



Characterization of a novel protease from *Anoxybacillus kamchatkensis* strain M1V with biotechnological interest

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Received: / Accepted: / Published:

Abstract:

A total of 5 proteolytic thermophiles bacteria were isolated from Hammam Righa hot spring in Algeria. Strain M1V was selected as the best producer of an extracellular protease, called SAPA, and was used for further studies. Sequence analysis of the 16S rRNA gene in addition to phenotypic tests led to the placement of this organism in the genus *Anoxybacillus* and species of *kamchatkensis*. Maximal protease production was detected after 48 h of incubation at 45 °C. This SAPA protease was purified and biochemically characterized, showing optimal activity at 70 °C, pH 11, and high levels of hydrolysis, substrate specificity, and catalytic efficiency than purified and commercial proteases. The protease activity was strongly inhibited by phenylmethanesulfonyl fluoride (PMSF), and diiodopropyl fluorophosphates (DFP). SAPA has a molecular mass of 28 kDa, and the N-terminal amino acid sequence determined showed similarity to serine proteases previously described.

Keywords: protease; *Anoxybacillus kamchatkensis*; Algeria, thermophilic; detergent;

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1. Introduction

With the discovery of extremophilic microorganisms which are able to grow in a broad variety of conditions, and more particularly since the isolation during the 1980s of many novel hyperthermophilic species, microbiologists quickly understood the strong potential of these microorganisms for industrial applications especially in the field of biocatalysis [1]. Since enzymes coming from extremophiles are in many cases more robust than the ones coming from their non-extremophilic counterparts, they can successfully replace existing enzymes in several processes. For particular interest, proteases which catalyze the hydrolysis of peptide and iso-peptide bonds

2. Results and Discussion

Screening of proteolytic strains:

Results of the assay for proteolytic enzyme production showed that the most active strain was M1V as shown in **Table 1**.

Identification of the microorganism:

The M1V isolate was subjected to various morphological, biochemical, microbiological, physiological, and molecular tests.

The 16S rRNA gene was also amplified by polymerase chain reaction (PCR) to identify the genus and species to which the strain M1V belong.

Optimization of protease production by M1V strain:

The optimized medium for the protease production is composed of (g/L): gruel, 8; soy peptone, 4; KH_2PO_4 , 1; K_2HPO_4 , 1, CaCl_2 , 0.2; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 and 1% trace elements at pH 7.4. Cultivations were performed on a rotary shaker (200 rpm) for 48 h at 45 °C and in 1000 mL. The highest extracellular protease activity (about 4,600 U/mL) in an optimized medium and it was, retained for all subsequent studies.

Protease purification

In this study, ammonium sulfate precipitation from 35 to 70% was initially used as part of the purification process of the enzyme. The pellet obtained from 35-70% ammonium sulfate precipitation was loaded and then subjected to

that join amino-acids together in proteins [2]. The present contribution was undertaken to investigate for the screening of a novel proteolytic bacteria.

Additionally, the optimization of the protease production by the selected bacteria. Additionally, extremophilic microorganisms and their biocatalysis may present novel industrial opportunities and biotechnological applications [3]. The purification and the compatibility with the liquid and solid detergents were also investigated. The nucleotide and amino-acid were also studied.

ion exchange chromatography FPLC using UNO Q-12 column. The peak of proteolytic activity from strain M1V was eluted at 120-160 mM NaCl and then purified by HPLC system using a ZORBAX PSM 300 HPSEC.

Biochemical characterization of SAPA

SAPA is active over a narrow range of pH (from 3 to 13), and temperature (30-90 °C) with an optimum pH 11 and 70 °C. Furthermore, thermostabilization was more effective of SAPA with calcium at 2 mM and glycerol at 100 g/L since the $t_{1/2}$ at 80 °C were determined to be, respectively 20 h, compared to 8 h without any additive. SAPA enzyme is extremely compatible and stable within the most commercial liquid detergents tested as presented in **Fig. 1**.

As shown in **Fig. 1** the visual comparison of the washed cloth revealed that the combinations of every enzyme individually with commercial detergents (Nadhif) yielded fairly good results of its ability to remove blood and chocolate stains.

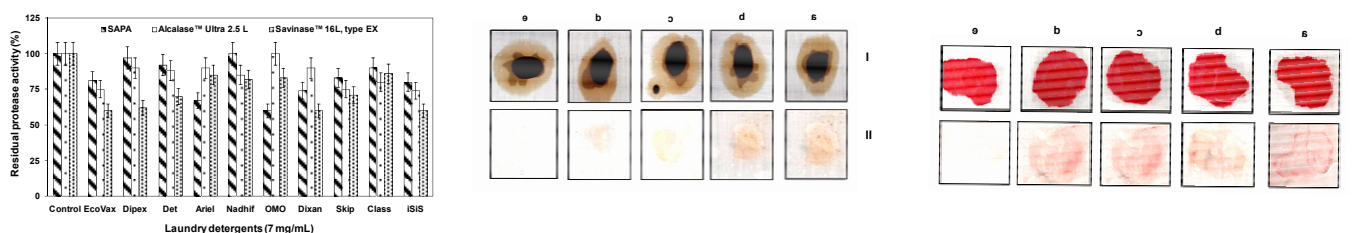
Cloning and sequencing of the *sapA* gene

A fragment of about 1.3 kb contain the *sapA* gene was amplified by PCR, which is, purified, cloned, sequenced and expressed.

