PROT, and TrEMBL). The sequence similarities and secondary structure representation were analysed with the new version 3.0 of Easy Sequencing in Postscript (ESPript) web server [17]. The functional protein analyses were conducted using the InterPro database and MEROPS, the peptidase data base from the European Bioinformatics Institute (EMBL-EBI). Molecular enzymatic signatures and conserved motifs were also recognized *via* the InterProScan tool by scanning the SAPN protein sequence against the PRINTS database. The recombinant N-terminal sequences were compared to those in the UniProtKB and SWISS-PROT protein databases. The 3D modeling of Pro-SAPA and Pro-SAPN was achieved with the SWISS-MODEL protein structure prediction tool. The final molecular model was built, analyzed, and visualized using COACH (a meta-server-based approach to protein-ligand binding site prediction) and PyMOL v. 0.99 software.

2.11. GenBank sequence depository

The *sapA* (1302 bp) and *sapN* genes (1348 bp) are deposited in the public GenBank repository below accession n°: MH979641 and MT292372, respectively.

2.12. Statistical analyses

The tests were executed at least 3 times independently, and the statistical analyses were performed. The results concerning the influence of pH, temperature, and hydrolysis degree on protease activity and stability were deemed to be statistically significant at $P<0.05$ and are presented with the standard error (±SE).

3. Results and discussion

3.1. Molecular characterization of the *sapA* and *sapN* genes

3.1.1. Sequencing of the sapA gene and amino-acid sequence inspection

The investigation of the nucleotide sequence of the *sapA* gene and its flanking DNA regions revealed the presence of an ORF of 1,143 bp that encoded a pre-pro-enzyme, consisting of 381 aa. It is started with an ATG codon at nucleotide position 1 and terminated with a TAA stop codon. The pre-pro-protease organized into three regions. Using the SignalP program, version 3.0 putative signal sequence prediction, we revealed the presence of a signal peptide of 29 aa (87 bp, from the residue of Met1 to the residue of Ala29). This peptide involved in the secretion of the enzyme is composed of a short, positively charged N-terminal sequence (3 Lys) followed by a large hydrophobic central domain of about 20 aa and a C-terminal domain containing a site cleavage (E28-A29-A30) recognized by the peptide signals peptidase. We also identified a sequence of 77 aa (231 bp) from the residue of Asn30 to the residue of Tyr106, which corresponds to the sequence of the pro-peptide. By homology to other serine proteases, we believe that this pro-domain plays a competitive inhibitor role for the enzyme in addition to its primary role in the correct folding of the enzyme. This role of intramolecular chaperone takes place via its IMC-domain and which will subsequently be self-proteolysed to generate the active mature protease of 275 aa (825 bp), ranging from the residue of Ala107 to the residue of Gln381 and having a predicted molecular mass, using the Expasy server, of 27 905.97 Da. A Shine-Dalgarno-like sequence was observed -12 to -8 bp upstream from the ATG codon. The presumed putative promoter region, at -42 (TTAACA) and -27 (TATATT) sequences, resembled to the consensus sequences determined for the promoter region by the lambda P_R RNA polymerase of *E. coli*. In addition, we have spotted the catalytic triad; Asp32, His64 and Ser221 in mature SAPA corresponding to the conserved residues (Signature of subtilases): 28-39, 64-75 and 216-226, characteristic of serine proteases. The SAPA protease has 3881 atoms with an atomic formula of $C_{1229}H_{1913}N_{337}O_{398}S_4$. SAPA has 17 negatively charged amino acids (Asp + Glu) and it has 13 positively charged amino acids (Arg + Lys) (Table 1). SAPA contains 42 amino acids Asx (16 Asn and 11 Asp), with a percentage of 9.8 mol%, while the SAPB proteases, subtilisin Carlsberg, and subtilisin Novo, and contain 12 respectively; 9.45; and 10.2 mol% of Asx.

This ORF was confirmed as the gene encoding SAPA, since, as determined by the Edman degradation method, the deduced amino-acid sequence was noted to include the 25 NH_2-terminal amino-acid sequence of SAPA. The *sapA* gene revealed 92% identity with subtilisin BSF1 from *Bacillus subtilis* A26, 95% identity with the sequence of peptidase S8 from *Bacillus subtilis*, 91% identity with subtilisin E from *Bacillus subtilis* WT 168, and 64% identity with subtilisin SAPB from *Bacillus pumilus* CBS [15, 18, 19]. The mature region of alkaline protease SAPA shares amino-acid residues identities of 259/275 (94%), 251/275 (91%), and 200/275 (73%), with the peptidase S8 (PepS8), subtilisin E, and subtilisin SAPB, respectively.

