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Full Length Research Paper

Inhibitory effects of extracts from Tunisian marine species on serine-protease from a local *Beauveria bassiana* strain

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The effect of marine biological extracts, collected from the Gabes gulf area located in Tunisia, was evaluated on the extracellular proteolytic activity of *Beauveria bassiana*. The majority of extracts exhibited a marked inhibitory activity against the protease. Depending on extract origin, the inhibition percentages differed when the extracts were dissolved either in ethanol or in water. The ethanol extract from C*aulerpa prolifera*, *Posidonia oceanica* algae and from the internal organs of both *Holothuria polii* and *Paracentrotus lividus* exhibited inhibition greater than 50%. However, when dissolved in water, only *C. prolifera, Hippospongia communis* and *P. oceanica* extracts led to an inhibition greater than 50%. Interestingly, only *C. prolifera* extract displayed a very important activity against the proteolytic enzyme and a total inhibition was obtained in the crude extract dissolved in water, in a dose dependent manner. This inhibitory activity was destroyed by boiling. The fractionation of inhibitor compounds from *C. prolifera* extract by C_{18} reversed-phase followed by gel-filtration chromatography allowed the isolation of fraction with 62% of inhibition. *C. prolifera* extract may therefore, be useful in developing new molecules with interesting potential of utilization in several biotechnological applications.

Key words: Marine species, extraction, inhibitors, protease, high performance liquid chromatography (HPLC).

INTRODUCTION

The interest in marine organisms as a potential and promising source of bioactive agents has increased during the last years. Interestingly, the marine environment is an abundant source of undiscovered compounds leading to the development of new bioactive molecules with different properties. Hence, a diverse group of marine products (animal, algae, fungi and bacteria) were screened for bio-activities such as antibacterial, anti-fungal, anticoagulant, antiviral and anti-enzyme activities (Lindequist and Schweder, 2001; Mayer and Hamann, 2002; Mayer and Hamann, 2004; Neifar et al, 2009; Newman et al, 2003). Enzyme inhibitors have received an increasing attention, not only because they constitute good tools for the study of enzyme structures and reaction mechanisms, but also for their potential utilization in pharmacology and agriculture (Mayer and Lehmann, 2000; Bode and Huber, 1992). For example, protease inhibitors play an important role in activating target proteases in the pathogenic process of human diseases such as arthritis, thrombosis, high blood pressure, muscular dystrophy, cancer, acquired immune deficiency syndrome (AIDS), etc. (Chiaki, 2004). Moreover, inhibitors can suppress several stages of carcinogenesis and mainly during its progression and metastasis.

Several reports focussed on the identification of

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Abbrviations: AIDS, Acquired immune deficiency syndrome; **TCA,** trichloroacitic acid; **SDS-PAGE,** sodium dodecyl sulphate-polyacrylamide gel electrophoresis; **TLC,** thin-layer chromatography.

protease inhibitors from marine sources (Chiaki, 2004; Pascal et al., 2007) and many of them showed the presence of serine proteases inhibitors in marine organisms belonging mainly to *Porifera* and *Cnidaria* phyla (Wunderer et al., 1976; Mebs et al., 1983; Kolkenbrock and Tsches, 1987; Delfin et al., 1996; Nakao et al., 1995; Nakao et al., 1998; Murakami et al., 2002; Hanessian et al., 2002; Hanessian et al., 2004). Such proteases are the most important group of industrial enzymes and are used in different processes, that is, in detergent, food, peptide synthesis, leather, pharmaceutical, silk degumming and recently, for recovery of silver from used x-ray films (Gupta and Lorenz, 2002). The entomopathogenic fungus *Beauveria bassiana* (Balsamo) Vuillemin is a virulent insect pathogen which can infect a wide range of insects and arthropods. The infection proceeds first by passing through the insect cuticle, the first physical barrier composed mainly of proteins and chitin, involving the combination of mechanical forces and hydrolytic enzymes, especially proteases and chitinases (Huang el al., 2004). Several proteases produced by different strains of this fungus have been reported and their role as pathogenic factors in the insect infection process is well documented (Bidochka and George, 1987; Urtz and Rice, 2000; Fang et al., 2002) as well as their classification as serine protease (North, 1982).

