Zebra blenny (*Salaria basilisca*) viscera as a source of solvent-stable proteases: characteristics, potential application in the deproteinization of shrimp wastes and evaluation in liquid laundry commercial detergents

Naourez Ktari · Hayet Ben Khaled · Islem Younes ·
Intidhar Bkhairia · Samiha Mhamdi · Ibtissem Hamza ·
Moncef Nasri

Revised: 23 May 2012 / Accepted: 16 August 2012 / Published online: 29 August 2012
© Association of Food Scientists & Technologists (India) 2012

Abstract The present study describes the characterization of crude protease extract from zebra blenny (*Salaria basilisca*) and its evaluation in liquid detergent and shrimp waste deproteinization. At least five caseinolytic proteases clear bands were observed in zymogram. The crude alkaline protease showed optimum activity at pH 8.0 and 60 °C, and it was highly stable over a wide range of pH from 6.0 to 11.0. Proteolytic enzymes showed extreme stability towards non-ionic surfactants (5 % Tween 80 and 5 % Triton X-100) and oxidizing agents (1 % sodium perborate), and relative stability towards anionic surfactant (1 % Sodium dodecyl sulfate (SDS)). They also showed high stability and compatibility with various laundry liquid detergents from Tunisian market. Furthermore, the crude enzyme was stable towards several organic solvents and retained more than 50 % of its original activity after 30 days of incubation at 30 °C in the presence of 50 % (v/v) dimethylsulfoxide (DMSO). Further, proteases from zebra blenny viscera were found to be effective in the deproteinization of shrimp wastes. The protein removal after 3 h at 40 °C with an enzyme/substrate ratio (E/S) of 5 U/mg protein was about 77 %.

Keywords *S. basilisca* · Alkaline proteases · Laundry detergents compatibility · Solvent-stable · Enzymatic deproteinization

Introduction

Proteases are the most important industrial enzymes accounting for approximately 40 % of the total industrial enzyme market (Gupta et al. 2002). They have diverse applications in a wide variety of industries such as detergent, food, pharmaceutical, leather, peptide synthesis, and for the recovery of silver from used X-ray films (Cowan 1996; Kumar and Takagi 1999). Recent advances in biotechnology have also demonstrated the capacity of proteases to produce fish protein hydrolysates (Ben Khaled et al. 2011; Ktari et al. 2012).

The detergent industry has now emerged as a major consumer of several hydrolytic enzymes acting at alkaline pH. Detergent proteases account for at least a quarter of all protease sales throughout the world (Anwar and Saleemuddin 1998; Gupta et al. 2002; Kumar and Takagi 1999). They are primarily used as detergent additives since they are biodegradable and increase performance/cost ratios (Gupta et al. 2002; Kumar and Takagi 1999). Moreover, although the enzymes selected for detergent composition have been subtilisins, they are not the ideal enzymes for detergents due to their low thermal stability and also because of their short shelf life (Samal et al. 1990). Thus, it is relevant to search for proteases from new sources presenting high thermal stability, alkaline activity and more compatible with washing systems (Banerjee et al. 1999). These properties have already been observed in trypsin-like enzymes.

Shrimp by-products have been identified as an animal protein source of a great potential and also as an important source of chitin and asthaxanthin (Shahidi and Synowiecki 1991). Only 65 % of the shrimp is edible. The remainder is discarded as inedible waste (cephalothorax and exoskeleton).

N. Ktari (✉) · H. B. Khaled · I. Younes · I. Bkhairia ·
S. Mhamdi · I. Hamza · M. Nasri
Laboratoire de Génie Enzymatique et de Microbiologie,
Université de Sfax- Ecole Nationale d'Ingénieurs de Sfax,
B.P. "1173", 3038 Sfax, Tunisia
e-mail: naourez.ktari@yahoo.fr

Over the years, techniques have been developed for the recovery and exploitation of these by products in valuable biopolymers such as chitin and chitosan (Bhaskar et al. 2007; Sini et al. 2007). These biomolecules are widely used in the food industry, pharmacy, textiles, chemical industries, etc. (Rinaudo 2006). Conventionally, to extract chitin from crustacean shells, chemical processing for demineralization and deproteinization has been applied by treatment with strong acids and bases to remove calcium carbonate and proteins, respectively (Roberts 1992). However, the use of these chemicals may destroy the chitin. To overcome the hazards from chemical treatments, alternative methods on use of microorganisms (Ghorbel-Bellaaj et al. 2011a; Jo et al. 2008) or proteolytic enzymes (Ghorbel-Bellaaj et al. 2011b; Manni et al. 2010) for the deproteinization of crustacean wastes have been reported.

