Purification and biochemical characterization of a novel thermostable and halotolerant subtilisin SAPN, a serine protease from *Melghiribacillus thermohalophilus* Nari2A[T] for chitin extraction from crab and shrimp shell by-products

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Abstract

The present study investigates the purification and biochemical characterization of a novel extracellular serine alkaline protease, subtilisin (called SAPN) from *Melghiribacillus thermohalophilus* Nari2A[T]. The highest yield of protease (395 IU/g) with white shrimp shell by-product (40 g/L) as a unique source of nutriments in the growth medium was achieved after 52 h at 55 °C. The monomeric enzyme of about 30 kDa was purified to homogeneity by ammonium sulfate fractionation, heat treatment, followed by sequential column chromatographies. The optimum pH and temperature values for subtilisin activity were pH 10 and 75 °C, respectively, and half lives of 9 and 5 h at 80 and 90 °C, respectively. The sequence of the 25 NH$_2$-terminal residues pertaining of SAPN exhibited a high homology with those of *Bacillus* subtilisins. The inhibition by DFP and PMSF indicates that this enzyme belongs to the serine proteases family. SAPN was found to be effective in the deproteinization (DDP %) of blue swimming crab (*Portunus segnis*) and white shrimp (*Metapenaeus monoceros*) by-products, with a degree of 65 and 82%, respectively. The commercial and the two chitins obtained in this work showed a similar peak pattern in Fourier-Transform Infrared (FTIR) analysis, suggesting that SAPN is suitable for the bio-production of chitin from shell by-products.

Article Highlights

- The purification of subtilisin (SAPN) from *M. thermohalophilus* Nari2A was carried out.
- The molecular weight and the NH$_2$-terminal sequence of the subtilisin were determined.
- Optimum pH and temperature values for activity were pH 10 and 75 °C respectively.
- SAPN was found to be effective in the deproteinization of crab and shrimp by-products.
- SAPN may be used as candidate for chitin extraction from crustacean by-products.

✉ Bassem Jaouadi
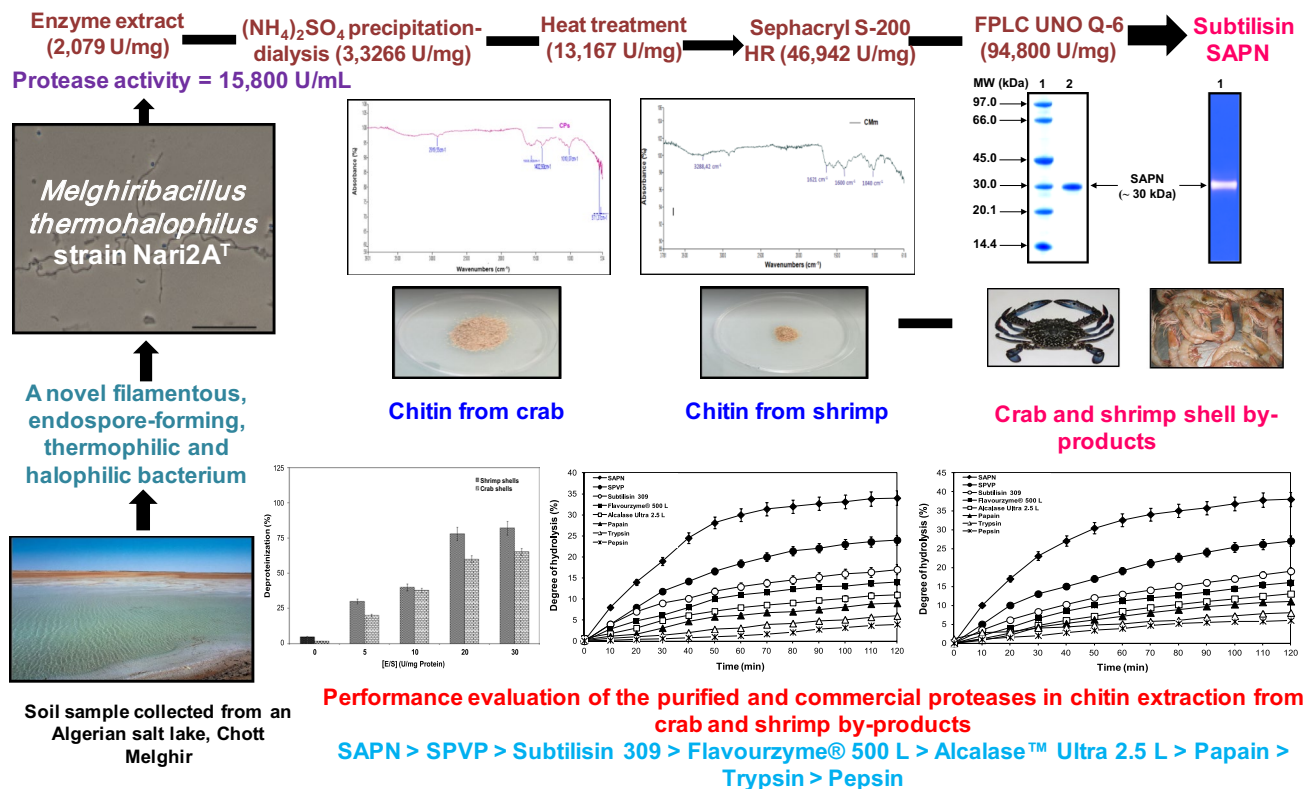 bassem.jaouadi@cbs.rnrt.tn; biotechecozym@gmail.com

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Graphic abstract

Enzyme extract (2,079 U/mg) — (NH$_4$)$_2$SO$_4$ precipitation-dialysis (3,3266 U/mg) — Heat treatment (13,167 U/mg) — Sephacryl S-200 HR (46,942 U/mg) — FPLC UNO Q-6 (94,800 U/mg) → Subtilisin SAPN

Protease activity = 15,800 U/mL

Melghiribacillus thermohalophilus strain Nari2A^T

A novel filamentous, endospore-forming, thermophilic and halophilic bacterium

Soil sample collected from an Algerian salt lake, Chott Melghir

MW (kDa)   1   2
97.0
66.0
45.0
30.0 — SAPN (~ 30 kDa)
20.1
14.4

Chitin from crab

Chitin from shrimp

Crab and shrimp shell by-products

Performance evaluation of the purified and commercial proteases in chitin extraction from crab and shrimp by-products

SAPN > SPVP > Subtilisin 309 > Flavourzyme™ 500 L > Alcalase™ Ultra 2.5 L > Papain > Trypsin > Pepsin

Keywords *Melghiribacillus thermohalophilus* · Subtilisin · *Portunus segnis* · *Metapenaeus monoceros* · Chitin

Introduction

Proteases are paramount hydrolytic enzymes, widely used in the industrial sector, accounting for ~65% of the aggregate worldwide enzyme market, and ranking first enzymes before amylases and lipases. The market revenue of global protease sale is expected to reach $ 2.21 billion by 2021 (www.FreedoniaGroup.com) (Singh et al. 2016). Subtilisin proteases are a member of the subtilase superfamily, generally found in bacteria, archaea, and eukaryotic microorganisms. They are the most important industrial enzymes and essential bioadditives in modern laundry detergents in terms of boosting washing performance by removing efficiently insoluble proteinaceous stains (Vojcic et al. 2015). They are also used for the degradation of proteinaceous recalcitrant solid fish bio-waste, abundantly available in nature, by converting them into low value-added products, such as chitinous materials, protein hydrolysate, or amino-acids. In fact, fishery-processing industries generate large amounts of bio-wastes and non-edible parts (Lopes et al. 2018; Mao et al. 2017). The disposal of these by-products has always been considered as a serious issue, causing a major environmental

and health problem. Of particular interest, cuticles of various crustaceans, especially crabs and shrimps, are the main sources of raw material for the production of chitin. This polymer, a long chain of *N*-acetyl-D-glucosamine residues linked by β-(1,4) bonds, is the most abundant renewable natural resource next to cellulose (Hammami et al. 2017). Owing to its numerous physico-chemical properties and biological activities, chitin is considered as an interesting bio-compound for several applications, with a great economic importance, including industrial, nutraceutical and medical sectors (Prameela et al. 2017; Wang et al. 2018). To date, a few studies on the use of proteolytic enzymes for the bio-deproteinization of shrimp wastes have been reported; however, little has been done with crab shell bio-wastes.

Blue swimming crab, *P. segnis*, and white shrimp, *M. monoceros,* belong to the order of *Decapoda* and are classified in the *Portunidae* and *Penaeidae* family, respectively. They are considered as two alien species in the Mediterranean Sea. *P. segnis* species is widespread in the west of the Atlantic Ocean. It proliferates easily and it has been introduced, recently, in the eastern Atlantic, in the north and east of the Mediterranean and Japan. Nowadays, it is

found, extensively, in the Mediterranean, especially, in Tunisian coasts, causing various damages regarding fishing. *M. monoceros* species is native to the Red Sea; it has entered the Mediterranean via the Suez Canal and is now quite common in landings from the Gulf of Gabes in Tunisian coasts (Safaie et al. 2016).

The assessment of bacterial diversity and bioprospection of Chott Melghir salt lake located in the south-east of Algeria revealed the presence of two novel species of the genus *Melghirimyces*, for which the names of *Melghirimyces algeriensis* NariEX[T], and *Me. thermohalophilus* Nari11A[T] were proposed and a novel species of a new genus, namely *Me. thermohalophilus* Nari2A[T] (Addou et al. 2013; Addou et al. 2012; Addou et al. 2015; Mohamed et al. 2019).

In this study, we present an overview on a recent research work in the field of subtilisin protease purification and characterization from the recently described polyextremophilic bacterium, *Me. thermohalophilus* Nari2A[T], which also represented a novel genus. The potential relevance of deproteinization of crab and shrimp by-products to extract chitin is also discussed.

