Appl Biochem Biotechnol (2010) 162:345–357
DOI 10.1007/s12010-009-8846-y

Extraction and Characterization of Chitin, Chitosan, and Protein Hydrolysates Prepared from Shrimp Waste by Treatment with Crude Protease from *Bacillus cereus* SV1

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Received: 30 May 2009 / Accepted: 29 October 2009 /
Published online: 5 December 2009
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Abstract Chitin is a polysaccharide found in abundance in the shell of crustaceans. In this study, the protease from *Bacillus cereus* SV1 was applied for chitin extraction from shrimp waste material of *Metapenaeus monoceros*. A high level of deproteinization 88.8%±0.4 was recorded with an E/S ratio of 20. The demineralization was completely achieved within 6 h at room temperature in HCl 1.25 M, and the residual content of calcium in chitin was below 0.01%. ^{13}C CP/MAS-NMR spectral analysis of chitin prepared by the enzymatic deproteinization of shrimp wastes was found to be similar to that obtained by alkaline treatment and to the commercial α-chitin. The degree of N-acetylation, calculated from the spectrum, was 89.5%. Chitin obtained by treatment with crude protease from *B. cereus* was converted to chitosan by N-deacetylation, and the antibacterial activity of chitosan solution against different bacteria was investigated. Results showed that chitosan solution at 50 mg/mL markedly inhibited the growth of most Gram-negative and Gram-positive bacteria tested. Furthermore, the antioxidant potential of the protein hydrolysates obtained during enzymatic isolation of chitin was evaluated using various *in vitro* assays. All the samples exerted remarkable antioxidant activities. These results suggest that enzymatic deproteinization of the shrimp shell wastes, using *B. cereus* SV1 protease, could be applicable to the chitin production process.

Keywords Shrimp waste · Enzymatic deproteinization · Hydrolysates · Chitin · ^{13}C CP/MAS-NMR · Chitosan

Introduction

Chitin is a natural polysaccharide synthesized by a great number of living organisms and functions as a structural polysaccharide [1]. According to the amount of chitin produced annually all over

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the world, it is the most abundant renewable natural resource after cellulose [2]. This polymer consists of a linear chain of linked 2-acetoamido-2-deoxy-β-D-glucopyranose units. In nature, chitin is found as ordered crystalline microfibrils forming structural components in the exoskeleton of arthropods or in the cell walls of fungi and yeast. It is also produced by a number of other living organisms in the lower plant and animal kingdoms, serving in many functions where reinforcement and strength are required. Despite the widespread occurrence of chitin, up to now, the main commercial sources of chitin are still crab and shrimp shells [3].

Several techniques to extract chitin from various crustaceans have been reported. Conventionally, preparation of chitin from marine waste materials involves demineralization and deproteinization with the use of strong acids and bases [1, 4–6]. However, these reagents can cause a partial deacetylation of the chitin and hydrolysis of the polymer, resulting in final inconsistent physiological properties. Alternatively, some efforts have been directed towards the reduction of chemical treatments in a more eco-friendly processes such as bacterial fermentation [7–9] and treatment by proteolytic enzymes [10, 11] which have been applied for the deproteinization of crustacean wastes.

Because chitin has a compact structure, it is insoluble in most solvents. Therefore, the chemical modifications of chitin were performed. The most common derivative is chitosan, derived by partial deacetylation of chitin [12]. In its crystalline form, chitosan is normally insoluble in aqueous solution above pH 7.0. However, in diluted acids (pH 6.0), the protonated free amino groups on glucosamine facilitate solubility of the molecule [13].

Chitin and chitosan are biomolecules of a great potential, possessing versatile biological activities, demonstrating excellent biocompatibility and complete biodegradability. Therefore, they have found extensive applications in pharmacy, medicine, agriculture, food and textile industries, cosmetics, and wastewater treatment [14–19]. Antimicrobial activity of chitosan and its derivatives against several bacterial species is considered as one of the most important properties linked directly to their possible biological applications. As a natural antimicrobial agent, the antibacterial and antifungal activities of chitosan have been widely reported [14, 20–22]. The antibacterial activity of these compounds is influenced by a number of factors such as degree of polymerization [23–25], level of deacetylation [26], type of microorganism [27, 28], and some other physico-chemical properties.

