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Purification, biochemical characterization, and molecular elucidation of a new biotechnologically compatible serine peptidase from *Virgibacillus natechei* strain FarD^{T}

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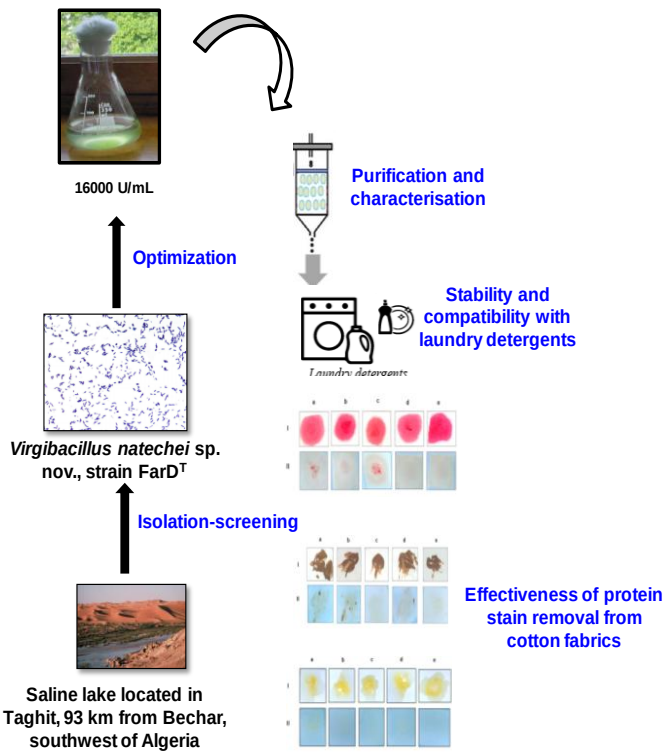
Graphical Abstract



Abstract.

A new peptidase designated as SAPV produced from a moderately halophilic *Virgibacillus natechei* sp. nov., strain FarD^{T} was investigated by purification to homogeneity followed by biochemical and molecular characterization purposes. Through optimization, it was determined that the optimum peptidase activity to be 16,000 U/mL in the optimized liquid medium that contains only white shrimp shell by-product as sole energy and carbon sources. The SAPV enzyme is a monomer protein with a molecular mass of 31 kDa. The sequence of its NH_2-terminal amino-acid residues showed homology with those of *Bacillus* peptidases S8/S53 superfamily. The SAPV showed optimal activity at pH 9 and 60 °C. The *sapV* gene was cloned, sequenced, and heterologously over expressed in the extracellular fraction of *E. coli* BL21(DE3)pLysS. The biochemical properties of the recombinant peptidase (rSAPV) were similar to those of native one. The highest sequence identity value (97.66%) of SAPV was obtained with peptidase S8 from *Virgibacillus massiliensis* DSM 28587, with 9 amino-acid residues of difference. Interestingly, rSAPV exhibited an excellent detergent stability and compatibility than Alcalase 2.4 L FG and Bioprotease N100L.

1. Introduction

Despite advances in understanding the diversity and systematics of bacilli, studying their hydrolytic enzymes with bioengineering interest and their characterization has received more attention. Of particular interest, *Virgibacillus* is a genus of Gram-positive bacteria belonging to the wider family of *Bacillaceae* within the *Firmicutes* phylum [1]. Most members of genus *Virgibacillus* are mostly isolated from saline environments like marine sediment, soil, fish sauce fermentation, and lake [2-5]. The genus showed the ability to produce a great variety of extracellular hydrolytic enzymes. For instance, *Virgibacillus* sp. strain SK37, *Virgibacillus halodenitrificans* strain RSK, *Virgibacillus* sp. strain CD6, and *Virgibacillus dokdonensis* strain VIT P14 have been shown to produce extracellular proteases [6-9]. However, information regarding stability and compatibility with laundry detergents, molecular modeling and structural characteristics as well as the docking study of proteases from *Virgibacillus* is still very limited. The assessment of bacterial diversity of an Algerian saline lake revealed the presence of a novel specie of a genus *Virgibacillus*, namely *Virgibacillus natechei* sp. nov., strain FarD^T with unusual phenotypic and genotypic characteristics [4]. In fact, the strain FarD^T was mesophilic, moderately halophilic and alkaliphilic. This strain grew in the presence of NaCl concentrations ranging from 1 to 200 g/L, with an optimum at 100 g/L. The temperature range for growth was (15-40 °C), with optimal growth occurring at 35 °C. The pH range for growth was from 6 to 12, with an optimum at 7 [4]. No enzymatic research regarding this new species has been found in the literature, and for the first time with the current study, a research into the purification, characterization, and biotechnological applicability of a new peptidase enzyme from strain FarD^T was investigated. Herein, the current research was undertaken to purify, characterize, and to express for the first time, a new peptidase secreted from the culture supernatant of the moderately halophilic bacterium *Virgibacillus natechei* strain FarD^T and explore its promising potential enzymatic performance as bioadditive laundry detergent composition.