Materials and methods

Substrates, reagents, and proteases used in this work

Commercial chitin was purchased from P-Biomedical, France. Hammerstein casein was supplied by Merck (Darmstadt, Germany). The UNO Q-6 column (12 mm × 53 mm) was purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). The commercial proteases, subtilisin 309, trypsin, pepsin, and papain, were purchased from Sigma Chemical Company (St. Louis, MO, USA). Subtilisin 309 is active at pH 10, with a maximum activity at 60 °C. Trypsin functions at pH 8 with maximum activity at 40 °C. Pepsin is active at pH 2 and 50 °C. The optimum pH of papain is 7, while the optimum temperature was determined to be 65 °C. Alcalase™ Ultra 2.5 L and flavourzyme® 500 L were purchased from Novozymes Biopharma DK A/S (Bagsvaerd, Denmark). Alcalase™ Ultra 2.5 L is a commercial bacterial *endo*-protease of the serine type (subtilisin Carlsberg or subtilisin A). It is active between pH 6.5 and 8.5. It functions between 45 and 65 °C, with a maximum activity at about 60 °C. Flavourzyme® 500 L, produced by *Aspergillus oryzae*, is active at pH 7, with a maximum activity at 50 °C.

Preparation and protein analysis of *P. segnis* and *M. monoceros* shells powders

The blue swimming crab, *P. segnis* and white shrimp, *M. monoceros* shells by-products were obtained in fresh conditions from a fishery market located at Sfax, Tunisia. To obtain a fine powder, they were cooked at 100 °C for 20 min [1:2 ratio (*w/v*)] in distilled water, then desiccated and milled (Hamdi et al. 2017). After drying, they were stored at room temperature until further use. Total nitrogen content was measured by Kjeldahl method and the corrected protein concentration was obtained by subtracting chitin nitrogen from total nitrogen and multiplying by the factor 6.25 (Hamdi et al. 2017; Hammami et al. 2017).

Microorganism

Melghiribacillus thermohalophilus Nari2A[T] was previously isolated from the soil of Chott Melghir, an Algerian salt lake located at north-east of Biskra city, Algeria (34°00′00″–34°30′ 01″N 6°07′30″–6° 30′02″E), in the Saharan region. This strain is a novel thermophilic and a moderately halophilic bacterium. The temperature range for growth was (35–62 °C), with optimal growth occurring at 50–55 °C. The NaCl concentration range for growth was (0.5–17%, *w/v*), with an optimum at 7–10% (Addou et al. 2015). An attractive halo of casein degradation was revealed around the colony growth onto skimmed milk agar plates, after an incubation at 55 °C, as described previously (Mechri et al. 2017a, b). Strain Nari2A[T] was maintained at 4 °C on ISP 2 agar plates (Addou et al. 2015). The protease-producing isolate was propagated on ISP 2 agar plates at 55 °C and conserved in ISP 2 medium added to 20% glycerol stored at − 80 °C.

SAPN production

The pre-culture of *Me. thermohalophilus* Nari2A[T] was carried out in a 250 mL Erlenmeyer flask with baffles containing 25 mL of ISP 2 liquid medium composed of (g/L): glucose, 4; yeast extract, 4; malt extract, 4; CaCO₃, 2; and 10% (*w/v*) NaCl then incubated at 55 °C overnight. This pre-culture was used to inoculate the culture, using the optimized medium at pH 7.4, containing only 40 g/L shrimp waste shells in Erlenmeyer flask with baffles.

The flasks were incubated at 55 °C for 52 h with shaking at 160 rpm in 500 mL Erlenmeyer flasks with a working volume of 50 mL. The obtention of clear supernatant crude protease solution and bacterial growth of strain Nari2A[T] were determined as described recently (Rekik et al. 2019).

Assay of proteolytic activity

As described previously by Mechri et al. (2017b) and Zaraî Jaouadi et al. (2012), the subtilisin activity was measured spectrophotometrically at 280 nm by the detection of aromatic amino-acids released from casein as a substrate

in 100 mM glycine–NaOH supplemented with 2 mM $CaCl_2$ (buffer A) at pH 10, after an incubation of 15 min at 75 °C.

SAPN purification

The SAPN production was undertaken using a fresh culture supernatant of strain Nari2A[T], in the optimal medium. To remove microbial cells, 500 mL of a 52 h culture were centrifuged for 30 min at 10,000g. 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 $CaCl_2$ (buffer B). 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 the buffer B. Protein contents (A_{280} nm) and subtilisin activity were determined. 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 $CaCl_2$ (buffer C). The column was washed extensively with the same buffer. The proteins were eluted with buffer C containing a linear gradient of NaCl from 0 to 500 mM at a rate of 30 mL/h. Fractions of each peak were collected manually and estimated by measuring the absorbance at 280 nm and the protease activity on casein. Pooled fractions, containing SAPN, were concentrated for further analyses as detailed previously (Mechri et al. 2017a, b).

Analytical methods and proteins measurement

The total protein contents were determined using bovine serum albumin (BSA) as a standard. As described previously, three electrophoresis techniques, casein zymography, SDS-PAGE, and native-PAGE were used to analyze the SAPN purity, determine its molecular weight, and confirm its activity, respectively (Zaraî Jaouadi et al. 2012). The molecular weight was also confirmed by size exclusion HPLC, using a Shodex Protein WK 802-5 column (8 mm × 300 mm), pre-equilibrated with buffer A and native protein markers [in kDa] of 669: thyroglobulin ($R_t = 9.150$ min), 158: aldolase ($R_t = 10.174$ min), 43: ovalbumin ($R_t = 11.067$ min), 17: myoglobin ($R_t = 13.800$ min), and 1.35: vitamin B12 ($R_t = 18.767$ min).

Edman degradation

Bands of purified subtilisin SAPN separated on SDS gels were transferred to a ProBlott membrane (Applied Biosystems, Foster City, CA, USA), and the NH_2-terminal sequence analysis was performed as detailed (Mechri et al. 2017b).

Physico-chemical characterization of the purified subtilisin SAPN

Influence of specific inhibitors and metallic ions on SAPN stability

Pre-incubation of SAPN with some specific inhibitors and reducing agents was investigated, as detailed recently (Rekik et al. 2019). The effects of different divalent metallic ions at an optimum concentration of 2 mM, on SAPN, were investigated by adding them to the reaction mixture after pre-incubating SAPN for 1 h at 40 °C.

Determination of the effects of pH on SAPN activity and stability

The full determination of pH on SAPN activity was investigated by measuring activity at 75 °C over a wide pH range from 3 to 13, using buffers at 100 mM each. The pH stability of SAPN enzyme was investigated by examining the residual activity after incubation of SAPN in different buffers with pH ranging from 8 to 11 at 40 °C for 24 h. The remaining subtilisin activity was then evaluated at the optimum pH as described above.

Determination of the effects of temperature on SAPN activity and stability

To study the effect of temperature on SAPN activity, an assay was conducted at temperatures ranging from 40 to 100 °C, at pH 10 with intervals of 5 °C. To evaluate its thermostability, SAPN was incubated for 24 h at temperatures, ranging from 60 to 90 °C, at pH 10, in the presence and absence of 2 mM $CaCl_2$. Taking the non-heated SAPN as 100% activity, aliquots were withdrawn at desired time intervals to test the remaining activity.

Effect of osmolytes, KCl and NaCl, on SAPN protease activity

SAPN was pre-incubated for 1 h with various concentrations of KCl and NaCl from 0.5 to 10 M at pH 10. The activity of SAPN without osmolytes was taken as 100%. The residual subtilisin activity was measured under the standard conditions.

Determination of the effect of polyols on SAPN stability

Polyols are used for stabilization of enzymes in soluble form in enzymatic reactions; thus, various polyols, at 100 g/L, were tested to evaluate their effects on subtilisin stability at 80 °C in buffer A with a 1 h pre-incubation in each polyol. The activity of SAPN without any polyol and in the absence

of calcium was taken as 100%. The residual protease activity was measured under the standard conditions.

Substrate specificity determination

As described previously, a number of natural, modified protein substrates as well as esters and synthetic peptides conjugated with *p*-nitroanilide (*p*NA) were used to investigate the substrate specificity profile of the subtilisin SAPN (Zaraî Jaouadi et al. 2013). The substrate, showing the highest activity was taken as the 100% reference.

Performance evaluation of the purified subtilisin SAPN

Kinetic measurements of SAPN, SPVP, SAPB, subtilisin 309, and subtilisin A proteases

The Michaelis constant (K_m) and the maximum rate (V_{max}) of the enzyme-catalyzed reaction were determined through a double reciprocal (Lineweaver Burk) plot of subtilisin activity towards casein, azo-casein, BTEE, and FAAF, as substrates at different concentrations, ranging from 0.10 to 50 mM at 50 °C and pH 10 for 10 min with Hyper32 program.

Determination of the degree of hydrolysis of proteins in crab and shrimp bio-wastes

The hydrolysis of proteins in crab and shrimp bio-wastes was carried out at 75 °C and pH 10 (for SAPN); 60 °C and pH 10 (for SPVP), 60 °C and pH 7 (for alcalase™ Ultra 2.5); 60 °C and pH 10 (for subtilisin 309); 50 °C and pH 7 (for flavourzyme® 500 L); 40 °C and pH 8 (for trypsin); 50 °C and pH 2 (for pepsin); and 65 °C and pH 7 (for papain). An amount of 2 g of each by-product was dissolved in 100 mL of assay buffer and then treated with 2000 U of commercial and purified enzymes. The pH was titrimetrically maintained constant using the pH–Stat set at the desired pH value of each enzyme by continuous addition of 4 N NaOH. The hydrolysis level was expressed as described previously (Hamiche et al. 2019).

Deproteinization of blue swimming crab blue and white shrimp bio-wastes by SAPN

The SAPN efficiency in the deproteinization of blue swimming crab (*P. segnis*) and shrimp (*M. Monoceros*) shells by-products was conducted using different enzyme/substrate [*E/S*] [unit of enzyme per milligram protein] ratios ranging from 0 to 30. The two shells homogenates (2 g) were mixed with 50 mL distilled water at 75 °C. The pH was adjusted to

10 with 5 N NaOH. The reactions were stopped by heating the solutions at 90 °C for 24 h to inactivate SAPN. The solid phase was washed and then pressed manually through four layers of gauze (Younes et al. 2014). The degree of deproteinization (DDP %) was calculated as described elsewhere (Hammami et al. 2017).

Chitin structural analysis with FTIR

Infrared spectra of commercial and extracted chitins were determined using a Nicolet FTIR spectrometer equipped with a horizontal attenuated total reflection (ATR) accessory. The internal crystal reflection was made from zinc selenide and had a 45° angle of incidence to the IR beam. The spectrum was acquired at a 4/cm resolution, and recorded between 4000 and 500/cm (mid-IR region). The data were analyzed by the OPUS 3.0 data collection software program (Bruker, Ettlingen, Germany).