3.1.2. Sequencing of the sapN gene and amino-acid sequence inspection

A PCR fragment of about 1.4 kb coding *sapN* gene was cloned within the pCR™-Blunt vector using One Shot® TOP10 chemically competent *E. coli* host strain, therefore leading to pMS30, a recombinant vector for DNA sequencing. The *sapN* gene comprised 1140 bp-ORF and encoded a serine alkaline protease (subtilisin SAPN) comprising 379 aa. The 1140 bp-ORF instigated by an ATG codon and terminated by a TAA end codon. An empirical ribosomal binding site (RBS) formed by 4 bp to 9 bp upstream to the initiation site (ATG) and the regulator sequences putative promoter elements, at -35 (ATATTA) and -10 (AATTAT) were identified. This ORF is proven as the gene encoding SAPN, seeing

that the inferred putative amino-acid sequence integrated the 25 aa residues of the NH_2-terminal residues was the same as determined with Edman's degradation [9].

As stated for the assumed amino-acid residues, SAPN has a recognized pre-pro-sequence domain of 105 aa before maturation to the final subtilisin (274 aa). Indeed, the signal peptide depict to the inceptive 29 aa (from M1 to A2) shows typical features for targeting subtilisins such as a short positive N-terminal sequence (3 Lys) followed by a highly hydrophobic region of about 20 aa and a C-terminal domain containing a site of cleavage (A29-N30-Q31) which is broken by signal peptide peptidase during maturation. The pro-peptide sequence corresponds to 76 aa (from N30 to K105). This pro-domain would play a role of competitive inhibitor for the enzyme in addition to its primordial role in the correct folding of SAPN. In fact, once folding is completed, subtilisin begins an intramolecular chaperone (IMC) degradation to finalize the predecessor's maturation and which will subsequently be auto-proteolysed to generate the active mature subtilisin of 274 aa (A106 to of Q379), with a theoretical mass of 27434.58 Da and pI of 7.18.

Mature SAPN showed over 95.99% identity to subtilisin MP1 (GenBank accession n°: HM147766) with 10 aa of difference (F14D, P17Q, V23G, R77T, S78T, I109G, E147V, I148V, M233I, and G234L). However, SAPN showed 91.94% homology with subtilisin subC (GenBank accession n°: CAA62666.1) from *B. licheniformis* strains with a divergence of 22 aa. In addition, 66.91% of homology (91 aa of difference) with that of protease SAPA from *Anoxybacillus kamchatkensis* M1V and 40.66% homology (162 aa of difference) with that of SAPV produced by *Virgibacillus natechei* $FarD^T$ were noticed [10, 20]. These enzymes have the same linear order of the catalytic triad. Furthermore, mature SAPN contains conserved essential catalytic triad constituting the active site (D32, H63, and S220) and three conserved subtilisin motifs at positions: 23-42, 59-72, and 217-233 as described by Varshney *et al.*, [21]and confirmed by the InterProScan tool according to the PRINTS database. The presence of three critical residues in the catalytic site which are D32, H63, and S220, and based on statistically significant similarities of the amino-acid sequence with other serine alkaline proteases, SAPN should be assigned to the S8 subtilisin family. As noticed from resources for information on proteases, subtilisin-like clan of serine alkaline proteases is composed of two distinct families which are subtilases S8 and sedolisins S53. In the S8 family, the catalytic residues are ordered as follows: D32, H63/64, and S220/221. But in the S53 family, H63/64 is substituted by a glutamate residue (E63/64) [22].

It is particularly interesting to note that despite the high similarity percent, SAPN showed some singulars feature. It might be deduced that the replacement in mature SAPN form, noted to vary between MP1 and further subtilisins, contributed to upgrading the alkaline pH and thermal stability, as well as the catalytic efficiency of subtilisin. The SAPN has 5480 atoms with an atomic formula of $C_{1724}H_{2733}N_{473}O_{539}S_{11}$ with an extinction coefficient of 33350 M^{-1} cm^{-1} at 280 nm. It contains 37 amino acids Asx (22 Asn and 15 Asp), with a percentage of 9.8 mol%. SAPN has 27 negatively charged amino acids (Asp + Glu) and 32 positively charged amino acids (Arg + Lys) (Table 1).