In the present paper, we describe the isolation and characterization of chitin from shrimp waste of *Metapeneaus monoceros* obtained by treatment with crude enzyme from *Bacillus cereus* SV1 and its deacetylation into chitosan. Moreover, the antibacterial activity of the acid-soluble chitosan and the antioxidant properties of protein hydrolysates obtained during extraction of chitin of shrimp waste were investigated.

Materials and Methods

Raw Material and Reagents

The shrimp (*M. monoceros*) shells were obtained in fresh condition from the shrimp processing plant located in Sfax, Tunisia. Prior to use, the shrimp shells were washed thoroughly with distilled water. The shells were then stored at −20 °C until further analysis. The shrimp waste powder (SWP) was prepared in our laboratory according to the method described by Jellouli et al. [29].

Casein was supplied by Sigma Company (Sigma Co., St Louis, USA). Sodium hydroxide, hydrochloric acid, and acetic acid were from Panreac Quimica (all the other chemicals were of analytical grade).

Bacterial Strain

The strain used in this study was isolated from an oil sewage station from a fishing port in Sfax, Tunisia. It was identified as *B. cereus* SV1 based on its morphological and physiological characteristics and 16S rRNA sequence analysis [30].

Enzyme Production

The protease production was conducted in SWP medium consisting of (grams per liter): SWP 40.0, ammonium chloride 2.0, K_2HPO_4 0.5, KH_2PO_4 0.5, and $MgSO_4 \cdot 7H_2O$ 0.1 (pH 8.0). Cultivations were performed on a rotatory shaker (150 rpm) for 72 h at 30 °C, in 250-mL conical flasks with a working volume of 25 mL. The culture was centrifuged, and the supernatants were used for estimation of proteolytic activity by the method of Kembhavi et al. [31].

Chemical Analysis

The moisture and ash content were determined according to the AOAC [32] standard methods 930.15 and 942.05, respectively. Total nitrogen content of shrimp protein hydrolysates was determined by using the Kjeldahl method. Crude protein was estimated by multiplying total nitrogen content by the factor of 6.25. Lipids were determined gravimetrically after Soxhlet extraction of dried samples with hexane. The shrimp waste powder was analyzed for the selected minerals (Ca, Na, K, Mg) by high performance liquid chromatography (HPLC) and for Fe, Mn, Cu, and Zn by atomic absorption.

Deproteinization of Shrimp Wastes by SV1 Protease

Shell wastes were mixed with water at a ratio of 1:2 (*w*/*v*), minced then cooked for 20 min at 90 °C. The cooked sample was then homogenized in a Moulinex® blender for about 2 min. The pH of the mixture was adjusted to 8.0. Then, the shrimp waste proteins were digested with SV1 crude enzyme using different enzyme/substrate (E/S) ratio (unit of enzyme per milligram of protein). After incubation for 3 h at 40 °C, the reaction was stopped by heating the solution at 90 °C during 20 min to inactivate the enzyme. The shrimp waste protein hydrolysates were then centrifuged at 5,000×*g* for 20 min to separate insoluble and soluble fractions. The solid phase was washed and then dried for 1 h at 60 °C. The supernatant was used for analysis of protein concentration, lyophilized, and then used as shrimp waste protein hydrolysate (SWPH).

Deproteinization was expressed as percentage and computed by the following equation as described by Rao et al. [33].

$$\%\text{Deproteinization} = \frac{[(P_O \times O) - (P_R \times R)] \times 100}{P_O \times O}$$

where P_O and P_R are protein concentrations (%) before and after hydrolysis; while, O and R represent the mass (grams) of original sample and hydrolyzed residue in dry weight basis, respectively.

Deproteinization by Alkali

Shrimp wastes were mixed with 1.25 M NaOH solution at a ratio of 1:20 (*w*/*v*). The mixture was allowed to react at room temperature for 4 h. After filtration, the solid residue

was washed with deionized water until a neutral pH, and then dried at 65 °C in an oven. The dried residues were used for the analysis of deproteinization.