Statistical analysis

All results derive from at least three independent replicates, and the control without proteolytic enzyme was under the identical standard experimental conditions. Results were expressed as the mean of the replicate determinations and standard deviation ($\pm$ SD). Statistical significance and interpretation of results were estimated as detailed recently by the authors (Mechri et al. 2017a).

Results

Producing Nari2A[T] protease using powder crustaceans by-products

For the optimization of protease production by strain Nari2A[T] in the inexpensive medium with the powder of *P. segnis* or *M. monoceros* bio-wastes, a classical method "one-factor-at-a-time (OFAT)", which involves changing one independent variable, while fixing others at certain levels, was adopted. Strain Nari2A[T] was cultured in Erlenmeyer flasks (500 mL) in an initial medium, containing 20 g/L of powder bio-waste from shrimp or crab for 52 h at 55 °C on a shaker incubator (160 rpm). Powder from shrimp by-product as the sole energy, carbon, and nitrogen sources gave an acceptable level of proteases production, with (7500 U/mL) followed by the powder from crab by-product with (3200 U/mL). Therefore, the effect of various concentrations of powder from white shrimp bio-waste on protease production by strain Nari2A[T] was studied. The optimum for the production of protease activity (15,800 U/mL) was obtained with 40 g/L of shrimp by-product with a significant yield of 395 IU/g of bio-waste (Fig. 1).

Fig. 1 Time course of *Me. thermohalophilus* Nari2A[T] cell growth (empty triangle) and subtilisin production (filled triangle) using casein as substrate. Cell growth was monitored by measuring the absorbance at 600 nm and converted to cell dry weight (g/L). Each point represents the mean ($n = 3$) ± standard deviation
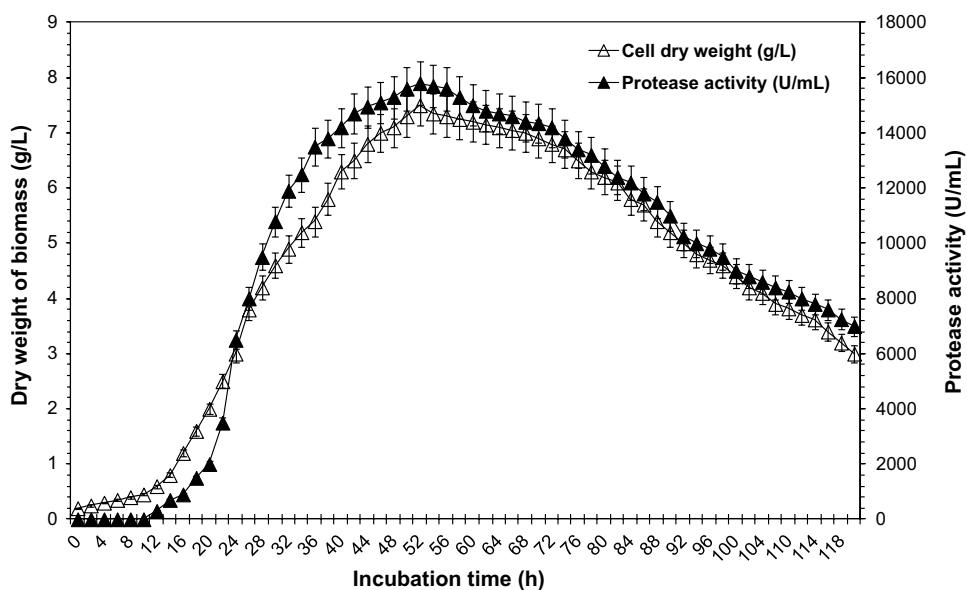
Table 1 Purification steps of subtilisin SAPN from *Me. thermohalophilus* Nari2A[T]

Purification step[a]	Total activity (units)[b] × 10³	Total protein (mg)[b,c]	Specific activity (U/mg of protein)[b]	Activity recovery rate (%)	Purification factor (fold)
Crude extract	7900 ± 92	3800 ± 80	2079	100	1
(NH₄)₂SO₄ fractionation (35-60%)-Dialysis	6399 ± 58	1924 ± 31	3,3266	81	1.6
Heat treatment (80 °C for 30 min)	4661 ± 43	354 ± 18	13,167	59	6.4
Sephacryl S-200 HR	3239 ± 30	69 ± 5	46,942	41	22.6
FPLC UNO Q-6	2370 ± 13	25 ± 1	94,800	30	45.6

[a]Experiments were conducted three times and ± standard errors are reported

[b]One unit of protease activity was defined as the amount of enzyme required to release 1 µg tyrosine per minute under the experimental conditions used

[c]Amounts of protein were estimated with a Dc protein assay kit purchased from Bio-Rad Laboratories (Hercules, CA, USA)

SAPN purification

The various purification steps are summarized in Table 1. The subtilisin activity recovery rate of the supernatant was 81%, even at a final ammonium sulfate concentration of 60%. By means of heat treatment, the specific activity of the SAPN enzyme increased by 6.4 times compared to that of crude enzyme. The clear supernatant obtained after heat treatment was loaded on a Sephacryl S-200 HR column. The fractions containing SAPN activity eluted at ~ 1.6 void volume (V_0) were pooled together (data not shown). In the last step, the protein eluted at 160–200 mM NaCl from the UNO Q-6 FPLC column (Fig. 2a). Under optimum assay conditions, using casein as substrate, the purified SAPN had a specific activity of 94,800 U/mg, with a yield of 30%.

Molecular weight determination of subtilisin SAPN

The purity of the purified SAPN enzyme eluted from the UNO Q-6 column (Fig. 2a) was checked by PAGE under non-reducing (Fig. 2b) and reducing (Fig. 2c) conditions and confirmed by gel filtration chromatography (Fig. 2d). The molecular mass of the SAPN enzyme was estimated to be about 30 kDa. This preparation was a homogeneous, as it exhibited a unique elution symmetrical peak at the retention time (R_t) = 12.455 min, corresponding to a single protein of nearly 30 kDa by HPLC gel filtration chromatography (Fig. 2c). The SDS-PAGE analysis of the pooled fractions showed one band, with an apparent molecular mass of about 30 kDa (Fig. 2d). The occurrence of only one clearance zone on the blue background in the zymography technique revealed the presence of caseinolytic activity and, therefore, of SAPN activity (Fig. 2e).

Fig. 2 Purification of the protease SAPN from *Me. thermohalophilus* Nari2A[T]. **a** Chromatography profile of the purified subtilisin activity of SAPN enzyme on FPLC system using UNO Q-6. The column (15 mm × 68 mm) was equilibrated with buffer B. Adsorbed material was eluted with a linear NaCl gradient (0–500 mM) in buffer B at a flow rate of 60 mL/h, and assayed for protein content at 280 nm. **b** Assessment of homogeneity and molecular weight analysis of purified SAPN protein on native-PAGE, Lane 1, protein markers (molecular masses in kDa). Lane 2, purified SAPN enzyme from *Me. thermohalophilus* Nari2A[T] (30 µg). **c** Chromatography profile of SAPN on a Shodex Protein WK 802-5 column (8 mm × 300 mm), pre-equilibrated with buffer A. Proteins were separated by isocratic elution at a flow rate of 30 mL/h with buffer A. Protein peak, with a retention time (R_t) of 12.455 min, contained the protease activity. **d** 12% SDS-PAGE of the purified protease SAPN. Lane 1, Amersham LMW protein marker (GE Healthcare Europe GmbH, Freiburg, Germany). Lane 2, purified SAPN (30 µg) obtained after UNO Q-6 FPLC chromatography. **e** Zymogram caseinolytic activity staining. Purified subtilisin SAPN (30 µg)
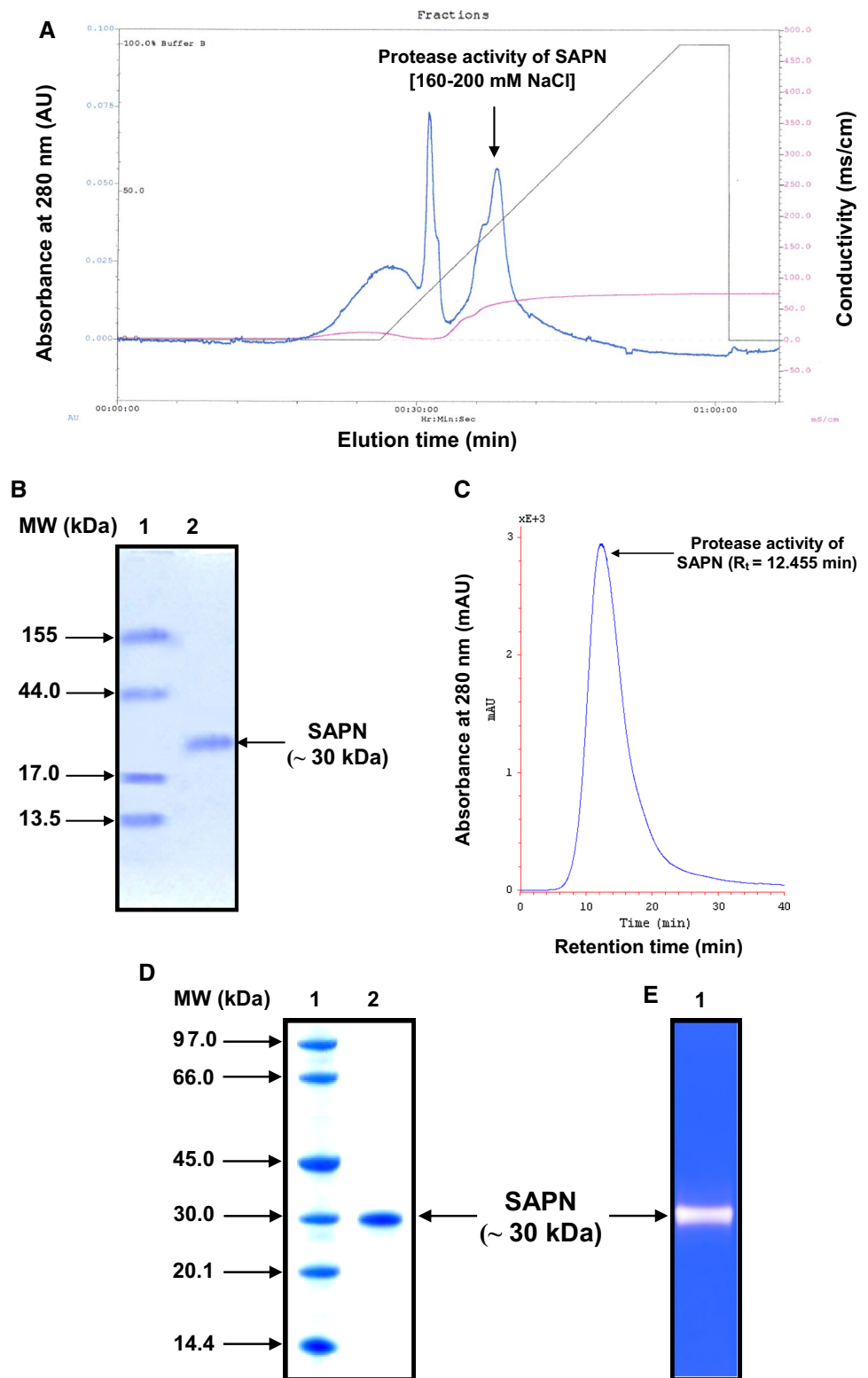
NH$_2$-terminal amino-acid sequence determination of subtilisin SAPN