We have isolated a novel strain of *B. bassiana* which produces high titre of serine alkaline protease. The aim of this study is to investigate the inhibitory activities of extracts obtained from various marine species on *B. bassiana* proteases.

MATERIALS AND METHODS

Fungal strain, culture conditions and protease production

The strain P2 from *B. bassiana* was isolated in the Laboratory of Molecular Genetic of Eukaryotes (CBS, Tunisia). For submerged cultivation, 10^6 spores/ml were used as inoculums. Culture was grown on Mandels medium (Mandels and Weber, 1969) supplemented with 1% (w/v) skimmed milk for 6 days at 30°C on a rotatory shaker (150 rpm). The culture was centrifuged at 7.000 x g for 10 min and the resulting supernatants, which contain an extracellular protease exhibiting casein-degrading activity, was used for inhibition tests by marine extracts.

Marine species

Seven marine species were collected from Gabes gulf area (Tunisia). These included two green algae (*Caulerpa prolifera* and *Ulva rigida*), sea cucumber (*Holothuria polii*), sponge (*Hippospongia communis*), two phanerogams (*Cymodocea nodosa* and *Posidonia oceanica*) and sea urchin (*Paracentrotus lividus*). After collection, samples were rinsed with water and kept at -20°C until use.

Chemical characterization of marine species

According to the AOAC (1990) methods, water content was quantified by drying samples at 100°C, lipid by Soxhlet extraction, nitrogen by Kjeldahl procedure (protein was calculated using a rate of 6.25% nitrogen to protein) and ash by incineration in a muffle furnace at 550°C.

Extract preparation

After drying on filter paper at room temperature, 5 g of each marine product was extracted with 100 ml of ethanol by stirring at 37°C for 24 h. In the case of sea urchin, (*P. lividus*) and sea cucumber (*H. polii*), the internal organs and the body wall were extracted separately. After centrifugation (30 min, 30,000×g), the supernatant was evaporated under vacuum. Organic extracts were dissolved in 5 ml of ethanol or in distilled water and assessed for their inhibitor activity on protease.

Measurement of protease activity

The method of Kembhavi et al. (1993) was used to determine the proteolytic activity of the fungal extracellular enzymes using casein as a substrate. Enzyme solution (10,000 U) was incubated with 0.5 ml of 1% casein, dissolved in 100 mM Tris-HCl buffer pH 8, in a test tube on a shaker for 15 min at 60°C. The reaction was stopped by adding 0.5 ml of 20% trichloroacitic acid (TCA) and the samples were allowed to stand for 15 min at room temperature. After centrifugation (15 min, 10,000×g), the absorbance of the resulting supernatants was measured at 280 nm.

Protease inhibition assays

To measure the inhibitory effects of marine extracts (dissolved in ethanol or in water), protease solution was pre-incubated (30 min) with each marine extract and the residual protease activity was determined, as indicated earlier. A control without marine extracts was run in parallel. Experiments without pre-incubation were also conducted. The dose effect of the inhibitor on protease activity was conducted by adding increasing volumes of the marine extract. The thermal stability of the inhibitor was also tested by assaying a sample that had been incubated only at 100°C for 10 min. Experiments was conducted in duplicate.