Table 1
Amino acid composition of SAPA and SAPN.

Enzyme	**SAPA**		**SAPN**	
Amino-acids	Number	(%)	Number	(%)
Ala	34	12.4	55	4.5
Arg	4	1.5	7	1.8
Asn	16	5.8	22	5.8
Asp	11	4	15	4.0
Cys	0	0	0	0.0
Gln	9	3	8	2.1
Glu	6	2.2	12	3.2
Gly	35	12.7	39	10.3
His	6	2.2	8	2.1
Ile	16	5.8	16	4.2
Leu	14	5.1	21	5.5
Lys	9	3.3	25	6.6
Met	4	1.5	11	2.9
Phe	3	1.1	11	2.9
Pro	15	5.5	12	3.2
Ser	33	12	40	10.6
Thr	17	6.2	20	5.3
Trp	4	1.5	2	0.5
Tyr	13	4.7	15	4.0
Val	26	9.5	40	10.6

The protein amino-acid sequence has long been thought to be allied to enzymes thermostability. The statistical analyses highlighted trends toward substitutions such as G→A and K→R. Equally, more charged (17.67%), hydrophobic (45.1%), and aromatic

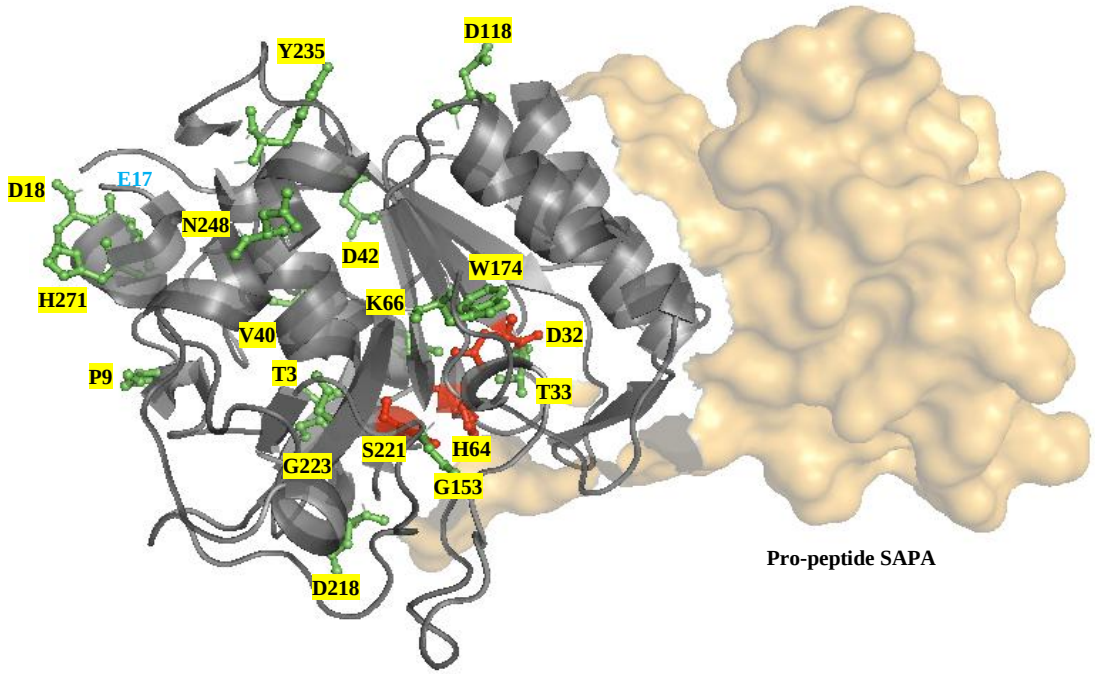
(9.5%) residues are found in SAPN than in mesophilic subtilisin. As explained in earlier research on thermophilic enzymes, a higher alanine content (14.5%) reflected the fact that alanine was the best helix-forming residue [23].