The unique sequence determined for the first 25 NH$_2$-terminal amino-acids of the blotted purified SAPN confirmed that it was pure. This sequence was compared to protein sequences in the GenBank non-redundant protein database and the Swiss-Prot database, using the BLASTP and tBlastn search programs, respectively. As illustrated in Table 2, the NH$_2$-terminal sequence of SAPN shared greatest

Table 2 Alignment of the NH$_2$-terminal amino-acid sequence of the purified SAPN enzyme from *Me. thermohalophilus* Nari2A^T with the sequences of other *Bacillus* proteases

Enzyme	Origin	NH$_2$-terminal amino acid [a,b]	Identity (%)	References
SAPN	*Me. thermohalophilus* Nari2A^T	AQTVPYGIPLIKAFKVPAQGFKVAN	-	(This study)
MP1	*B. licheniformis* MP1	AQTVPYGIPLIKADKVQAQGFKGAN	88	(Jellouli et al. 2011)
SAPHM	*B. licheniformis* K7A	AQTVPQGIPLIKAEKVQAQGFDGARV	86	(Hadjidj et al. 2018
SAPB	*B. pumilus* CBS	AQTVPYGIPQIKAPAVHAQGY	76	(Zaraî Jaouadi et al. 2012)
SPVP	*Aeribacillus pallidus* VP3	APSGPYGPQGIKADKVHAQGFKGAN	73	(Mechri et al. 2017a)
SAPRH	*B. safensis* RH12	AQTVPYGIPQIKAPAIHABGYKG	64	(Rekik et al. 2019)
SAPLF	*Lysinibacillus fusiformis* C250R	VPSGPYGPIDIKADKVIEDGFKMDEYF	63	(Mechri et al. 2017b)
Subtilisin E	*B. subtilis* 168	AQSVPYGISQIKAPALHSQGY	58	(Jain et al. 1998)
Substilisin Carlsberg	*B. licheniformis* NCIB 6816	AQSVPYGISQIKAPALHSQGYTGSN	58	(Jacobs et al. 1985)
SAPDZ	*B. circulans* DZ100	AQTVPYGMAQIKDPAVHGQGYKGAN	57	(Benkiar et al. 2013)
Subtilisin Novo	*B. amyloliquefaciens* DC-4	AQSVPYGVSQIKAPALHSQGY	54	(Peng et al. 2004)

[a] Amino-acid sequences for comparison were obtained using the program BLASTP (NCBI, NIH, USA) database

[b] Residues not identical with the subtilisin SAPN from *Me. thermohalophilus* Nari2A^T are shaded

sequence identity with *Bacillus* proteases, particularly those from *B. licheniformis* strain MP1 (88% identity with SAPN). Besides, it indicated 73 and 63% identities with proteases from *Aeribacillus pallidus* strain VP3 and *Lysinibacillus fusiformis* strain C250R, respectively (Table 2).

Physico-chemical characterization of purified subtilisin SAPN

Effects of inhibitors and metallic ions on stability of subtilisin SAPN

The profile of natural and synthetic inhibitors at an [inhibitor/enzyme] molar ratio = 100, as well as chelating agents and group-specific reagents, which affect the activity of subtilisin SAPN, are shown in Table 3. The SAPN activity was strongly inhibited by DFP and PMSF, eminent inhibitors of serine proteases, indicating that SAPN enzyme belongs to that subclass. In contrast, chymotrypsin and trypsin alkylating agents, as well as acid-, thiol-, zinc-, and cysteine-protease inhibitors had almost no effect on the activity of the purified SAPN. In this work, in the presence of 10 mM EDTA and 1 mM EGTA, SAPN retained, respectively, 67 and 86% of its activity, which suggests that no metal cofactors were required.

Table 3 also comprises the list of various metallic ions that were tested for an effect on SAPN activity. The addition of Ca^{2+} increased SAPN activity by 300%. Surprisingly, Cu^{2+} and Fe^{2+} increased the proteolytic activity, respectively, by 140 and 260%. SAPN activity was essentially unaffected by Mg^{2+} and Mn^{2+}. Among all the tested metallic ions, only Hg^{2+}, Co^{2+} and Cd^{2+} completely inhibited the purified protease SAPN.

Effects of pH on subtilisin activity and stability

The pH effect on the SAPN protease activity was determined in the range of pH 3–13, using casein as a substrate under the standard assay condition (Fig. 3a). The enzyme exhibited activity at all of the tested pH values, with a maximum activity at pH 10.

The effect of pH on the stability of SAPN was studied at 75 °C. Figure 3c indicates that the stability of the SAPN activity is very high in a wide pH range.

Effects of temperature on subtilisin activity and stability

To determine the temperature at which the purified enzyme SAPN showed optimum activity, the protease activity was monitored at various temperatures viz., 40, to 100 °C, as described in Fig. 3b. The enzyme showed activity at all the tested temperature values, and the maximum activity was obtained at 75 °C, in the presence of 2 mM Ca^{2+}. Without 2 mM Ca^{2+}, SAPN was optimally active at 65 °C.

For investigating the thermal stability of the purified protease, it was incubated at temperatures between 60 and 90 °C, as depicted in Fig. 3d. The enzyme did not display significant activity loss at the end of the 3 h incubation, with and without 2 mM Ca^{2+}. A slight decrease in activity was determined after 4 h of incubation at 90 °C; the protease activity was reduced to 65 and 25%, respectively, with and without Ca^{2+}.

Effect of polyols on the thermal stability

Among the polyols examined, the best results were obtained with PEG 1000 (Fig. 3e). Furthermore, SAPN

Table 3 Effects of various inhibitors, reducing agents, and metallic ions on SAPN stability. SAPN assayed without any inhibitor or reducing agent was taken as control (100%)

Inhibitor/reducing agent/metallic ions	Concentration	Residual protease activity (%)[a]
None	–	100 ± 2.5
PMSF	5 mM	0 ± 0.0
DFP	2 mM	0 ± 0.0
SBTI	3 mg/mL	108 ± 2.7
TLCK	1 mM	105 ± 2.8
TPCK	1 mM	116 ± 2.9
Benzamidine	5 mM	113 ± 2.8
DTNB	10 mM	104 ± 2.6
NEM	2 mM	110 ± 2.8
Iodoacetamide	5 mM	119 ± 2.10
Leupeptin	2 mM	99 ± 2.3
Pepstatin A	1 mM	107 ± 2.8
MIA	50 μM	104 ± 2.2
EPNP	5 mM	102 ± 2.6
1,10-Phenanthroline monohydrate	10 mM	114 ± 2.7
EDTA	10 mM	67 ± 1.8
EGTA	2 mM	86 ± 3
Ca^{2+} ($CaCl_2$)	2 mM	300 ± 7.7
Fe^{2+} ($FeCl_2$)	2 mM	260 ± 5.2
Cu^{2+} ($CuCl_2$)	2 mM	140 ± 2.9
Mn^{2+} ($MnCl_2$)	2 mM	100 ± 2.5
Mg^{2+} ($MgCl_2$)	2 mM	100 ± 2.5
Zn^{2+} ($ZnCl_2$)	2 mM	210 ± 4.7
Co^{2+} ($CoCl_2$), Hg^{2+} ($HgCl_2$), or Cd^{2+} ($CdCl_2$)	2 mM	0 ± 0.0

The dialyzed and non-treated enzyme was considered as 100% for metallic ion assay. Residual SAPN activity was assayed at pH 10 and 75 °C

PMSF phenylmethanesulfonyl fluoride, *DFP* diisopropylfluorophosphate, *SBTI* soybean trypsin inhibitor, *TLCK* Nα-p-tosyl-ʟ-lysine chloromethyl ketone, *TPCK* Nα-p-tosyl-ʟ-phenylalanine chloromethyl ketone, *DTNB* 5,5′-dithio-bis-2-nitro benzoic acid, *NEM* N-ethylmaleimide, *MIA* monoiodoacetic acid, *EPNP* 1,2-epoxy-3-(4-nitrophenyloxy) propane, *EDTA* ethylene-diaminetetraacetic acid, *EGTA* ethylene glycol-bis (β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid

[a]Values represent the means of three replicates, and ± standard errors are reported

thermostabilization was more effective with 2 mM calcium and PEG 1000 at 100 g/L, since the $t_{1/2}$ at 80 °C was determined to be, respectively, 13 h, compared to 7 h in the absence of any additive (Fig. 3f).
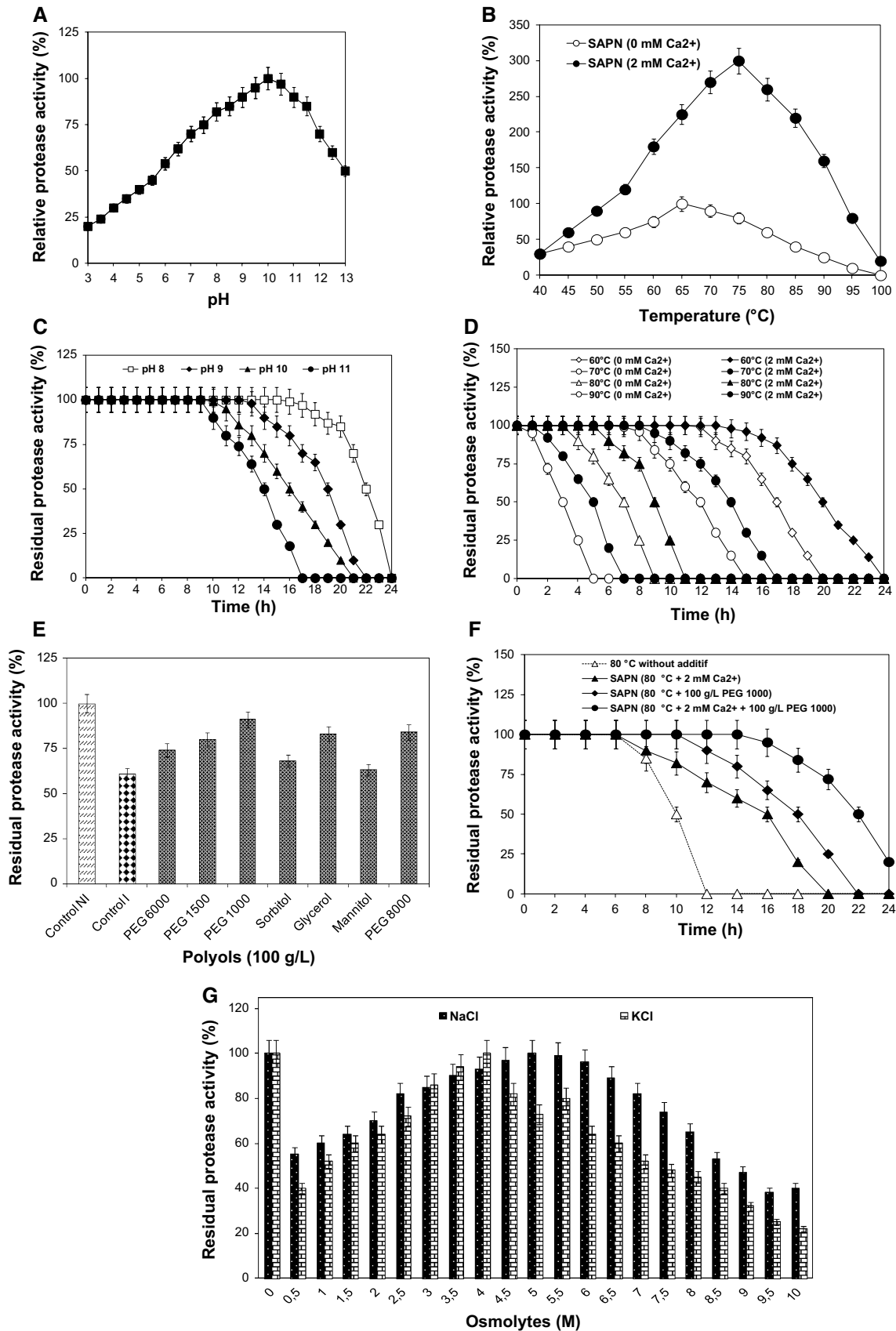
Effect of KCl and NaCl on the thermal stability

The effect of osmolytes on the enzyme stability was studied increasing NaCl and KCl concentrations from 0 to 10 M (Fig. 3g). The optimal residual activity was reached at 5 M NaCl and 4 M KCl and was about 100%. Beyond 5 M NaCl, protease activity decreased progressively and retained 40% of its relative activity at 10 M NaCl. The relative activities of SAPN at 5 M KCl were about 22% of initial activity.

Substrate specificity

As given in Table 4, SAPN protease reacted with all proteins tested. It was able to hydrolyze casein, albumin, gelatin, ovalbumin, and keratin to varying degrees. However, it was not able to hydrolyse collagen type I and collagen type II. Furthermore, SAPN was not able to hydrolyze BAEE, TAME, and BCEE. Besides, it has a high hydrolysis rate towards BTEE and ATEE.

Synthetic peptide substrates, conjugated with *p*-nitroanilide, were also used to determine the pattern of specificity. The highest specificity of the protease was observed towards Suc-F-A-A-F-*p*NA followed by Suc-A-A-P-F-*p*NA. In addition, the relative activity of SAPN enzyme is 45% with Suc-A–A-P-L-*p*NA and 63% with Suc-L-L-V-Y-*p*NA

when alanine and proline residues are present in P_1 position. SAPN exhibited no detectable activity on Ac-Y-V-A-D-*p*NA.

Performance evaluation of the purified subtilisin

Determination of kinetic parameters

The kinetic parameters for casein, azo-casein, BTEE, and FAAF hydrolysis were determined under optimal experimental conditions for each enzyme, according to the Michaelis–Menten equation using Lineweaver–Burk plot.

The k_{cat}/K_m value of each enzyme (SAPN, SAPB, SPVP, subtilisin 309, and subtilisin A) is illustrated in Table 5. In the present report, SAPN showed a maximum turnover number (k_{cat}) of 63,200/min for casein and a low K_m of 0390 mM at 75 °C and pH 10 (Table 5). Therefore, catalytic efficiency (k_{cat}/K_m) was found to be 162,051/min/mM.

SAPN subtilisin has relatively higher K_m for casein, azo-casein, BTEE, and FAAF. When casein was used as a substrate, SAPN was noted to exhibit k_{cat}/K_m values that were 2.6, 3.5, 9.7, and 17.1 times elevated than those of SPVP, SAPB, subtilisin 309, and subtilisin A, respectively. When azo-casein was used as a modified protein, SAPN was noted to exhibit k_{cat}/K_m values that were 3.3, 6.3, 10.9, and 13.7 times elevated than those of SPVP, SAPB, subtilisin 309 and subtilisin A, respectively.

A kinetic study was also performed using FAAF to investigate the effects of amino-acid residues adjacent to phenylalanine as a specific tetra-peptide substrate for subtilisin. FAAF was also the preferred substrate for SAPN with k_{cat}/K_m that were at least 2.0, 4.6, 12.8, and 18.4 fold higher, respectively, than those observed for SPVP, SAPB, subtilisin 309, and subtilisin A.

Determination of the degree of hydrolysis of the proteins in the by-products

The potential protein hydrolysis ability of each commercial and purified proteolytic enzyme, namely SAPN, SPVP, alcalase™ Ultra 2.5, subtilisin 309, flavourzyme® 500 L, trypsin, pepsin, and papain was tested by investigating the extent of hydrolysis. The hydrolysis curves of *M. monoceros* or *P. segnis* by-products, after 120 min of incubation, are shown in Fig. 4a, b. The purified SAPN from strain Nari2A[T] was the most efficient among the proteases. In fact, as shown in Fig. 4a, SAPN was the most efficient, with 34% protein hydrolysed used during hydrolysis of blue swimming crab *P. segnis*, followed by SPVP (24%), subtilisin 309 (17%), flavourzyme® 500 L (14%), alcalase Ultra 2.5 L (11%), papain (9%), trypsin (6%), and pepsin (4%). As shown in Fig. 4b, the purified SAPN was the most efficient, with 38% SAPN used during hydrolysis of white shrimp *M. monoceros*, followed by subtilisin 309 (19%), flavourzyme® 500 L (16%), alcalase Ultra 2.5 L (13%), papain (11%), and trypsin (8%), with pepsin being the least efficient (6%) one.

Chitin extraction from blue swimming crab blue and white shrimp bio-wastes

Deproteinization of swimming crab *P. segnis* or white shrimp *M. monoceros* by-products using SAPN from Nari2A[T] was first carried out at pH 10 and 75 °C. As shown in Fig. 4c, in the control experiments without SAPN, there was hardly any deproteinization of white shrimp and blue crab shells. The addition of 5 U of SAPN protease/mg proteins for white shrimp shells and blue swimming crab improved the efficiency of proteins removal and eliminated around 30 and 20% of proteins, respectively. However, the deproteinization ratio with $E/S = 5$ U/mg was 80%, and the percentage of protein removal increased with increasing E/S ratio, reaching about 93% with E/S ratio of 10 U/mg.

However, the percentage of protein removal increased with increasing E/S ratio and reached about 60 and 78% for blue swimming crabs and white shrimp shells, respectively, beyond a ratio of $E/S = 20$ U/mg. Additional increase in SAPN concentration did not improve significantly the deproteinization rate.

Chitin characterization by FTIR

The two extracted chitins were subjected to FTIR spectroscopy analysis, and compared with the commercial one, as shown in Fig. 5. The spectra of the *P. segnis* and *M. monoceros* samples exhibited typical α-chitin features, without bands at 1540/cm with bands above 3000/cm, with peaks at 2919, 2922, 1621, 1600, 1429, 1403, 1010, and 1040/cm.