Demineralization

Demineralization was carried out in a dilute HCl solution. Solid fractions obtained after hydrolysis by SV1 crude protease were treated with 1.5 M HCl in 1:10 (*w*/*v*) ratio for 6 h at room temperature (25 °C) under constant stirring. The chitin product was filtered through four layers of gauze with the aid of a vacuum pump and washed to neutrality with deionized water and then freeze-dried. The residual minerals were estimated by HPLC.

^{13}C CP/MAS-NMR Spectroscopic Analysis

Chitin structural analysis was carried out by ^{13}C NMR (nuclear magnetic resonance) with CP/MAS technique (cross-polarization, magic-angle-spinning) using a BRUKER-ASX300 instrument. NMR spectra were recorded at a ^{13}C frequency of 75.5 MHz (field of 7.04 T). CP/MAS sequence was used with the following parameters: the ^{13}C spin lattice relaxation time was 5 s; powdered samples were placed in an alumina rotor used for the double air-bearing-type MAS system and spun as fast as 8 kHz. Contact time was 8 ms.

The degree of acetylation (DA) of the samples was determined by dividing the intensity of the resonance of the methyl group carbon by the average intensity of the resonances of the glycosyl ring carbon atoms. The DA was calculated using the following relationship [34].

$$\mathrm{DA}(\%) = \frac{100 \times I[\mathrm{CH3}]}{(I[\mathrm{C1}] + I[\mathrm{C2}] + I[\mathrm{C3}] + I[\mathrm{C4}] + I[\mathrm{C5}] + I[\mathrm{C6}])/6}$$

(*I* is the intensity of the particular resonance peak)

Antioxidant Activity of Shrimp Waste Protein Hydrolysates

DPPH Radical-Scavenging Assay

The DPPH (1, 1-diphenyl-2-picrylhydrazyl) radical-scavenging activity of SWPH was determined as described by Bersuder et al. [35]. A volume of 500 μL of each sample at different concentrations (0–2 mg/mL) was mixed with 500 μL of 99.5% ethanol and 125 μL of 0.02% DPPH in 99.5% ethanol. The mixtures were then incubated for 60 min in the dark at room temperature, and the reduction of DPPH radical was measured at 517 nm using a UV-visible spectrophotometer (T70, UV/VIS spectrometer, PG Instruments Ltd, China). In its radical form, DPPH has an absorption band at 517 nm which disappears upon reduction by an antiradical compounds. Lower absorbance of the reaction mixture indicated higher DPPH free radical-scavenging activity. Butylated hydroxyanisole (BHA) was used as a standard. DPPH radical-scavenging activity was calculated as follows:

$$\text{Radical} - \text{scavenging activity } (\%) = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

The control was conducted in the same manner, except that distilled water was used instead of sample. The test was carried out in triplicate.

Reducing Power Assay

The ability of hydrolysates to reduce iron (III) was determined according to the method of Yildirim et al. [36]. Sample solutions (1 mL) containing different concentrations of SWPH (0–2 mg/mL) were mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixtures were incubated for 30 min at 50 °C, followed by addition of 2.5 mL of 10% (*w*/*v*) trichloroacetic acid. The reaction mixtures were then centrifuged for 10 min at 1,650×*g*. Finally, 2.5 mL aliquot of the supernatant solution, from each sample mixture, was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% (*w*/*v*) ferric chloride. After a 10-min reaction time, the absorbance of the resulting solution was measured at 700 nm. Higher absorbance of the reaction mixture indicated higher reducing power. Values presented are the mean of triplicate analyses.

Antioxidant Assay Using the β-Carotene Bleaching Method

The ability of the protein hydrolysates to prevent the bleaching of ß-carotene was determined as described by Koleva et al. [37]. A stock solution of β-carotene/linoleic acid was prepared by dissolving 0.5 mg of β-carotene, 25 μL of linoleic acid, and 200 mg of Tween-40 in 1 mL chloroform. The chloroform was completely evaporated under vacuum in a rotatory evaporator at 45 °C, then 100 mL distilled water was added, and the resulting mixture was vigorously stirred. The emulsion obtained was freshly prepared before each experiment. Aliquots (2.5 mL) of the β-carotene/linoleic acid emulsion were transferred to test tubes containing 0.5 mL of each sample at different concentrations. The tubes were immediately placed in a water bath and incubated at 50 °C for 2 h. Thereafter, the absorbance of each sample was measured at 470 nm. A control consisted of 0.5 mL of distilled water instead of the sample solution. BHA was used as positive standard. Tests were carried out in triplicate.