Isolation of inhibitor compounds

Chromatographic experiments were performed using a Hewlett-Packard 1050 liquid chromatograph (*Knauer* S-1000*)* equipped with a variable UV–vis detector type 2500**,** a gradient elution pump and a Rheodyne 7125 injection loop of 500 µl (Rheodyne *Knauer*). The chromatographic column was C_{18} column *Knauer* (8 mm × 250 mm) for reversed-phase chromatography and Shodex KW-802.5 column (8 mm × 300 mm) for gel filtration chromatography. Separation was first accomplished using C_{18} reversed-phase column equilibrated at a flow rate of 0.8 ml/min, with a mobile phase consisting of water/formic acid (19 v/1 v). The elution was performed with methanol and monitored at UV absorbance of 280 nm. Fractions were collected and tested for their effect on protease activity. The fraction having the highest protease inhibitory activity was next subjected to the gel filtration chromatography and equilibrated with water. The elution was also performed with water at a flow rate of 0.8 ml/min at UV absorbance of 280 nm. The molecular weight of inhibitors was estimated by comparing their elution time with that of molecular weight markers (thyroglobulin, 670 kDa; bovine gamma-globulin, 158 kDa; chicken ovalbumin, 44 kDa; equine myoglobin, 17 kDa). The sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) method was performed to visualize proteins

Table 1. Proximate chemical composition of the marine products (w/ dw).

Marine products	Protein	Lipid	Ash	Carbohydrates*
U. rigida	18.71	1.35	10.55	69.39
C. prolifera	19.60	11.70	2.98	65.72
H. polii	57.31	1.93	28.62	12.14
H. communis	61.32	7.20	19.01	12,47
C. nodosa	21.91	2.36	11.73	64.00
P. oceanica	6.90	2.63	17.23	73.24
P. lividus	6.64	1.77	53.00	38.60

*Carbohydrates = (100 % - (proteins + lipids + ash)).

Table 2. Inhibition of protease activity by marine extracts dissolved in ethanol or in water was determined in duplicate.

Marine extracts*	Inhibition (%)	
	Ethanol	Water
U. rigida	6.08 ± 0.57	27.65 ± 1.42
C. prolifera	100 ± 0	100 ± 0
H. polii Internal organs	61.52 ± 4.33	20.18 ± 1.50
H. polii Body wall	32.79 ± 1.06	12.45 ± 0.59
H. communis	18.24 ± 1.17	51.12 ± 0.90
C. nodosa	38.68 ± 3.60	36.73 ± 1.88
P. oceanica	54.52 ± 2.34	50.28 ± 3.22
P. lividus Internal organs	58.02 ± 1.61	29.32 ± 2.58
P. lividus Body wall	41.26 ± 1.10	20.69 ± 2.33

*Dose of extracts: 200 µl.

as described by Laemmli (1970). Low molecular weight calibration standards ranging in size from 97 to 14.4 kDa were used and protein bands were visualised by Coomassie brillant blue R-250.

RESULTS

Biochemical composition of the marine products

The approximate composition of the marine products collected from the Gabes gulf area varied among species (Table 1). The crude protein contents of the two green algae were 18.7 and 19.60% w/dw (dw for dry weight) for *U. rigida* and *C. prolifera*, respectively. The sea cucumber *(H. polii*) and the sponge (*H. communis*) contained the highest protein contents (57.31 and 61.32% w/dw, respectively) while the lowest ones were the phanerogam species *P. oceanica,* (6.90% w/dw) and the sea urchin *P. lividus*, (6.64% w/dw). Concerning the lipids content, the lowest values were observed for *U. rigida (*1.35%*)* and *H. pollii* (2.63%), while the highest one (11.7% w/dw) was found in *C. prolifera*. The ash contents ranged from 2.98% w/wd (for *C. prolifera*) to 53% w/dw (for the sea urchin, *P. lividus*). Carbohydrates constituted the most abundant component in the case of the green algae (*U. rigida* and *C. prolifera*) and phanerogam species (*C. nodosa* and *P. oceanica*) with values exceeding 60% w/dw.

Screening of protease inhibitors

The inhibitory effects of the nine marine products extracted using ethanol and dissolved either in ethanol or in water, were screened against protease of *B. bassiana*. Inhibitory activities were exhibited by all extracts and generally, higher inhibition percentages were found for extracts dissolved in ethanol (Table 2). Indeed, an anti-protease activity greater than 50% was obtained in four extracts from *C. prolifera* (100%), *P. oceanica* (54.52%) and internal organs of both *H. polii* (61.52%) and *P. lividus* (58.02%). However, when dissolved in water, only *C. prolifera*, *P. oceanica* and *H. communis* extracts showed an inhibition rates superior to 50%. In addition, the extract of sponge, *H. communis*, exhibited more inhibition, while dissolved in water and the protease inhibition passed from 18.24% (in ethanol) to 51.12% (in water). Interestingly, only *C. prolifera* extract dissolved in both ethanol and water displayed a total inhibitory activity against the enzyme.