2. Materials and Methods

2. 1. Materials

The raw material of shrimp shell was obtained in fresh conditions from a fishery market located at Sfax, Tunisia. The used comparative enzymes were: Thermolysin type X, Alcalase 2.4 L FG, Bioprotease N100L, and SPVP from *Aeribacillus pallidus* strain VP3 [10].

2.2. Methods

2.2.1. Isolation and identification of peptidase-producing of strain FarD^T

According to the phenotypic, morphologic, and molecular analysis, strain FarD^T is considered to represent a novel species of the genus *Virgibacillus* in the family *Bacillaceae*, order *Bacillales*, for which the name *Virgibacillus natechei* sp. nov., is proposed. The type strain of *Virgibacillus natechei* is FarD^T (DSM 25609^T or CCUG 62224^T) [4]. After incubation at 35 °C, a halo of casein degradation was revealed around the colony growth onto skimmed milk agar plates, as well described previously by the authors [10, 11].

2.2.2. Peptidase production

The pre-culture of strain FarD^T was carried out in a 1 L Erlenmeyer flask containing 100 mL of Sehgal and Gibbons liquid medium (SG) at pH 7 supplemented with 100 g/L NaCl and incubated at 35 °C for overnight. This pre-culture was used to inoculate the culture using the optimized enzyme liquid medium (ELM) at pH 7.4 containing only 60 g/L white shrimp shell by-product in Erlenmeyer flask. The initial A_{600} nm of the culture was 0.1.

2.2.3. Peptidase activity Assay

The peptidase activity was assayed using Kembhavi method [12]. One unit (U) of peptidase was defined as the amount of enzyme releasing 1 µg of tyrosine released under the assay conditions detailed. Peptidase activity present in the laundry detergent solution was determined through the method proposed by Boulkour Touioui et al. [13] which used the *N*,*N*-dimethylated casein (DMC) as a substrate and 2,4,6-trinitrobenzene sulfonic acid (TNBSA) as a colour indicator. One unit of protease activity was defined as the amount of enzyme required to catalyze the cleavage of 1 µmole of peptide bond from DMC per minute under the experimental conditions used. The absorbance was measured at 450 nm.

2.2.4. Peptidase purification procedure and analytical methods

500 mL of a 36 h culture of strain FarD^T was centrifuged for 20 min at 10,000×*g*. Proteins were precipitated to 20% with solid $(NH_4)_2SO_4$ and then centrifuged at 10,000×g for 20 min. The obtained supernatant was saturated up to 80% with $(NH_4)_2SO_4$, centrifuged and the precipitate was re-suspended in a minimal volume of buffer B composed with: 50 mM MOPS and 2 mM $CaCl_2$ at pH 7, and dialyzed overnight. Next, the obtained supernatant was loaded and applied to a HPLC system using a ZORBAX PSM 300 HPSEC column previously equilibrated with buffer B. Protein concentration was estimated through the method of Bradford [14], using bovine serum albumin as a reference. The molecular weight of the purified peptidase SAPV was carried out by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli [15]. Casein zymography staining was estimated by incorporating azo-casein (10 g/L) into the separating gel before polymerization as detailed elsewhere [16].

2.2.5. Biochemical characterization

Pre-incubation of the purified SAPV with specific inhibitors, reducing agents various and divalent metal ions was investigated as well described by Jaouadi et al. [16]. For the effect of metal ions, the non-treated SAPV, or dialyzed, was considered as control 100%. For the inhibitors and reducing agents on SAPV stability, the peptidase solution without inhibitor was considered as control. Determination of the optimum pH of SAPV activity was performed at 60 °C with different buffer systems at 0.1 M each supplemented with 2 mM calcium. Using casein as a substrate, the effect of temperature on SAPV was investigated at 30-80 °C at pH 9.

2.2.6. Molecular study of SAPV protease

Based on the high homology found in peptidase S8 gene from *Virgibacillus massiliensis* DSM 28587, two primers were designed to generate ~1.4 kb PCR (Polymerase Chain Reaction) fragment encoding the peptidase SAPV. The PCR product was purified and cloned into pCR-Blunt, pUT57, and pTrc99A Vectors, previously digested with *Eco*RI, leading to the pSM40, pSM41, and pSM42 plasmids, respectively using the *E. coli* BL21(DE3)pLysS [16]. For the recombinant SAPV production, the *E. coli* BL21(DE3)pLysS/pSM41 and *E. coli* BL21(DE3)pLysS/pSM42 were induced by the addition of 5 mM IPTG after reaching an absorbance (A_{600}) of about 0.7. The enzyme crude extracts were obtained from the extracellular fraction by the modified procedure described by Abe et al. 1994 [17]. The extracellular recombinant enzyme (rSAPV) was purified to homogeneity using the same steps applied for the purification of the native enzyme.