Deacetylation of Chitin

The purified chitin was treated with 50% (*w*/*v*) NaOH at 80 °C for 4 h until it was deacetylated to chitosan. After filtration, the residue was washed with distilled water, and the crude chitosan was obtained by drying in a dry heat incubator at 50 °C overnight.

Antimicrobial Activity of Chitosan

The microorganisms used for antimicrobial activity are *Vibrio metschnikovii* (FJ752498), *Micrococcus luteus* (ATCC 4698), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Klebsiella pneumoniae* (ATCC 13883), *B. cereus* (ATCC 11778), *Bacillus mojavensis* (EU366229) isolated from marine water sample [38], *Bacillus pumilus* (EU719191) isolated from slaughter house polluted water [39], *Salmonella typhi*, and *Enterococcus faecalis* (ATCC 29212).

Antibacterial activity assays were performed according to the method described by Berghe and Vlietinck [40]. Chitosan was dissolved at 50 mg/mL in 0.1% acetic acid. The inoculum suspension (200 μL) of the tested microorganisms, containing 10^6 colony-forming units (cfu per milliliter) of bacteria cells were spread on Muller–Hinton agar. The inoculums were allowed to dry for 5 min. Then, bores (3-mm depth, 4-mm diameter) were made using a sterile borer and were loaded with 50 μL of each sample. Well with only acetic acid (without chitosan) was used as a negative control. Gentamycin was used as

positive reference. The petri dishes were kept, firstly, for 1 h at 4 °C, and then were incubated for 24 h at 37 °C. Antibacterial activity was evaluated by measuring the diameter of the growth inhibition zones in millimeters (including well diameter of 4 mm) for the test organisms and comparing to the controls. The measurements of inhibition zones were carried out for three sample replications, and values are the average of three replicates.

Statistical Analysis

All experiments were carried out in triplicate, and average values with standard deviation errors are reported. Mean separation and significance were analyzed using the SPSS software package (SPSS, Chicago, IL). Correlation and regression analysis was carried out using the EXCEL program.

Results and Discussion

Production of Chitins

We have earlier reported the production of an oxidant- and solvent-stable protease from *B. cereus* SV1 when grown in media containing only shrimp wastes powder as a sole carbon source. This indicated that the strain can deproteinize crustacean wastes to obtain its carbon and nitrogen requirements [41].

In the present study, chitins obtained by deproteinization of shrimp wastes with SV1 crude protease or by base extraction were carried out and chemical compositions of chitins were compared (Table 1). High enzymatic deproteinization of shrimp wastes was achieved and reached 88.8±0.4% with an E/S ratio of 20. Chemical deproteinization by NaOH 1.25 M of shrimp wastes of *M. monoceros* was investigated at different incubation times (data not shown). The best result was obtained after a 4-h incubation, and the protein content was decreased to 6.2%±1.4.

In the recovery of chitin from shrimp waste, associated minerals should be removed as a second stage. As a consequence, shrimp wastes deproteinized by enzymatic and alkaline treatments were subjected to mild acid treatment in order to remove minerals. The demineralization was completely achieved within 6 h at room temperature (25 °C) after treatment with 1.25 M HCl solution at a ratio of 1:10 (*w*/*v*). One of the factors determining the good quality of chitin is the low mineral content [42]. The two chitins obtained in this work present content of metals as low as those reported in other works [43, 44]. The

Table 1 Properties of the chitins prepared by enzymatic treatment (1) and by alkali (2).

%	Raw material	Chitin (1)	Chitin (2)
Moisture	66.2±1	4.2±0.7	3.96±0.5
Ash	34.69±0.19	0.433±0.05	0.441±0.07
Protein	40.83±1.22	10.78±0.2	6.2±1.38
Appearance	–	White flakes	Yellowish flakes
pH	7.6±0.2	6.95±0.2	6.5±0.2
Yield	–	16.55±1.5	20±2
Degree of acetylation (%)	–	89.57	77

residual contents of calcium, sodium, magnesium, and potassium in both chitins were about 0.01%, 0.0098%, 0.0019%, and 0.0011%, respectively.