Preliminary characterization of the inhibitory activity of

Table 3. Effect of the incubation (30 min) of the enzyme with the marine extracts dissolved in water on the inhibition rate, determined in duplicate.

Marine extracts*	Inhibition (%)	
	With incubation	Without incubation
U. rigida	27.59 ± 1.37	8.97 ± 2.34
*C. prolifera**	100 ± 0	0 ± 0
H. polii Internal organs	20.18 ± 1.86	5.52 ± 2.63
H. polii Body wall	12.45 ± 1.45	0 ± 0
H. communis	51.12 ± 4.21	17.76 ± 2.11
C. nodosa	36.73 ± 3.69	1.16 ± 0.25
P. oceanica	59.32 ± 3.50	0.18 ± 0
P .lividus Internal organs	29.32 ± 3.05	25.18 ± 2.48
P .lividus Body wall	20.69 ± 1.45	0 ± 0

* Dose of extracts: 200 µl extracts.

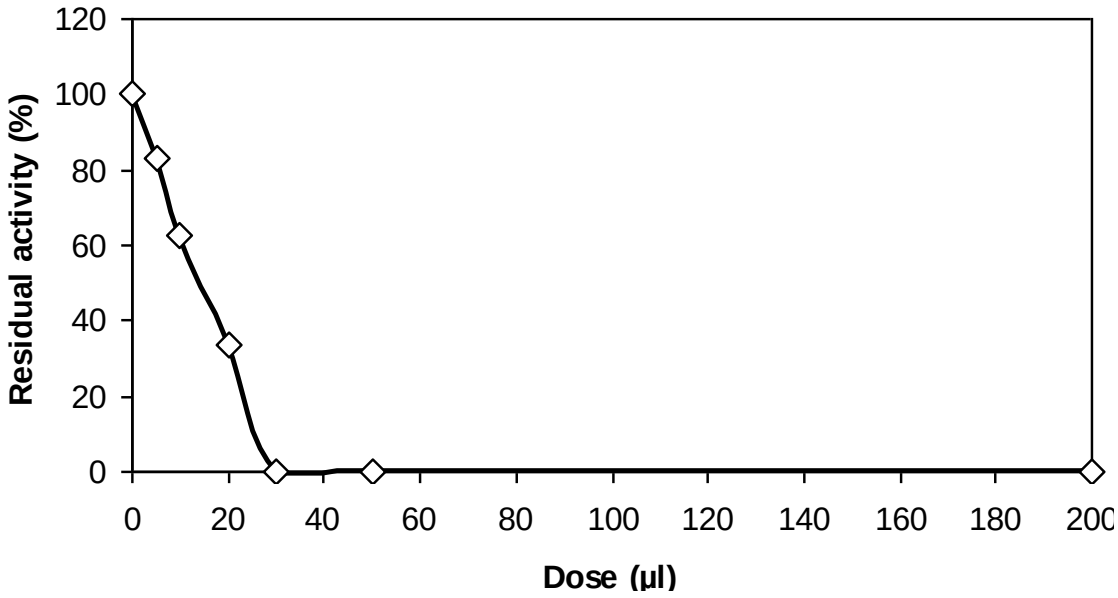

Figure 1. Effect of increasing dose of *C. prolifera* extract on protease activity (extracts dissolved in water).

these extracts was started by studying the effect of pre-incubation of the enzyme with the extract. According to Table 3, the incubation plays an important role in the inhibition process with rates ranged between 12.45 and 100% depending on the extract origin. Without incubation, all samples showed a considerable decrease of the inhibition rates. For example, in the case of *C. prolifera* extract, the protease inhibition passed from 100% (with incubation) to 0% (without incubation).