2.2.7. Comparison of stability and compatibility with laundry detergents

Using DMC as a substrate, the compatibility of rSAPV, Alcalase 2.4 L FG, and Bioprotease N100L enzymes with a wide range of commercialized detergents was assayed. So, to simulate washing conditions, detergents solutions were prepared in tap water (as laundry washing has to be done in tap water) at a concentration of 7 g/L and then heated for 1 h at 70 °C to destroy the indigenous protease or enzyme activity anterior to the addition of the enzymes. Then, residual peptidase activity was analyzed by performing the assay under standard protocol. The peptidase activity of a control (without any detergent), incubated under similar conditions, was taken as 100%.

2.2.8. The effectiveness of protein stain removal from cotton fabrics

To estimate the stain removal capabilities of rSAPV, Alcalase 2.4 L FG, and Bioprotease N100L proteases, a clean white cotton cloth pieces (5 cm × 5 cm) were soaked and dried with chocolate, egg, and blood. The used bloods were freshly obtained from a local municipal slaughterhouse and collected from cow into anticoagulant heparin tubes (Sfax municipal slaughterhouse, permission was obtained from this slaughterhouse to use these animal parts). The stained cloth pieces were shake-incubated separately for 1 h with litre beakers containing a total volume of 100 mL of: tap water, Class detergent (7 g/L, in tap water), and detergent added with 500 U/mL of rSAPV peptidase or with 500 U/mL of commercial proteases (Bioprotease N100L and Alcalase 2.4 L FG), followed by rinsing with water. Then, the washed cloth pieces were dried. Visual examination of various pieces was also carried out to show the effect of each used peptidase in the removal of proteinaceous stains. The untreated stained piece of cloth was taken as a control.

3. Results and Discussion

3.1. Producing strain FarD^T peptidase using powder from white shrimp shell by-product

Strain FarD^T was cultured for 36 h at 35 °C in Erlenmeyer flasks in optimized ELM media. A high level of peptidase production, (16,000 U/mL) was obtained with this bio waste as the sole carbon, and nitrogen source (Fig. 1). Thus, the powder from white shrimp shell by-product is a source for the development of the bacterium and the synthesis of metabolites as well.

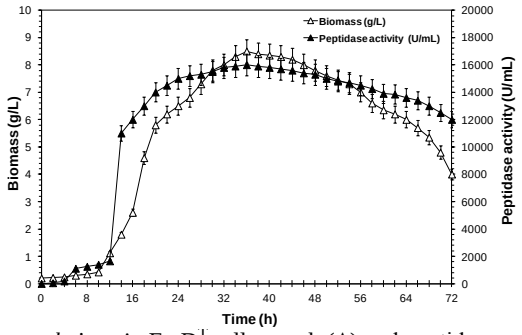


Fig. 1. Time course of *Virgibacillus natechei* strain FarD^T cell growth (Δ) and peptidase production (▲). Cell growth was monitored by measuring the absorbance at 600 nm and was converted to cell dry weight (g/L).

3.2. SAPV purification

The procedure was beginning with salting-out procedure carried out by fractional ammonium sulfate precipitation. Salting-out procedure by 20-80% ammonium sulfate led to and recovery yield of 83% and purification fold of 2.17, which is higher than the study have reported previously by the authors, in which recovery yield is 79% and purification fold is 2 [10]. Gel filtration dramatically decreased the yield to 60.8% and increased the purification fold to 33.82. The SDS-PAGE analysis of the pooled fractions from HPLC column showed one band, with apparent molecular mass, about 31 kDa (Fig. 2A). Zymogram activity staining revealed the presence of one prominent zone of caseinase activity for the purified peptidase (Fig. 2B).

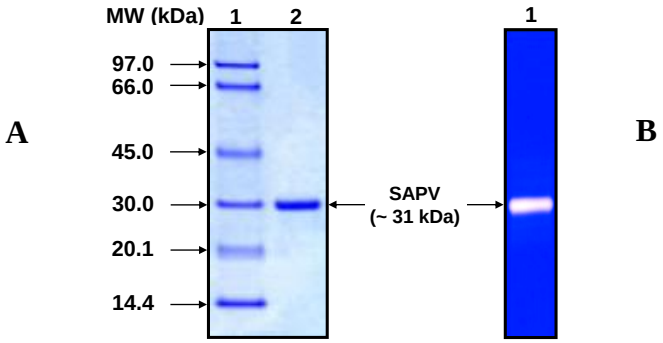


Fig. 2. (A) 12% SDS-PAGE of the purified peptidase SAPV. **(B)** Zymogram caseinolytic activity staining of peptidase activity.