The treatments employed to extract chitin from the shrimp wastes allowed the recovery of 20%±2 and 16.5%±1.5 (chemical and enzymatic deproteinization, respectively) of its initial mass as a water insoluble white fibrous material, which indicates that a good yield for the chitin extraction was attained and no pigments were present in the chitin. Other studies [42, 44] reported similar yields for the extraction of chitin. Canizares et al. [45] reported similar observations concerning the color of the obtained chitin. In fact, the discoloration of the chitin prepared by inversion of the two steps (i.e., demineralization followed by deproteinization) was less important. In addition, the HCl treatment may probably remove the residual acido-soluble proteins.

Chitin Characterization

The characteristics of the raw material and chitins that were prepared by enzymatic or alkaline treatments are shown in Table 1. The ground shrimp wastes before pre-treatment contained a relatively high content of protein (40.8±1.2%) and ash (34.7±0.2%). These results are comparable with those reported by previous studies [43, 46]. The demineralization conditions used in this study reduce the mineral content to permissible limits in the chitin. Indeed, the ash content was reduced to about 0.4%. This was lower than that found by Sini et al. [9]. This low ash content for both chitins indicated the suitability of removal of calcium carbonate and other minerals from the raw material. There were no significant differences in the moisture content, ash, and pH among the two chitins prepared ($p>0.05$). In contrast, the protein content was significantly higher in the chitin isolated with enzymatic deproteinization ($p<0.05$), but no complete removal of the residual protein associated with the chitin was achieved. This residual protein content must be corrected, and the percentage of the non-protein nitrogen fraction (nitrogen from chitin) must be withdrawn from the total nitrogen value as reported by Rødde et al. [47]. Although such deproteinization percentage is lower than that of chemical treatment, enzymatic deproteinization helps to avoid many drawbacks of chemical treatment such as heavy metal residues, over-hydrolysis, break down of chitin, etc.

^{13}C CP/MAS-NMR Spectroscopic Analysis

The structure of the chitin and its purity was evaluated by using ^{13}C CP/MAS solid-state NMR spectroscopy. ^{13}C CP/MAS-NMR spectra of the chitin samples prepared by enzymatic and alkaline treatments are shown in Fig. 1. Each spectrum consisted of eight well-defined resonances of C_1–C_6 carbons of the *N*-acetylglucosamine monomeric unit, which were observed between 50 and 110 ppm, indicating high structural homogeneity. The C=O signal appears as a sharp and symmetric profile indicating a unique conformational state, typical of α-chitin structure. In addition, the ^{13}C signals for C_3 (72.88 ppm) and C_5 (73.69 ppm) are clearly separated into two signals. These are similar to the commercial α-chitin and to chitins reported by Cardenas et al. [48] and Focher et al. [49]. However, for the β-chitin from *Illex argentinus* squid pens, the C_3 and C_5 merge into single resonance centered at 75.0 ppm [50]. Solid-state NMR studies support the identity of chitin prepared by the two methods described above. The chemical shift analysis of the two chitins prepared gave similar peak patterns to that of the commercial shrimp α-chitin.

The degree of acetylation is the most important characteristic of chitin, and its value depends on the raw material and the processes used for the deproteinization and