Effect of increasing doses and heat treatment of *C. prolifera* extract on protease inhibitor activity

The protease activity was determined in the presence of increasing doses of *C. prolifera* extract (Figure 1). 50% inhibition was obtained at a dose of 15 µl and a total inhibition started with a dose superior to 30 µl. The pre-heating of *C. prolifera* extract at 100°C for 10 min led to the loss of protease-inhibitory activity, suggesting a thermo labile character of the active component in the *C. prolifera* extract.

Isolation of inhibitor compounds from *C. prolifera* extract

The *C. prolifera* extract was separated into five fractions by C_{18} reversed-phase column as indicated in Figure 2a. Figure 2b shows the protease residual activity corresponding to each fraction. Only fraction F4 and F5 exhibited remarkable inhibitory capacity with 10.2 and

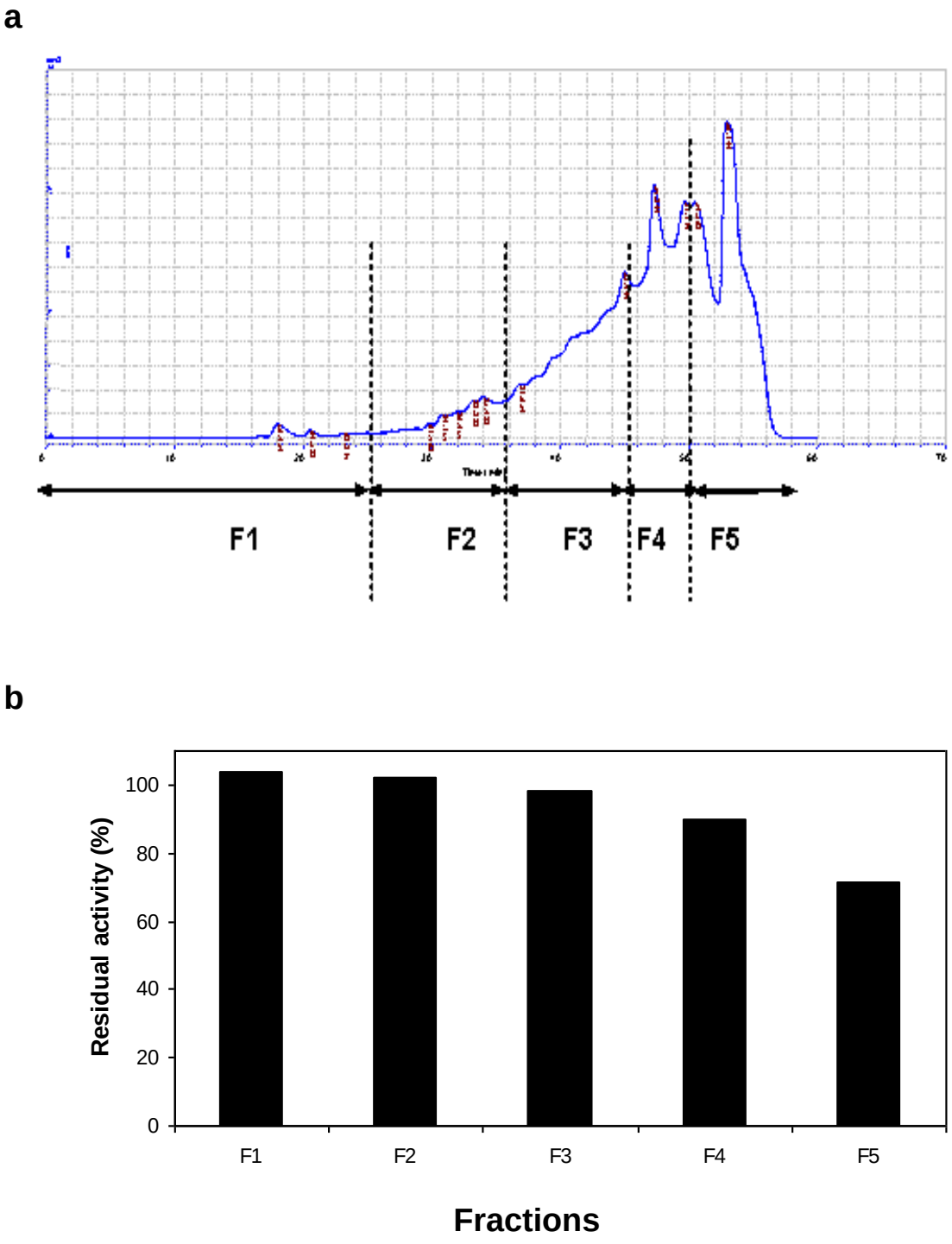

Figure 2. Purification of protease inhibitor from *C. prolifera* extract (dissolved in water) on C_{18} column (a) HPLC Chromatogram with in ordinates the absorption at 280 nm; (b) inhibition rates of protease by the obtained fractions (F1 to F5).

28.25% of inhibition, respectively. The fraction F5, with the strongest protease inhibitory activity was further subjected to gel filtration chromatography Shodex KW-802.5 column and five sub-fractions were collected (Figure 3a). Only sub-fractions SF1, SF2 and SF4 exhibited protease inhibition (Figure 3b). Sub-fraction SF2 and SF4 showed only 6.38 and 32.72% inhibition of the enzyme. However, sub-fraction SF1 yielded 62.12% of inhibition. The