3.3. SAPV characterization

SAPV possesses an optimum activity at pH 9 and 60°C. Irreversible inhibition of enzyme activity by diiodopropyl fluorophosphates (DFP) and phenylmethanesulfonyl fluoride (PMSF) confirmed its belonging to the serine peptidases. The addition of Ca^{2+} increased SAPV activity by 290%. Again, SAPV peptidase is improved by Fe^{2+}, Cu^{2+}, and Zn^{2+}. The heavy metals: Co^{2+}, Hg^{2+}, and Cd^{2+} completely inhibited SAPV activity.

3.4. Cloning and heterologous expression of the **sapV** *gene*

The PCR product corresponding to the entire SAPV coding region was cloned and sequenced leading to the pSM40. It revealed an open reading frame (ORF) of 1158 bp encoding 386 amino-acids.

The apparent molecular weight of the purified enzyme (31 kDa) determined by SDS-PAGE, and HPLC gel filtration chromatography was in good agreement with the predicted value. The typical triad catalytic residues (D32, H64, and S219) in the active site and three serine peptidase signatures (amino-acid residues 28-39, 64-75, and 216-226) [18] were also conserved in the *sapV* gene.

The amino-acid sequence deduced from the nucleotide sequence of the *sapV* gene was compared to those of other known subtilisin superfamily of serine peptidases S8/S53. The amino-acid composition

of SAPV indicated that it was devoid of cysteine and cystine residues. To express SAPV, the corresponding gene was cloned downstream of P*T7* and P*tac* promoters in pSM41 and pSM42, respectively, and then transformed into *E. coli* BL21(DE3)pLysS strain. No alkaline peptidase activity was found in the periplasmic fraction, neither in the intracellular fraction for all recombinant strains. Relatively elevated quantities of specific activity of 310 and 1250 U/mg were, nevertheless, detected in the extracellular fractions of *E. coli* BL21(DE3)pLysS/pSM41 and *E. coli* BL21(DE3)pLysS/pSM42, respectively. Founded on this investigation, the SAPV peptidase was most efficiently expressed with the construction of P*tac*-*sapV* (pSM42). The latest was, therefore, preserved for the purification of the recombinant peptidase (rSAPV). Extracellular rSAPV (pSM42) was purified using the same steps taken for the native enzyme from strain FarD^T. All the biochemical properties from rSAPV were almost analogous to those of the original one. The recombinant enzyme, rSAPV can be readily prepared on a big scale for biotechnological sectors.

3.5. Functional properties of the purified rSAPV

rSAPV peptidase is compatible with all the tested commercial detergents at concentration of 7 g/L. It exhibited better stability with Class followed by Dipex, Nadhif, Det, Dixan, Skip, iSiS, Ariel, EcoVax, and OMO. rSAPV retained 100% of its initial activity with Class (vs 86% for Bioprotease N100L and 80% for Alcalase 2.4 L FG) even after 1 h incubation at 40 °C. Protein stains like chocolate, egg, and blood have been hard to eliminate with commercial detergent. Some stains could only be dealt with at high temperatures and even then the stain was only partly removed. Detergent enzymes especially with proteases offered a key and are being persistently improved to digest proteinaceous stains. The ability of proteases to hydrolyse the various proteinaceous stains has attracted the interest of industrialists in detergent market. The visual comparison of the washed cloth revealed that the combinations of every enzyme individually with commercial detergents (Class) yielded fairly good results of its ability to remove blood, egg, and chocolate stains. In fact, a limited washing performance was observed with tap water only or with detergent (Class) only. The supplementation of SAPV enzyme or commercial proteases (Bioprotease N100L or Alcalase 2.4 L FG) in Class detergent seems to enhance the cleaning process as evidenced by rapid stain removal. Thus, SAPV peptidase was characterized by its strong hydrolytic effect against blood and chocolate which are recalcitrant stains. Similar study superintend on the protease from *Virgibacillus dokdonensis* VIT P14 increase the usefulness of proteolytic enzyme in removing the blood stains from cotton cloth [19]. Although reported the usefulness of thermostable alkaline peptidase from *Bacillus* and related genus for the removal of protein stains from cotton cloth in the presence and absence of detergents [10, 20, 21], we believe that the SAPV peptidase from is more effective.

4. Conclusions

Biochemical properties of SAPV enzyme demonstrated that it has a high optimum working temperature and pH, which could make the peptidase suitable for industries applications. This was supported by cloning amino-acid sequence inspection of the gene encoding SAPV peptidase, which is endowed with a number of characteristics that are highly valued for the detergent industry and peptide synthesis. These observations inspired us to explore other enzymes from strain FarD^T. The structure-function relationship of SAPV is now underway to improve the properties of this peptidase. Further works will be investigated in our laboratories to perform the crystallization of SAPV enzyme.

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