Table 4 Substrate specificity profile of SAPN enzyme from *Me. thermohalophilus* Nari2A[T]

Substrate	Concentration	Absorbance (nm)[a]	Relative protease activity (%)[b]
Natural protein			
Casein	30 g/L	600	100 ± 2.5
Albumin	30 g/L	600	96 ± 1.8
Gelatin	30 g/L	600	65 ± 1.2
Ovalbumin	30 g/L	600	23 ± 0.4
Keratin	30 g/L	600	30 ± 0.9
Modified protein			
Azo-casein	25 g/L	440	100 ± 2.5
Albumin azure	25 g/L	440	74 ± 1.6
Keratin azure	25 g/L	440	25 ± 1.4
Collagen type	10 g/L	440	0 ± 0.0
Collagen type II	10 g/L	440	0 ± 0.0
Ester			
BTEE	10 mM	253	100 ± 2.5
ATEE	10 mM	253	96 ± 2.4
BAEE	10 mM	253	0 ± 0.0
BCEE	10 mM	253	0 ± 0.0
TAME	10 mM	253	0 ± 0.0
Synthetic peptide (*p*NA)			
P4–P3–P2–P1–P'1			
Suc-F-*p*NA	5 mM	410	41 ± 1.7
Benz-Y-*p*NA	5 mM	410	0 ± 0.0
Met-*p*NA	5 mM	410	0 ± 0.0
Ac-L-*p*NA	5 mM	410	0 ± 0.0
Pro-*p*NA	5 mM	410	0 ± 0.0
Ac-A-*p*NA	5 mM	410	0 ± 0.0
Benz-R-*p*NA	5 mM	410	72 ± 1.9
Suc-Y-L-V-*p*NA	5 mM	410	0 ± 0.0
Suc-A-A–A-*p*NA	5 mM	410	0 ± 0.0
Suc-A-A-V-*p*NA	5 mM	410	14 ± 0.1
Suc-A-A-F-*p*NA	5 mM	410	27 ± 0.8
Benz-F-V-R-*p*NA	5 mM	410	0 ± 0.0
Suc-F-A–A-F-*p*NA	5 mM	410	100 ± 2.5
Suc-A-A-P-F-*p*NA	5 mM	410	97 ± 2
Suc-A-A-V-A-*p*NA	5 mM	410	84 ± 1.8
Suc-A-A-P-M-*p*NA	5 mM	410	71 ± 1.4
Suc-A-A-P-L-*p*NA	5 mM	410	45 ± 0.4

Table 4 (continued)

Substrate	Concentration	Absorbance (nm)[a]	Relative protease activity (%)[b]
Suc-L-L-V–Y-*p*NA	5 mM	410	63 ± 0.9
Ac-Y-V-A-D-*p*NA	5 mM	410	0 ± 0.0

BTEE *N*-benzoyl-ʟ-tyrosine ethyl ester, *ATEE* *N*-acetyl-ʟ-tyrosine ethyl ester monohydrate, *BAEE* *N*-benzol-ʟ-arginine ethyl ester, *BCEE* *S*-benzyl-ʟ-cysteine ethyl ester hydrochloride, *TAME* N_α-*p*-tosyl- ʟ-arginine methyl ester hydrochloride

N-succinyl-ʟ-Phe-*p*-nitroanilide; *N*-benzoyl-ʟ-Tyr-*p*-nitroanilide; *N*-acetyl-ʟ-Leu-*p*-nitroanilide; ʟ-Met-*p*-nitroanilide; ʟ-Pro-*p*-nitroanilide trifluoroacetate salt; *N*-acetyl-ʟ-Ala-*p*-nitroanilide; ʟ-Val-*p*-nitroanilide hydrochloride; *N*-benzoyl-ʟ-Arg-*p*-nitroanilide; *N*-succinyl-ʟ-Tyr-ʟ-Leu-ʟ-Val-*p*-nitroanilide; *N*-succinyl-ʟ-Ala-ʟ-Ala-ʟ-Ala-*p*-nitroanilide; *N*-succinyl-ʟ-Ala-ʟ-Ala-ʟ-Phe-*p*-nitroanilide; *N*-succinyl-ʟ-Ala-ʟ-Ala-ʟ-Val-*p*-nitroanilide; *N*- benzoyl-ʟ-Phe-ʟ-Val-ʟ-Arg-*p*-nitroanilide; *N*-succinyl-ʟ-Phe-ʟ-Ala-ʟ-Ala-ʟ-Phe-*p*-nitroanilide; *N*-succinyl-ʟ-Ala-ʟ-Ala-ʟ-Pro-ʟ-Phe-*p*-nitroanilide; *N*-succinyl-ʟ-Ala-ʟ-Ala-ʟ-Val-ʟ-Ala-*p*-nitroanilide; *N*-succinyl-ʟ-Leu-ʟ-Leu-ʟ-Val-ʟ-Tyr-*p*-nitroanilide; *N*-succinyl-ʟ-Ala-ʟ-Ala-ʟ-Pro-ʟ-Met-*p*-nitroanilide; *N*-succinyl-ʟ-Ala-ʟ-Ala-ʟ-Pro-ʟ-Leu-*p*-nitroanilide; *N*-acetyl-ʟ-Tyr-ʟ-Val-ʟ-Ala-ʟ-Asp-*p*-nitroanilide

[a]The activity of each substrate was determined by measuring absorbance at specified wave lengths, according to the relative method reported elsewhere (Zaraî Jaouadi et al. 2013)

[b]Values represent the means of three replicates, and ± standard errors are reported

Discussion

Hypersaline environments constitute typical examples of environments with extreme conditions due to their exposure to high and low temperatures, high salinity, high pH values and in some cases, ultrasonic vibrations, and low oxygen conditions. In halophilic or halotolerant bacterial proteins, the 'halo-adaptation' is achieved by multiple ways. A novelty in their biochemical significance, structure and characteristics, and a thorough understanding of robust proteases, exceptionally salt-stable subtilisin proteases, denoted as halolysin functioning, could be of prime importance (Mokashe et al. 2018).

The application of fish processing by-products as cheap substrate for the production of proteolytic enzyme could also reduce environmental pollution. *P. segnis* crab powder *and M. monoceros* shrimp by-products are two essential sources for the development of the strain Nari2A[T] and the synthesis

Table 5 Kinetic parameters of purified proteases: SAPN, SPVP, SAPB, subtilisin 309, and subtilisin A for the hydrolysis of a natural protein (casein), a modified protein (azo-casein), an ester (BTEE), and a synthetic peptide (FAAF)

Substrate	Enzyme	K_m (mM)	V_{max} (U/mg)	k_{cat} (/min)	k_{cat}/K_m (min/mM)	Relative catalytic efficiency to SAPN
Casein	SAPN	0.390 ± 0.01	$94,800 \pm 590$	63,200	162,051	1.00
	SPVP	0.455 ± 0.03	$41,255 \pm 465$	27,500	60,439	0.37
	SAPB	0.400 ± 0.02	$27,160 \pm 370$	18,106	45,265	0.28
	Subtilisin 309	0.760 ± 0.05	$18,901 \pm 295$	12,601	16,580	0.10
	Subtilisin A	0.810 ± 0.06	$11,517 \pm 113$	76,78	9479	0.05
Azo-casein	SAPN	0.489 ± 0.03	$66,250 \pm 499$	44,167	90,321	1.00
	SPVP	0.576 ± 0.05	$23,045 \pm 311$	15,363	26,671	0.29
	SAPB	0.605 ± 0.05	$12,870 \pm 118$	8580	14,181	0.16
	Subtilisin 309	0.880 ± 0.07	$10,912 \pm 107$	7274	8265	0.09
	Subtilisin A	0.960 ± 0.08	9456 ± 100	6304	6566	0.07
BTEE	SAPN	0.572 ± 0.05	$39,231 \pm 425$	26,154	45,723	1.00
	SPVP	0.682 ± 0.06	$10,076 \pm 101$	6717	9848	0.21
	SAPB	0.752 ± 0.05	4970 ± 90	3313	4405	0.09
	Subtilisin 309	0.899 ± 0.07	4752 ± 85	3160	3523	0.07
	Subtilisin A	0.987 ± 0.08	3698 ± 50	2465	2497	0.05
Suc-FAAF-pNA	SAPN	0.635 ± 0.05	$175,222 \pm 999$	116,814	187,960	1.00
	SPVP	0.701 ± 0.05	$95,833 \pm 596$	63,887	91,137	0.48
	SAPB	1.340 ± 0.12	$81,375 \pm 795$	54,250	40,485	0.21
	Subtilisin 309	1.685 ± 0.16	$36,958 \pm 402$	24,638	14,621	0.08
	Subtilisin A	1.921 ± 0.21	$29,350 \pm 381$	19,566	10,185	0.05

SPVP is a serine alkaline protease, produced by the thermophilic *Aeribacillus pallidus* VP3, and it was previously purified and characterized by the authors (Mechri et al. 2017a). The optimum pH of SPVP is 10, while the optimum temperature was determined to be 60 °C

[a]Values represent the means of three replicates, and ± standard errors are reported

of its metabolites. Previously, a significant production was noted for the proteolytic enzymes by *B. pumilus* strain A1 and *B. mojavensis* strain A21 (1267 and 214 U/mL, respectively, on a medium containing 50 g/L powder of shrimp by-product) (Ghorbel Bellaaj et al. 2012). Similarly, Annamalai et al. documented a production of halo-stable proteases from halophilic bacterial strains using marine bio-wastes (Annamalai et al. 2014a, b).

The protease activity of SAPN is assayed and the protein concentration is determined. This relatively good recovery rate, indicated that ammonium sulfate precipitation is efficient in proteins precipitation (Amid et al. 2012). The high level of specific activity strongly suggested the potential prospects of SAPN in various industrial applications.

A variable molecular weight of bacterial serine proteases was determined; viz the alkaline protease from *B. licheniformis* MP1 with MW of 49 kDa (Jellouli et al. 2011) and the SPVP enzyme from *Aeribacillus pallidus* VP3 with MW of 29 kDa (Mechri et al. 2017a). On the other hand, a protease from *Bacillus* sp. ZJ1502 has an extremely low molecular weight of 14 kDa (Yu et al. 2019). Serine proteases that have larger molecular weight are also available in the literature. Taking the example of the one secreted from *B. halotolerans* strain CT2 had high molecular weight of 250 kDa (Dorra

et al. 2018). These illustrated results potently suggest that SAPN protease from strain Nari2A[T] is a monomeric protein comparable to those previously reported for other proteases from *Bacillus* strains (Mechri et al. 2017b; Zaraî Jaouadi et al. 2012).

The NH2-terminal sequence of SAPN showed uniformity. It differed to *B. licheniformis* strain MP1 by 3 amino-acid residues at positions 14, 17, and 23. Moreover, it differed to subtilisin Novo from *B. amyloliquefaciens* strain DC-4 by ten amino-acid residues at positions T3S, I8V, P9S, L10Q, F14P, K15A, V16L, P17H, A18S, and F21Y. These results revealed that SAPN protease is a new subtilisin enzyme.