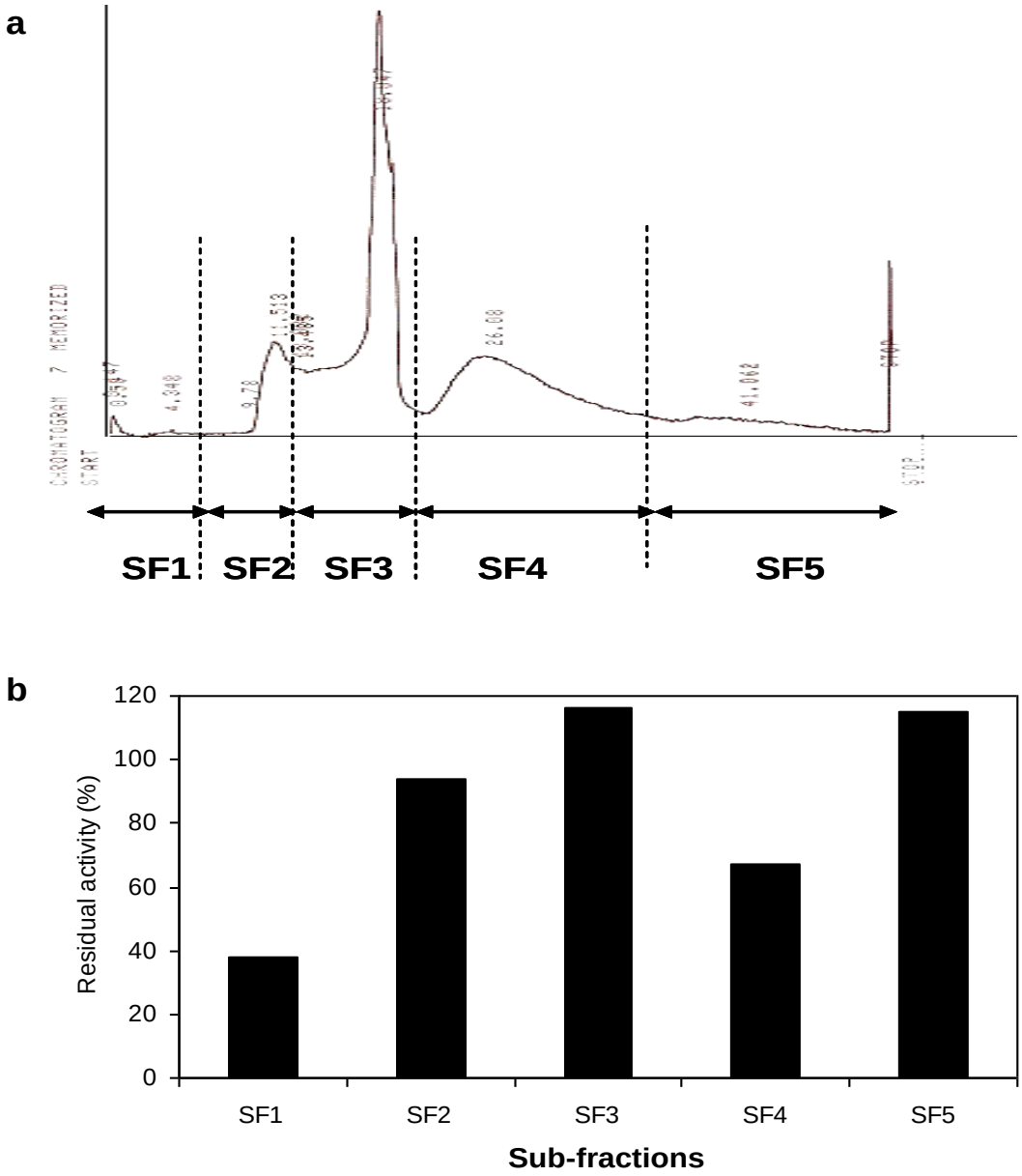

Figure 3. Gel-filtration, of the Fraction F5 from C_{18} column, on Shodex KW-802.5 column (a) HPLC Chromatogram with in ordinates the absorption at 280 nm; (b) inhibition rates of protease by the obtained sub-fractions (SF1 to SF5).

estimation of the molecular weight of the active components indicated that, in the sub-fraction SF1, it corresponded to high molecular weight compound of more than 670 kDa but in the sub-fraction SF4, it should have less than 17 kDa.

Analyse of sub-fractions obtained from gel chromatography by SDS-PAGE.

The SDS-PAGE analysis of sub-fractions SF1-SF5 is provided in Figure 4. The pattern of proteins is different from well to well but it seems that the proteins visible in SF1 and SF4 fractions