3.2. Homology modeling simulation

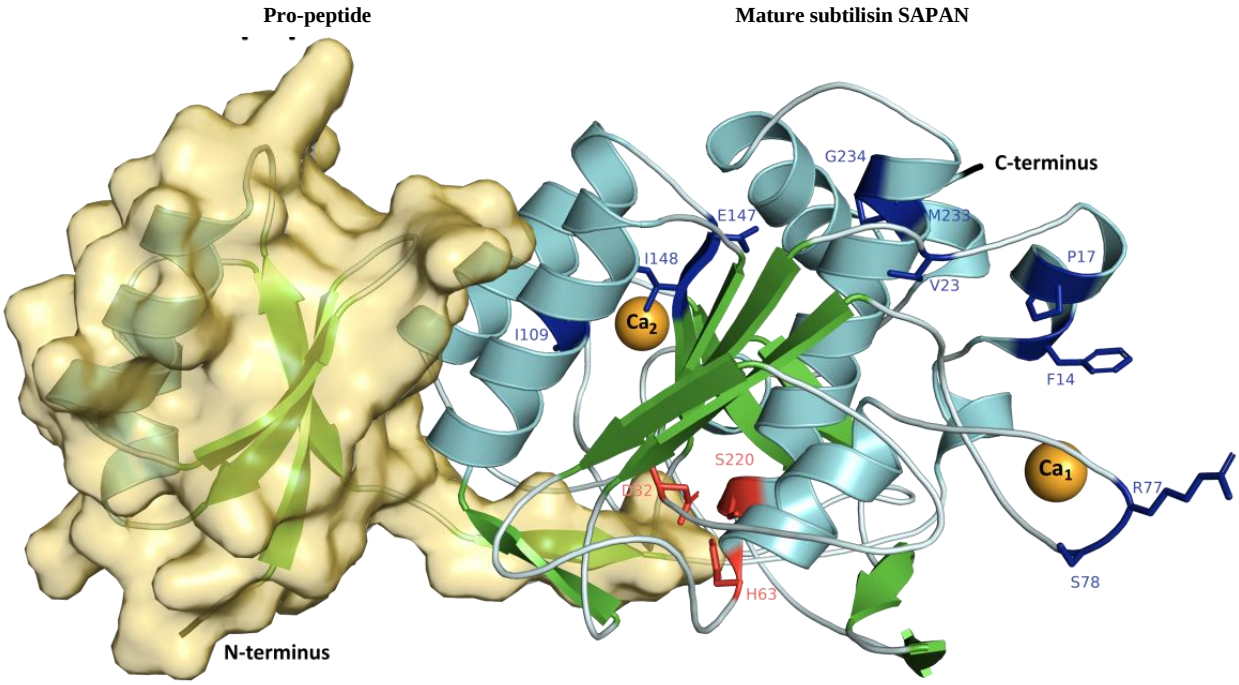
To provide an insight into the biochemical properties and further inspect structure-function correlations, the structure model of the SAPA enzyme was built, with the crystal structure of the subtilisin E (PDB ID: 1SCJ) as a template (Figure 4.A). The structure model of the SAPA has a globular configuration with an α/β hydrolase fold with a single compact domain that consists of 9 α-helices and 11 β-strands. The core structure consists of nine β-strands β-sheet [β2-β3-β1-β4-β5-β6-β7-β11-β10] (strand β10 is anti-parallel) ringed by α-helices [α1, α2, α3, α6, α7, α8, α9, and anti-parallel β8-β9 strand] on one side and α-helices α4 and α5 on the other side. Asp32, His64, and Ser221 are positioned in a catalytic triad-like structure. Whereas no crystal structure has yet been investigated for SAPA, the significantly strong sequence identity of 91% suggests that its 3D structure is likely to be similar to that of subtilisin E (PDB ID: 1SCJ). The model of SAPA was build using the 3D structure of the subtilisin E-pro-peptide. Both proteases shared very analogous overall folding and the root mean square deviation (RMSD) determined after the superposition of their Cα-atoms was approximately 0.5 Å.

The available 3D structure of Pro-subtilisin E (PDB ID: 3WHI) displaying 65% sequence identity with Pro-SAPN was used for building the 3D model of Pro-SAPN from *Melghiribacillusthermohalophilus*Nari2A^{T}. The root-mean-square deviation implying α-carbons between the initial and the optimized model was 0.85 Å, which indicates a good degree of similarity of the superimposed model and template; this suggests the reliability of the predicted structure for its use in further studies. The Ramachandran diagram of the final Pro-SAPN model generated using the MolProbity program revealed that 93% of the amino-acids are located in favored regions. The structural model of Pro-SAPN presents a globular form with a characteristic α/β folded hydrolase (Figure 4.B). This structure shows a unique and compact domain having eleven beta-strands and nine alpha-helices. It is arranged in a central structure comprising eight parallel beta-strands namely: β1-β7, β11, and the anti-parallel β10-strand, all bordered by seven alpha-helices, namely α1-α3 and α6-α9, in addition to the anti-parallel β8-β9-strands on one side and the α4- and α5-helices on the other side.