PMSF is known to sulphonate the essential serine residue on the active site of the SAPN enzyme, resulting in a decrease in enzyme inactivation (Rani et al. 2012). This group of salt-stable serine or subtilisin-like serine protease, denoted as 'halolysin', is isolated from different halophilic *Bacillus* and related groups such as *Alkalibacillus* sp. strain NM-Fa4, *Oceanobacillus iheyensis* strain O.M.A18, *B. halodurans* strain US193, and *Halobacillus* sp. strain SCSIO 20089 (Daoud et al. 2018; Mesbah and Wiegel 2014; Purohit and Singh 2014; Yang et al. 2013). The inhibition profile was similar to that observed for the purified SAPHM produced by the alkaline protease of *B.*

Fig. 4 Performance evaluation of the purified subtilisin SAPN in *M. monoceros* and *P. segnis* proteins hydrolysis. **a** Hydrolysis curves of white shrimp *M. monoceros* treated with various purified and commercial proteases. The proteases used were: SAPN (filled diamond), SPVP (filled circle), subtilisin 309 (empty circle), flavourzyme® 500 L (filled square), alcalase™ Ultra 2.5 L (empty square), papain (filled triangle), trypsin (empty triangle), and pepsin (asterisk). Each point represents the mean ($n=3$) ± standard deviation. **b** Hydrolysis curves of blue swimming crab, *P. segnis* proteins treated with the same purified and commercial proteases. Each point represents the mean ($n=3$) ± standard deviation. **c** Effects of the *E*/*S* ratio on the deproteinization of white shrimp *M. monoceros* and blue swimming crab, *P. segnis* by-products. Protein hydrolysis was performed for 3 h at pH 10 and 75 °C. Each point represents the mean ($n=3$) ± standard deviation
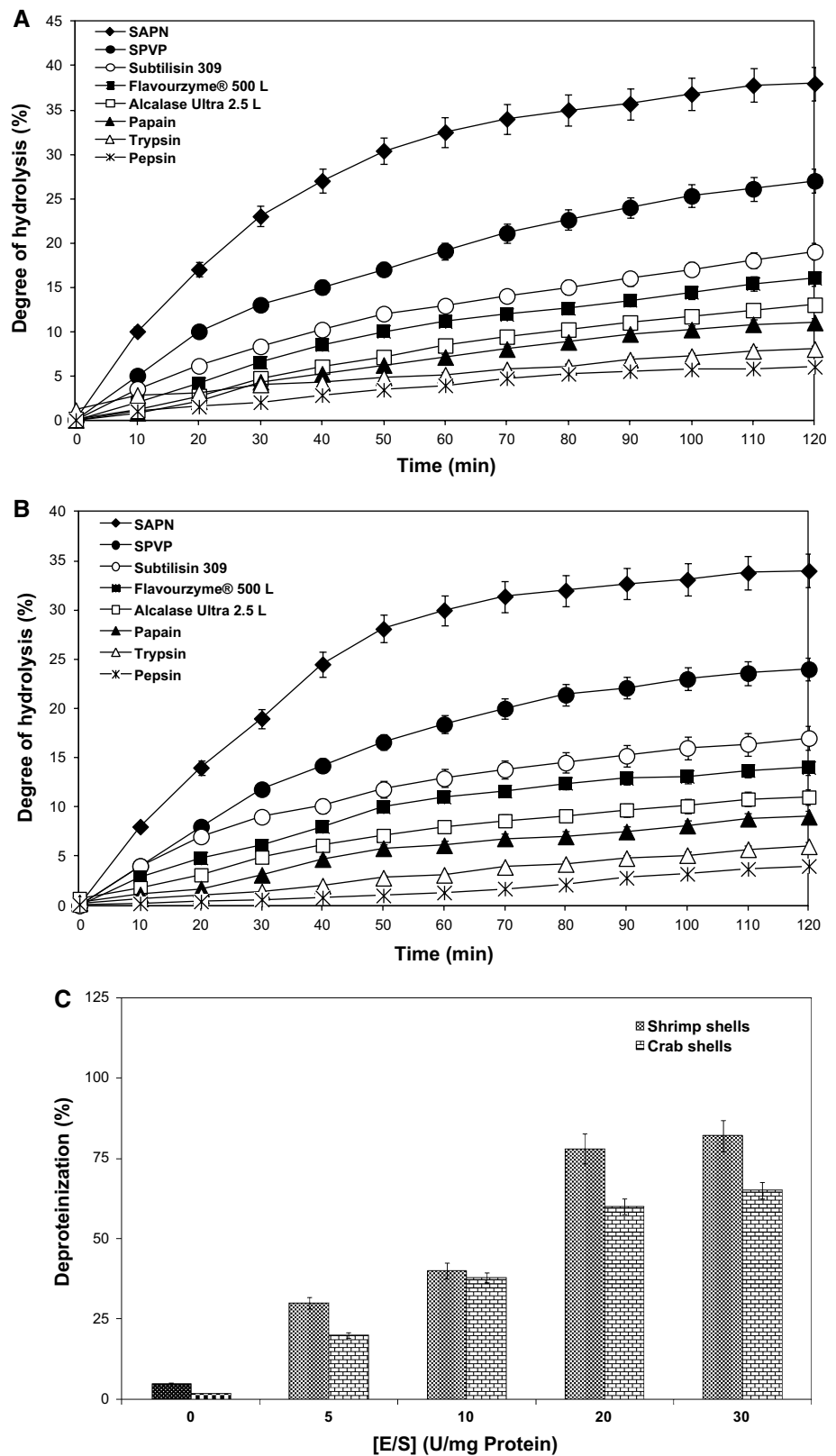
licheniformis K7A (Hadjidj et al. 2018). Furthermore, aspartic and cysteine *endo*-proteases are rarely found in halophilic and halotolerant bacteria.

The moderate inhibition by EDTA and EGTA suggested that SAPN enzyme is not a metalloprotein. Similar result was reported for alkaline proteases recovered from *Bacillus*

Fig. 5 FTIR analysis of chitins. **a** Commercial chitin. **b** Swimming crab chitin (CPs) treated with subtilisin SAPN. **c** White shrimp *M. Monoceros* chitin (CMm) treated with subtilisin SAPN
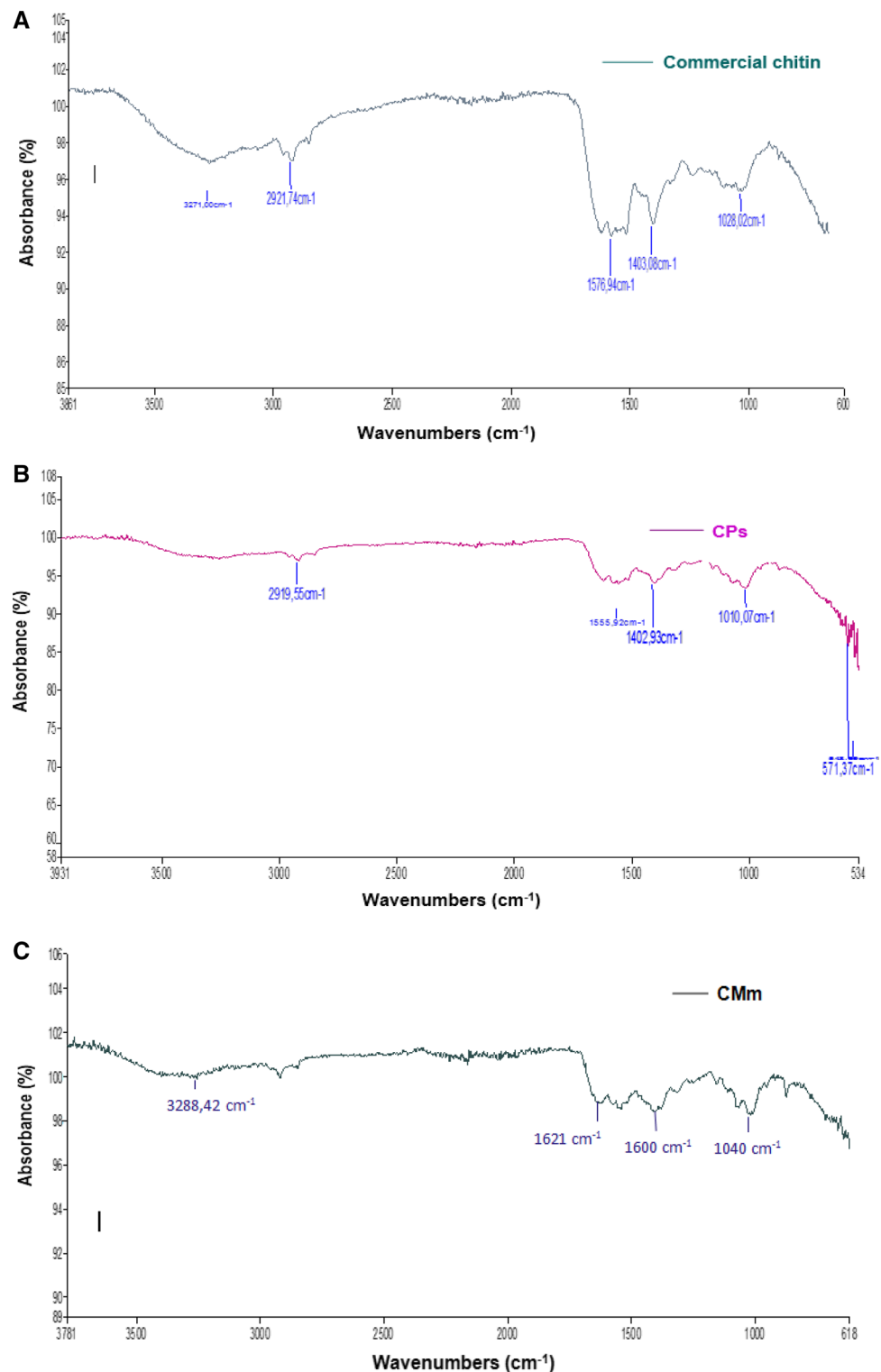
strains (Hadjidj et al. 2018; Mechri et al. 2017b). The stability of the enzyme in the presence of EDTA is advantageous for the use of SAPN as detergent additive. The fact that the proteolytic enzyme shows high stability in the presence of EDTA and EGTA is of great significance with respect to detergent industry use. In fact, the chelating agents such as EDTA and EGTA are among the most commonly found components in detergent formulations, used to soften water and assist in removing stains. The majority of enzymes of halophilic or halotolerant bacteria require the presence of Ca^{2+}. In fact, two calcium-binding sites are present in other members of the subtilisin superfamily, including subtilisin

SAPRH (Rekik et al. 2019). Metal-binding sites can function to both stabilize and activate enzymes. The inhibition of SAPN activity by cobalt and cadmium which are two heavy metals could be perhaps ascribed to their binding to catalytic residues in the active site of the enzymes. Such an observation was made for the purified protease KERUS strongly inhibited by cobalt, nickel, and mercury (Zaraî Jaouadi et al. 2013). Rekik et al. explored the engineering of binding affinity at metal ion binding sites for the stabilization of subtilisin conformation (Rekik et al. 2019). Rather, SAPB, purified from *B. pumilus* strain CBS, is strongly stabilized by calcium and magnesium ions at specific sites in its tertiary structure, preventing autolysis and thermal unfolding (Zaraî Jaouadi et al. 2012). It is worth noting, in this context that, considering potential industrial application, the activity of SAPN in the presence of metallic ions is a key property.