Extracellular recombinant enzyme, rSAPA was purified using the same strategy for the native enzyme from *An. kamchatkensis* strain M1V.

Table 1. Screening of proteolytic strains.

Strains	Protease activity (U/mL)
ATAM	400
M1V	3000
B5GN	1600
HB14	1800
BA1	2500

Figure 1. Washing performance analysis test of SAPA.

3. Materials and Methods

Experimental

Isolation and growth conditions of protease-producing bacterial strain:

Hammam Righa is a heating system design for bungalows situated in geothermal area. 1 L sterile thermal glass bottles. Samples dispersed in sterile distilled water and heated 80 °C for 30 min to kill vegetative cells. The heat-treated samples were then plated onto skimmed milk agar plates as well detailed previously [3]. The plates were then incubated at 45 °C, overnight, to obtain a halo of casein degradation. The colonies with a clear zone formed by the hydrolysis of casein were evaluated as protease producers and several positive strains were isolated. Bacteria were maintained at 4 °C on Lysogeny broth (LB).

Classical and molecular identification of the microorganism:

The morphological, cultural, physiological, and biochemical characteristics of the bacterium were investigated as well described by a previous detailed study [4].

Assay of proteolytic activity:

Protease activity assay was carried out following the method described by Kembhavi et al. [5], using Hammerstein casein (Merck, Darmstadt, Germany) as a substrate. The proteolytic activity present in the laundry detergent solution was evaluated by the method suggested by Boulkour-

Touioui et al. [6] using N,N-dimethylated casein (DMC) as a substrate.

Protease purification:

All purification procedures were performed at 4 °C. In the first step, proteins were precipitated to 35% with solid $(\text{NH}_4)_2\text{SO}_4$ and then centrifuged at $10,000\times g$ for 20 min. The obtained supernatant was saturated up to 70% with $(\text{NH}_4)_2\text{SO}_4$, re-centrifuged, re-suspended in a minimal volume of 25 mM PIPES buffer at pH 6 supplemented with 2 mM CaCl_2 (Buffer B), and dialyzed overnight against the repeated changes of the same buffer. Hence, the obtained sample was loaded onto a UNO Q-6 column. The column was washed extensively with the above-mentioned buffer. The proteins were eluted with the same buffer, containing a linear of NaCl gradient 0 to 500 mM at a rate of 30 mL/h. Fractions of each peak were collected manually and estimated by measuring absorbance at 280 nm and the protease activity on casein. Pooled fractions, containing protease activity, were applied to HPLC system using a Zorbax PSM 300 (26.2 mm \times 250 mm), Agilent Laboratories, pre-equilibrated with 25 mM HEPES buffer at pH 8 supplemented with 2 mM CaCl_2 (Buffer C). The protease activities and protein contents were assayed after each purification step.

Gene cloning and expression of the protease

Two external oligonucleotides were synthesized based on the high degree of sequence homology published for the alkaline protease, peptidase S8 from *B. subtilis* used for the isolation and determination of the *sapA* encoding gene sequence.

4. Conclusions

The biochemical properties of SAPA described, here, indicate that its biocatalysts are promising candidates for biotechnological

applications such as a cleaning bio-additive in laundry detergent formulations. These observations inspired us to explore the protease activity of other microbial strains of Hammam Righa spring (ATAM, B5GN, HB14, and BA1).

Acknowledgments

The authors would like to thank to Mrs. N. Masmoudi, Mrs. N. Kchaou, and Mr. K. Walha (Analysis Unit-CBS) for their technical assistance. We would also like to express their gratitude to Pr. H. Belghith and Dr. W. Saibi (CBS) for their constructive suggestions and discussions. Special thanks are also due to Pr. W. Hariz from the English Department at the Sfax Faculty of Science, University of Sfax (Sfax, Tunisia) for language polishing services and constructively proofreading.

Author Contributions

Conceived and designed the experiments: BJ.

Performed the experiments: SM, KB.

Analyzed the data: NZJ, HR, HH, SB, ABD.

Contributed reagents/materials/analysis tools: MBE, MOB.

Wrote the text of the paper: SM, KB, BJ.

Critical revision of manuscript: NZJ, HH, SB, ABD, BJ.

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Conflicts of Interest

The authors declare that they have no conflict of interest.

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