In the Pro-SAPN model, two calcium binding sites were predicted by the COACH tool using the 3D structure of Pro-subtilisin E (PDB ID: 3WHI) as template. The Pro-SAPN model shows the first calcium binding site (Ca_1 or high-affinity site), involving the oxygen atoms of 6 potential residues, namely Q2, D41, L75, N77, S79, and V81. In the Pro-subtilisin E (PDB ID: 3WHI), the Ca_1 is hepta coordinate with the oxygen atoms at the vertices of a pentagonal bipyramid. The second calcium binding site (Ca_2 or low-affinity site) potentially involves 3 residues, which are A169, Y171, and V174. The Ca_2 in subtilisin Carlsberg (PDB ID: 2SEC) is presumably occupied by a K^+ ion in subtilisin BPN′. A third calcium binding site in subtilisin Carlsberg (Ca_3) has a relatively high occupancy and low *B* factor, but the mean ligand distance of 2.85 Å is again more typical of a K^+ ion.



(A)



(B)

Figure 4: 3D-model of SAPA (A) and SAPN (B)

3.3. Genes cloning and expression

To express SAPA and SAPN, the corresponding gene was cloned downstream of P*T7* or P*tac* promoters then introduced to the *E. coli* strain BL21(DE3)pLysS. The intracellular and periplasmic fractions of all recombinant strains displayed no alkaline protease activity. Relatively high specific activities of 370 $U.mg^{-1}$ and 735 $U.mg^{-1}$ were, however, detected in the extracellular fractions of BL21(DE3)pLysS/pSM21 and BL21(DE3)pLysS/pSM22, respectively. Again, a relatively high specific activities of 2,517 $U.mg^{-1}$ and 9,375 $U.mg^{-1}$ were, however, detected in the extracellular fractions of BL21(DE3)pLysS/pSM31, and BL21(DE3)pLysS/pSM32, respectively. Based on this study, SAPA and SAPN were respectively most efficiently expressed with the P*tac-sapA* construction (pSM22) and P*tac-sapN* constructions (pSM32) which were, therefore, retained for the purification of the recombinant proteases (rSAPA and rSAPN). Our previous experimental results also indicated that the strong promoter P*trc* expression system allows high-level expression of protease activity [24].

The extracellular rSAPA and rSAPN were purified using the same strategy employed respectively for the native enzyme from *Anoxybacillus kamchatkensis*M1V and *Melghiribacillus thermohalophilus* Nari2A^{T}. The SDS-PAGE of purified rSAPA (Figure 5.A) and rSAPN (Figure 5.B) enzymes revealed a single band of 28 kDa and 30 kDa, respectively in size with reference to the standard LMW marker, which is similar to the native one. This is due to the absence of post-translational modification in Prokaryotic system.