did not correspond to the molecular weights suggested from the gel filtration chromategraphy. We checked in another shorter migration that there are no small proteins in SF4. These results would suggest that, the inhibiting molecules could be either of protein nature (but their amount is too low that they are not visible on the gel) or of non-protein nature. In this context, polysaccharides have been characterised in algae that exhibit potential activities such as antioxidant, anti-proliferative and anti-coagulation protease (Melo and Mourão 2008; Yuan et al., 2006; Shanmugam et al., 2001).

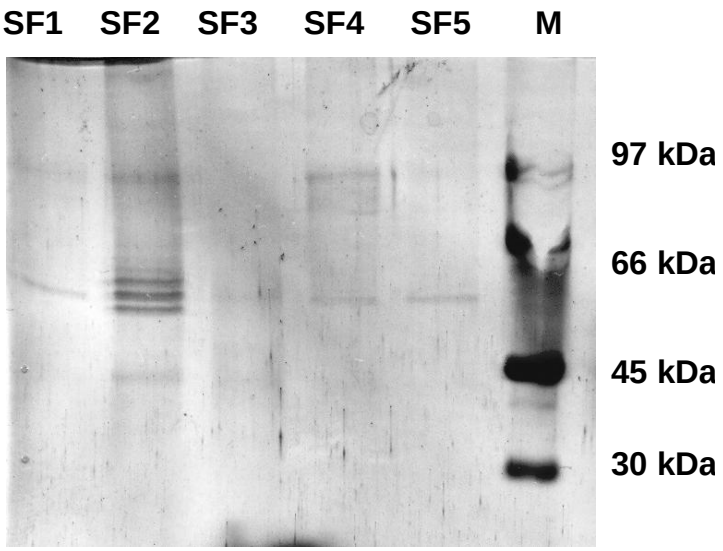

Figure 4. SDS-PAGE of sub-fractions SF1, SF2, SF3, SF4 and SF5 obtained from gel chromatography. M, molecular weight markers.