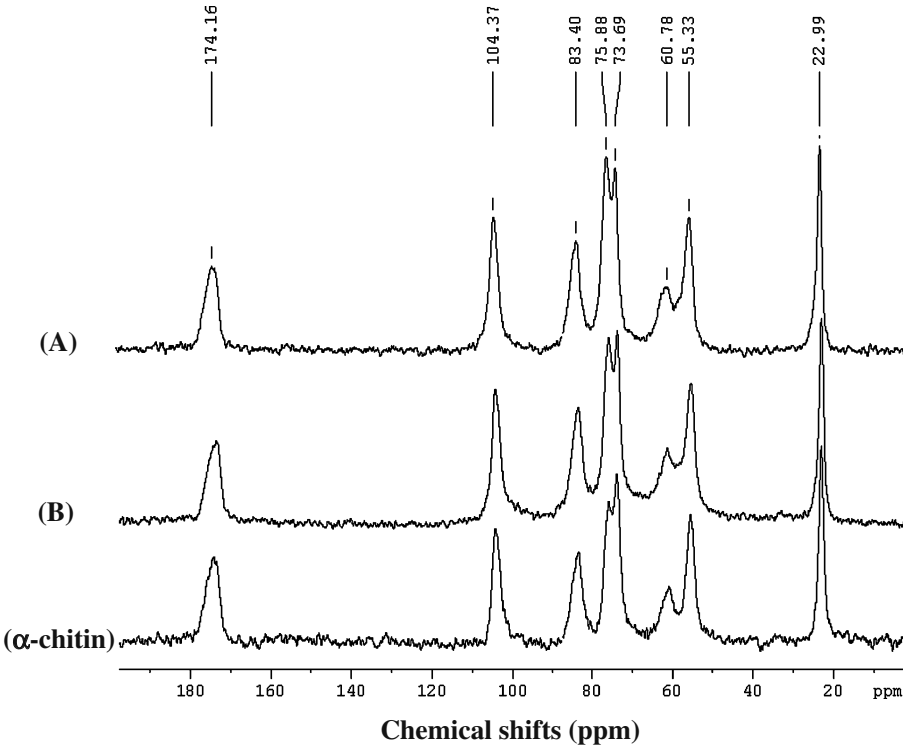

Fig. 1 ^{13}CP/MAS NMR solid-state spectra of chitins deproteinized by SV1 protease (**a**), and alkali treatment (**b**), and commercial α-chitin

demineralization. Solid-state ^{13}C CP/MAS-NMR spectroscopy appears to be suitable for the evaluation of the degree of acetylation [51] and is known to be very sensitive to changes in the local structure. The degree of acetylation from the NMR spectra of chitin isolated by enzymatic deproteinization (89.6%) was higher than that of chitin obtained by alkaline treatment (77%; Table 1). Observed differences in the degree of acetylation values were caused by deacetylation of polysaccharide occurring in the base solution during the deproteinization process. These values are similar to those obtained for the chitin produced using *B. subtilis* fermentation [9] and chitin from shrimp *Crangon crangon* shells deproteinized by Alcalase® [10]. Compared with the commercial α-chitin [52], the two obtained chitins were more deacetylated, especially chitin prepared by chemical deproteinization. Regardless of the procedure of deproteinization employed, the ^{13}C CP/MAS-NMR spectra exhibited the same main bands for the two prepared chitin.

Preparation of Chitosan and Its Antimicrobial Activity

Chitosan is soluble in aqueous acidic solutions due to the presence of amino groups. Chitosan has been confirmed to possess a broad spectrum of antimicrobial activities [53]. Several reports show that chitosan displays antibacterial activity only in an acid environment [21, 54], but a recent report investigates antibacterial activity of water-soluble chitosan [22].

The major procedure for obtaining chitosan is based on the alkaline deacetylation of chitin with strong alkaline solution. In this study, chitosan was prepared from chitin obtained by a treatment with NaOH 50% for 4 h at 80 °C. The antimicrobial activity of chitosan dissolved in acetic acid 0.1% was investigated against four Gram-positive and six Gram-negative bacteria. As shown in Table 2, both chitosans prepared by enzymatic (A) and alkaline treatments (B) markedly inhibited the growth of all Gram-negative and Gram-positive bacteria tested.

Both chitosans showed higher inhibition activity against *E. coli* than the other bacteria tested. This indicated that the two prepared chitosans might have the same inhibition mechanism. Gram-negative bacteria, with lipopolysaccharide at the outer surface providing negative charges, seemed to be very sensitive to chitosan [55] while the sensitivity of Gram-positive bacteria that can have variable amounts of negatively charged teichoic acids at their outer surface varied greatly. However, No et al. [56] showed stronger bactericidal effects of chitosan towards Gram-positive than Gram-negative bacteria.

Determination of the Antioxidant Activity of SWPH

To further enhance the utilization of the shrimp wastes, the antioxidant activities of the five hydrolysates, obtained during the enzymatic extraction of chitin by crude protease from *B. cereus* SV1, were investigated.

DPPH Radical-Scavenging Activity

The antioxidant activity was evaluated using the DPPH, a stable free radical that shows maximum absorbance at 517 nm in ethanol. When DPPH encounters a proton-donating substance such as an antioxidant, the radical would be scavenged and the absorbance reduced [35].