SAPN had a good activity in pH range from neutral to alkaline; however, the activity was higher in alkaline pH. In line with the current study, the serine protease from *Lysinibacillus fusiformis* C250R has an optimum pH at 10, as the same of SAPN (Mechri et al. 2017b). Proteases from *Caldicoprobacter guelmensis* D2C22[T] and *B. licheniformis* K7A also show an optimum of 10 (Bouacem et al. 2015; Hadjidj et al. 2018).

Bacterial proteases are active over a wide range of pH, depending on the source of the bacteria from which they have been isolated. The range of pH stability of SAPN from strain Nari2A[T] will be very useful in terms of industrial applications, particularly, in commercialized laundry detergents. An alkaline pH in the wash water can considerably improve the cleaning ability of the detergent. They are generally optimal in an alkaline pH range of 9–11. Other serine proteases with an optimum temperature of 75 °C have been reported in the literature. The optimum pH range of alkaline proteases is generally between 55 and 65 °C (Hadjidj et al. 2018), with a few exceptions at higher temperature optima of 70 and 80 °C (Bouacem et al. 2015; Mechri et al. 2017a; Omrane Benmrad et al. 2018). All the results obtained strongly suggested the high thermostable nature of the enzyme, thus ideal for the applications in alkaline environments. Results of the study that was performed demonstrated that the SAPN protease is much more stable at 70 and 80 °C than the serine protease SAPRH purified from *B. safensis* strain RH12 (Rekik et al. 2019).

The addition of each polyol increased the stability of SAPN from Nari2A[T] towards 80 °C after pre-incubation at 1 h over the control. Rather, literature studies have confirmed that the addition of substances, such as polyols, enhanced the stability of several proteolytic enzymes (Omrane Benmrad et al. 2018; Shakilanishi et al. 2017). In fact, studies have been conducted, revealing that the effectiveness of PEG on proteins highly depends on the polymer molecular weight and on the protein structure.

Moreover, PEG with high molecular weights is believed to prevent protein–protein interactions, leading to stabilization (Nirmal and Laxman 2014). A thorough study of the process of deactivation pertaining to proteases has shown that improving thermal stability is essential for efficient operation in numerous industrial applications (Mechri et al. 2017b; Rekik et al. 2019). The increase in the thermal stability by adding polyols was probably due to the reinforcement of the hydrophobic interactions among nonpolar amino-acids inside the enzyme molecules, thus increasing their resistance to inactivation. The investigation of SAPN substrate specificity is interesting, because no quantitative measure of absolute substrate specificity exists. Rather, specificity must be discussed in relative terms, wherein ratios of catalytic parameters with multiple substrates are presented to ascertain patterns of reactivity (Li et al. 2018). Purified proteases with high specificity for substrates can be used in industrial processes in such similarly to chemical catalysts, and their recovery and reuse would facilitate a better cost–benefit ratio. The specificity of proteolytic enzyme modulated by the classification of amino-acids as well as by functional group, aromatic, aliphatic or sulphur-containing, determines the specificity of the enzyme to the substrate close to the bond being hydrolysed (Hadjidj et al. 2018). In fact, subtilisin that preferentially hydrolyzes peptide links, contributed by hydrophobic (Ala, Val, Leu, Ile, Met, Phe, Tyr, and Trp), cationic (Lys and Arg), anionic (Asp and Glu), and polar (Ser and Gln) side chains at P1 as well as proline at P1, which are known to exist. As expected, among natural and modified proteins, the casein and azocasein were hydrolysed to a much higher degree than the albumin, gelatin, ovalbumin, keratin, albumin azure, and keratin azure, which is in accordance with other investigations of substrate specificity profiles of subtilisins (Rekik et al. 2019). In fact, they are found to be more active against casein compared to albumin, ovalbumin, and keratin, since albumin and ovalbumin are present in several stains. These results indicate the utility of the purified enzyme SAPN for detergent formulation. Thus, it can also be used in pharmaceutical and cosmetic purposes, or in the leather industry, where collagen should not be attacked (Zaraî Jaouadi et al. 2013). SAPN was noted to exhibit both esterase and amidase activities on N-terminal and C-terminal protected L-tyrosine. This enzyme has no esterase and amidase activities on N-terminal and C-terminal protected L-arginine and cysteine. This behavior has already been described for other subtilisins from *Bacillus* origins towards esters (Hadjidj et al. 2018; Mechri et al. 2017b). In contrast to these results, another serine keratinolytic protease, KERUS, did not show protease activity towards ATEE and BTEE. Several biologically active peptides are broken by the enzyme at sites consistent with

the specificity deduced from studies with model synthetic substrates. The higher subtilisin activity with Suc-F-A-A-F-*p*NA and Suc-A-A-P-F-*p*NA substrates shows that the SAPN enzyme specifically prefers hydrophobic substrates, with aromatic residues occupying the P1 and P4 positions. Mechri et al. (2017a) reported that another serine protease, SPVP, showed similar activity towards Suc-F-A-A-F-*p*NA and Suc-A-A-P-F-*p*NA, while it did not show activity towards Suc-A-A-A-*p*NA and a very weak activity towards Suc-A-A-V-*p*NA, which suggested that SPVP protease largely preferred hydrophobic substrates, with aromatic residues occupying the P1 and P4 positions of *p*NA substrates. In contrast to these results, other serine keratinolytic proteases, such as KERUS and KERCA and KERQ7, did not show protease activity towards Suc-F-A-A-F-*p*NA and Suc-A-A-P-F-*p*NA (Zaraî Jaouadi et al. 2013). Proline at the P2 position was also noted to promote hydrolysis by SAPN enzyme, a feature that was not observed for other subtilisins. Hence, the substrate specificity profiles of the proteolytic enzyme SAPN were closely similar to subtilisins, not only in terms of specificity for position P1, but also with regard to the effects of amino-acid residues neighboring the cleavage site.

The shape of the hydrolysis curve (Fig. 4) is similar to those previously published for cuttlefish muscle, keratin, and casein (Balti et al. 2015; Hamiche et al. 2019; Omrane Benmrad et al. 2016). These findings indicate that SAPN can be useful for the preparation of protein and peptides hydrolysates. In fact, an optimal release of protein from blue swimming crab *P. segnis* or white shrimp *M. monoceros* while leaving chitin intact is a promising industrial and biotechnology opportunity. The quality of chitin depends on the extraction process. In the cuticles of crustaceans, chitin is closely associated with lipids, proteins, and minerals (Arbia et al. 2013). These compounds should be removed to achieve the purity degree of chitin, necessary for biological and industrial applications (Younes et al. 2016). Chitin from crustaceans can be recovered chemically or enzymatically. Since chemical deproteinization results in high amounts of bases and acids waste, enzymatic deproteinization using proteolytic enzymes is considered as the most favorable approach and an alternative treatment to alkali digestion (Doan et al. 2019; Dun et al. 2019).

Despite proteins, probably related to chitin by covalent bonds, which require a chemical or enzymatic treatment for their recovery, some proteins may be associated with the chitinous matrix by electrostatic forces or hydrogen bonds that can be dissociated by heat treatment (Hamdi et al. 2017; Jellouli et al. 2011). Other proteins are probably linked to chitin by hydrogen bonds and electrostatic forces, whose removal requires enzymatic treatment or additional chemical.

The ability of the subtilisin SAPN to rapidly and efficiently degrade recalcitrant solid by-product may be harnessed for developing an eco-friendly and cost-effective process for solid bio-waste management, especially from crustacean shells. The absence bands at 1540/cm proved the efficacy of enzymatic deproteinization, and thereby, the purity of the extracted chitin (Lassoued et al. 2015). In the stretching vibration region of OH and NH, groups above 3000/cm are assigned to the vibrational modes involved in intermolecular hydrogen bonding CO–HN and the intramolecular bonds –NH groups, respectively (He et al. 2012). The peaks at 2919 and 2922/cm were assigned to the stretching vibration of $-CH_3$ and $-CH_2$, respectively (Hamdi et al. 2017). In addition, the splitting of the amide I band in the blue swimming crab and shrimp chitins spectra was observed, with the occurrence of peaks at 1621 and 1600/cm, attributed to the intermolecular hydrogen bonds CO–HN and at 1555/cm due to the intramolecular hydrogen bond $CO–HOCH_2$ (Liu et al. 2012; Mohammed et al. 2013). Rather, a band at 1429/cm, attributed to CH_2 group occurred in the FTIR spectra of the extracted chitin from shrimp by-product. The peaks at 1403, 1010, and 1040/cm for the characteristic peaks of β-(1-4)-glucoside bond in chitin are both shown in Fig. 5a–c, which demonstrated that the enzymatic deproteinization of the two bio-wastes by SAPN did not destroy the structure of glucoside molecule (He et al. 2012).

Conclusions

This study describes in this study allowed the production, purification and characterization of an extracellular protease secreted by *Me. thermohalophilus* strain Nari2A[T], aiming at its utilization in the production of chitin from blue swimming crab, *P. segnis*, and white shrimp, *M. monoceros*. The process developed in this investigation allowed to produce a novel subtilisin with interesting properties in a process with alternate substrates from crustacean's by-products, thus, creating an outstanding alternative to managing them. Moreover, these investigations inspired us to explore the enzymatic biodiversity of other soil microorganisms of Chott Melghir. Further works are needed to be translated from laboratory and fundamental experiment to pilot an industrial-scale application. Therefore, the extracellular overproduction of SAPN, its thermostable nature, and its use in deproteinization of crab and shrimp shell wastes may find potential applications in the valorization of shrimp and crab bio-wastes to produce chitin.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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