These molecules may have high or small molecular weight.

DISCUSSION

The aim of this study is to evaluate the possible inhibitory effect of marine extracts on the serine proteases of the fungus *B. bassiana*. Different degrees of inhibition of the activity of *B. bassiana* protease were obtained in the presence of the nine marine extracts. Our findings indicated that, the approximate composition (lipids, proteins, ash and carbohydrates) of the studied marine products varied depending on the nature of species. Other than the origin of the extract, two parameters influenced greatly the inhibition rates: The solute in which the extract was dissolved (either ethanol or water) and the inhibition test (either with or without pre-incubation of the extract with the enzyme). These marine species should contain active compounds at various qualitative and quantitative levels but the most promising was the *C. prolifera* ethanol extract.

Our results confirmed those reported by others who found that marine organisms produced large variety of secondary metabolites leading to the development of new bioactive compounds of various chemical classes (Newman et al., 2003; Cragg and Newman, 2005). However, secondary metabolites production is associated with physical (light, temperature, etc.) and biological factors (community composition, biological stage, sex, etc.), season and geographical location (Maréchal et al., 2004). For example, it was reported that the production of phlorotannins by a number of brown algae is positively affected by light intensity and fouling pressure (Pavia and Toth, 2000; Moreno et al., 2003).

Interestingly, among all marine extracts, only *C. prolifera* extract exhibited a complete inhibition of the test protease. However, it seems that the *C. prolifera* extract contained thermolabile compounds, which could be destroyed by heat treatment (100°C) causing the loss of the protease inhibitory effect.

The first step of separation of *C. prolifera* extract using C_{18} chromatography resulted in five fractions showing lower efficient inhibition rates against protease. Only two fractions, F4 and F5 showed protease inhibition with different rates. The gel filtration chromatography of F5 fraction resulted in the isolation of a major compound with protease inhibition rate of 62.12% (contained in the sub-fraction SF1) and a second less active fraction SF4 (32.72%). SF1 should be a large molecule, while SF4 is a small one and could be of proteic nature due to its mass and absorption at 280 nm. Nevertheless, in this context, it is very important to note also that some non-protein compounds such as condensed polyphenols (tannin) had an inhibitory action on certain digestive enzyme such as proteases (Barwell et al., 1989).

The inhibition of protease by algae is poorly documented. To the best of our knowledge, the algae specie, *C. prolifera,* has not been screened earlier for their anti-protease activity. Recently, plant proteins that inhibit various types of enzymes have been extensively studied. The most commonly occurring inhibitors are the proteinase and amylase inhibitors (Saxena et al., 2010). In other studies, extracts obtained from *C. prolifera* exhibited a lipase inhibitory activity. Moreover the separation of *C. prolifera* extract by thin-layer chromategraphy (TLC) resulted in eight fractions showing efficient inhibition rate against dog gastric lipase, compared with the crude extract. In the case of human pancreatic lipase, TLC fractionation reduced the inhibitory rates, suggesting

that the effect of algal extract on lipases may be caused by a synergetic action of several compounds within the extract (Ben Rebah et al., 2008). However, further researches are needed to identify the inhibitor compounds that may be useful tools in enzymology studies (enzyme structures and reaction mechanisms) and/or in drug development with interesting potentials in pharmacology and agriculture.

Conclusions

The majority of marine species collected from the Gabes gulf area exhibited a marked inhibitory activity against the protease of the fungus *B. bassiana.* Many factors such as extract preparation, species, enzyme incubation and heat treatment could affect the inhibitory rates. Interestingly, the alga specie, *C. prolifera*, showed the highest inhibitory activity. The isolation of inhibitor compounds from *C. prolifera* extract by C_{18} reversed-phase column flowed by Gel-filtration Shodex KW-802.5, allowed the isolation of fraction with almost 62% inhibition rate.

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