Figure 2 shows the DPPH radical-scavenging activity of the SWPH at various concentrations. The results clearly indicated that all the hydrolysates exhibited a radical-

Table 2 The diameters of inhibition zones against Gram-positive and Gram-negative bacteria.

	Diameter of inhibition zones (mm)	
	Chitosan A	Chitosan B
B. cereus	11.4±1.4	11.5±2.1
B. mojavensis	16±2.8	21±4.2
B. pumilis	11±1.4	14±1.4
E. coli	25.5±3.5	29.5±2.9
P. aeruginosa	11.5±2.1	13±1.4
V. metschnikovii	11.7±2.8	13.5±2.1
K. pneumonia	20±2.8	15.5±2.1
S. typhi	14±4.2	18.5±2.1
M. luteus	13±1.4	17±2.1
E. feacalis	13.5±2.1	13±1.4

Chitosan A is obtained by enzymatic DP of shrimp wastes and chitosan B by chemical DP of shrimp wastes

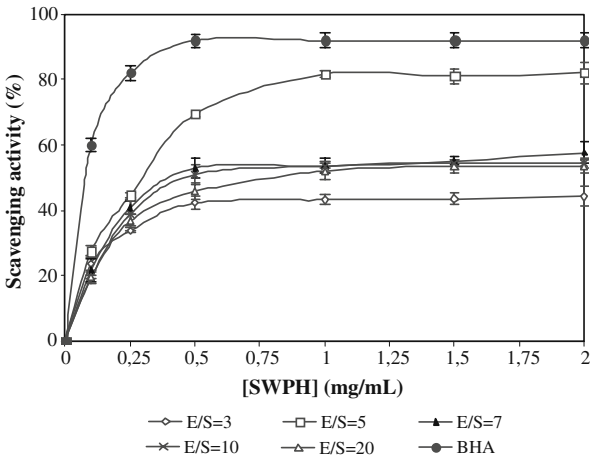

Fig. 2 DPPH radical-scavenging activity of SWPH at different concentrations. BHA was used as positive control

scavenging activity, and the hydrolysate obtained with an E/S ratio of five (H_5) exhibited the highest radical-scavenging activity. The radical-scavenging activity showed a dose-dependency with nearly 82% radical-scavenging activity at a concentration of 2 mg/mL. However, all hydrolysates showed lower radical-scavenging activity than BHA at the same concentrations. The effective concentration for 50% radical-scavenging activity (EC_{50}) of the H_5 determined using the regression equation is 0.3 mg/mL±0.02. The obtained results suggest that shrimp wastes hydrolysates probably contained peptides, which were electron donors and could react with free radicals to convert them to more stable products and terminate the radical chain reaction.

Thus, the enzymatic hydrolysis of shrimp waste at the conditions of this work has provided a complex mixture of compounds able to quench DPPH, such as the carotenoid derivatives released by the hydrolysis of protein–astaxanthin complexes of the shrimp shells and other low-molecular-weight peptides and free amino acids.

Reducing Power

The reducing power assay is often used to evaluate the ability of antioxidant to donate electron or hydrogen [36]. Different studies have reported that there is a direct correlation between antioxidant activities and reducing power of certain bioactive compounds. In this study, the ability of protein hydrolysates to reduce Fe^{3+} to Fe^{2+} was determined. For the reducing power assay, the presence of reductants (antioxidant) in tested samples results in the reduction of Fe^{3+}/Ferric cyanide complex to ferrous form. Figure 3 shows the reducing power activities of the different hydrolysates compared with BHA. Reducing power of all hydrolysates increased with increasing concentrations (0.1 to 2 mg/mL). The higher activity was found in hydrolysate produced with an E/S ratio of five. Several works reported that the reducing power increased with increasing amount of samples [57, 58]. BHA, as positive control, was found to have more efficiency at the same concentrations than protein hydrolysates.

The obtained results clearly demonstrated that some peptides, which exhibited moderate reducing power, are electron donors capable of neutralizing free radicals, converting them into more stable non-reactive species and thus terminating the free radical-initiated chain reactions.