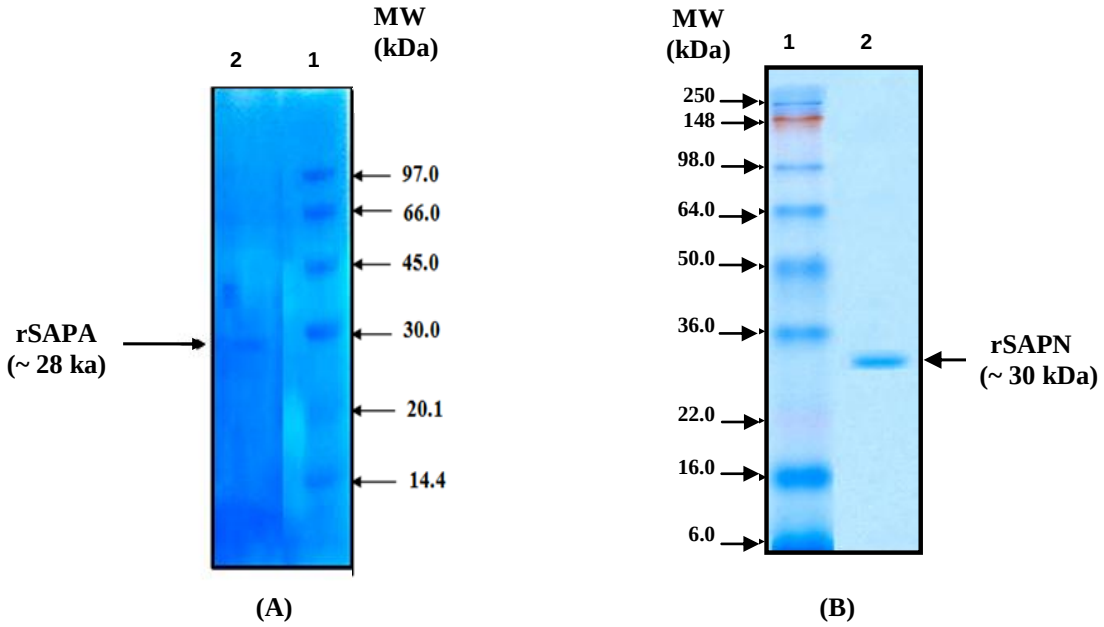


Figure 5: SDS-PAGE of rSAPA (A) and rSAPN (B).

3.4. Biochemical characterization of the rSAPA and rSAPN

All the biochemical characteristics identified from rSAPA and rSAPN were almost similar to those of the original enzymes. The large-scale preparation of rSAPA and rSAPN as a biocatalyst for biotechnological application can, therefore, be easily performed and may offer new promising opportunities for the enhancement of several biotechnological bioprocesses. Particularly, the pH and temperature optima of rSAPA were respectively of 11°C and 70°C. While, the rSAPN showed a optima pH and temperature equal to 10°C and 75°C. The relative rSAPA activity at pH 9 and 10 were, respectively, 90% and 98%. At pH levels of 5 and 6, the rSAPA activity was 25% and 42%, respectively. rSAPA was completely stable in the pH range of 8 to 12.

The half-life times of rSAPA at 60%, 70%, and 80°C increased to 32 h, 22 h, and 14 h in the presence 2 mM $CaCl_2$. The pH stability study revealed that rSAPN enzyme was completely stable in a pH range from 7 to 12 at 40°C. rSAPN displayed a half-live of 9 h for SAPN at 80°C. The substrate specificity profile of rSAPA and rSAPN revealed that these enzymes were able to degrade natural substrates with varying degrees. They were more active against casein compared to albumin, gelatin, ovalbumin, and keratin. When assayed with modified protein, there enzymes showed high level of hydrolytic activity with all the substrates tested with highest specificity for azo-casein, albumin azure, and keratin azure, however, displaying no activity towards collagen types I and II-FITC conjugates.

3.5. Hydrolysis assessment of shrimp and crab by-products

The potential protein hydrolysis ability of each enzyme was tested by investigating the extent of hydrolysis. As shown in table 2, rSAPN was the most efficient, with 34% protease used respectively during hydrolysis of blue swimming crab and white shrimp. These findings indicate that rSAPN can be useful for the preparation of protein and peptides hydrolysates. In fact, an optimal release of protein from blue swimming crab *Portunus segnis* or white shrimp *Metapenaeus monoceros*, which are two alien species in the Mediterranean Sea, while leaving chitin intact, is a promising industrial and biotechnology opportunity.

Table 2
Hydrolysis assessment of shrimp and crab by-products

Enzyme	**rSAPA**		**rSAPN**	
By-product	Shrimp	Crab	Shrimp	Crab
DH (%)	38	32	29	34

4. Conclusions

The gene cloning, amino-acid sequence inspection and homology modeling of two proteases SAPA and SAPN, respectively from M1V and Nari2A^T strains has been well detailed in this study. These recombinant enzymes are endowed with some attribute that are deeply appreciated for biotechnology applications. The structural interpretation provided insights into the catalytic mechanism of rSAPA and rSAPN. In view of the outstanding traits and properties of rSAPA and rSAPN, additional studies of these enzymes, especially at the structure-function relationship level, are needed. The exploration of the scale-up production and the storage stability of rSAPA and rSAPN under lyophilized and atomized forms are also required. Finally, the immobilization of these activities within organic and hybrid supports be the next challenges.

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