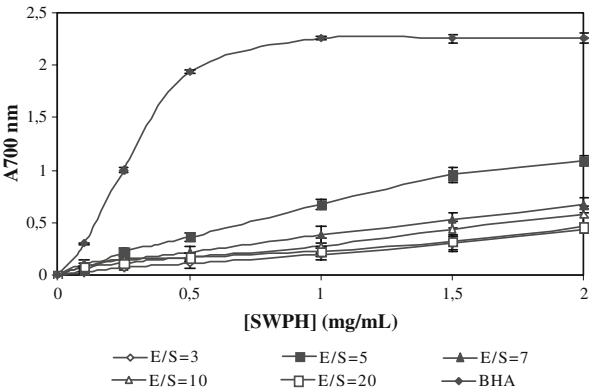

Fig. 3 Reducing power activity of SWPH at different concentrations. BHA was used as positive control

Antioxidant Activity Measured by the β-carotene Bleaching Method

The antioxidant assay using the discoloration of β-carotene is widely used to measure the antioxidant activity of bioactive compounds because β-carotene is extremely susceptible to free radical-mediated oxidation of linoleic acid [59, 60]. Furthermore, β-carotene is used as a coloring agent for beverages, and its discoloration would markedly reduce the quality of these products. In this test, β-carotene undergoes rapid discoloration in the absence of antioxidant, which results in a reduction in absorbance of the test solution with increasing reaction time. The presence of antioxidant hinders the extent of bleaching by neutralizing the linoleic free radical formed.

As shown in Fig. 4, all hydrolysates inhibited the oxidation of β-carotene to different degrees and the antioxidant activity increased with increasing SWPH. Hyrolysate obtained by a ratio of ten showed significantly ($p<0.05$) higher ability to prevent the bleaching of β-carotene than the other hydrolysates ($IC_{50} = 0.9\ mg/mL \pm 0.01$). However, all SWPH showed lower antioxidant activity than BHA at the same concentrations. These results demonstrated that SWPH have strong effects against the discoloration of β-carotene.

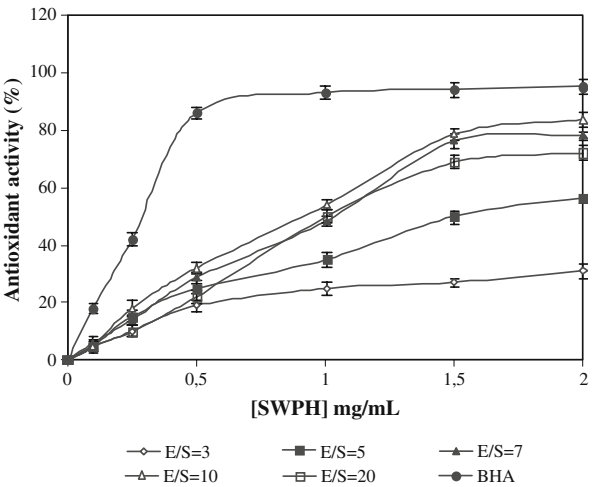

Fig. 4 Determination of antioxidant activity using the β-carotene bleaching method of SWPH at different concentrations

Conclusion

In this paper, enzymatic deproteinization process was applied using a crude protease from *B. cereus* SV1. To our knowledge, the use of *B. cereus* for this purpose has never been demonstrated before. SV1 crude protease was found to deproteinize up to 88% of the shell proteins with an E/S ratio of twenty. The chemical deproteinization rate was 92.6±0.9%. Chitins obtained by enzymatic and alkaline deproteinizations were then converted to chitosan, which were found to exhibit remarkably antibacterial activities. In addition, SWPH recuperated during extraction of chitin were found to possess antioxidant activity.

The obtained results, demonstrated that the crude protease from *B. cereus* SV1 could be used effectively in the deproteinization of shrimp wastes to produce chitin and protein hydrolysates with antioxidant properties. Further studies should be done regarding the relationship between the deacetylation of chitin and the antimicrobial activity.

Acknowledgements This work was supported by grants from Ministry of Higher Education, Scientific Research and Technology-Tunisia. The authors express their thanks to Hajji Rachid (Laboratoire de Chimie Industrielle II, Ecole Nationale d'Ingénieurs de Sfax) for the solid-state NMR analysis. The authors would like to thank Mr. Hajji Ayedi from the Faculty of Letters and Human Sciences of Kairouan for his help with English.

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