Fish viscera have been known as a potential source of different enzymes, especially proteases (Gildberg 1992; Shahidi and Kamil 2001). A variety of digestive proteolytic enzymes has been isolated from the internal organs of fish. The most important digestive enzymes from fish viscera are pepsin, secreted from gastric mucosa, and serine proteases trypsin and chymotrypsin secreted from the pancreas, pyloric caeca and intestine (Simpson 2000). Acidic proteases from fish stomachs display high activity between pH 2.0 and 4.0, while alkaline digestive proteases, such as trypsin, are most active between pH 8.0 and 10.0.

Zebra blenny, (*S. basilisca*) is very common benthic fish in Tunisian coasts. This species is abundant in the Gulf of Gabes. It is brownish green in colour and reaches a maximum length of 18 cm. *S. basilisca* was described as living on sea grass beds, also within the Mediterranean (Heymer 1985) where spawning occurs in water temperatures between 18 and 22 °C. In Tunisia, *S. basilisca* is one of the most abundant marine resources.

The present paper describes some biochemical characterization of the alkaline crude enzyme preparation from *S. basilisca* as well as its compatibility with commercial laundry detergents, oxidants, surfactants agents and organic solvents and its application in the deproteinization of shrimp wastes.

Materials and methods

Zebra blenny viscera

Zebra blenny (*S. basilisca*) was purchased from the fish market of Sfax City, Tunisia. The samples were packed in polyethylene bags, placed in ice with a sample/ice ratio of approximately 1:3 (w/w) and transported to the research laboratory within 30 min. After the fish was washed with water, their viscera were separated, rinsed thoroughly with

cold distilled water and then stored in sealed plastic bags at –20 °C until they were used for enzyme extraction.

Preparation of crude alkaline enzyme extract

Viscera from *S. basilisca* (100 g) were washed with distilled water, and then homogenized for 60 s with 200 ml of extraction buffer (10 mM Tris-HCl, pH 8.0). The homogenate was centrifuged at 8,500×g for 30 min at 4 °C. The pellet was discarded and the supernatant was collected and used as the crude protease extract. All enzymatic assays were conducted within a week after extraction.

Polyacrylamide gel electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by Laemmli 1970, using 5 % (w/v) stacking and 15 % (w/v) separating gels. Samples were prepared by mixing the crude enzyme extract at 1:5 (v/v) ratio with the SDS-PAGE sample buffer (10 mM Tris-HCl (pH 8.0), 2.5 % SDS, 10 % glycerol, 5 % β-mercaptoethanol and 0.002 % bromophenol blue). The samples were heated at 100 °C for 5 min before loading in the gel. After electrophoresis, the gel was stained with 0.25 % Coomassie Brilliant Blue R-250 in 45 % ethanol-10 % acetic acid and destained with 5 % ethanol-7.5 % acetic acid.

Detection of protease activity by zymography

Protease activity staining was performed on SDS-PAGE according to the method of Garcia-Carreno et al. (1993) with a slight modification. The sample was not heated before electrophoresis. After electrophoresis, the gel was submerged in buffer A (100 mM Tris-HCl buffer (pH 8.0)) containing 2.5 % Triton X-100, with shaking for 30 min to remove SDS and allow enzyme renaturation. Triton X-100 was removed by washing the gel three times with buffer A. The gel was then immersed in casein solution (1 % (w/v) in buffer A) for 20 min at 50 °C. Finally, the gel was stained with 0.25 % Coomassie Brilliant Blue R-250 in 45 % ethanol and 10 % acetic acid and destained with 5 % ethanol and 7.5 % acetic acid. The development of clear bands on the blue background of the gel indicated the presence of protease activity.

Protease activity assay

Protease activity in the crude alkaline enzyme crude was measured by the method of Kembhavi et al. (1993) using casein as a substrate. A 0.5 ml aliquot of the crude enzyme, suitably diluted, was mixed with 0.5 ml of buffer A containing 1 % substrate (w/v), and incubated for 15 min at 60 °C.

The reaction was stopped by the addition of 0.5 ml of 20 % (w/v) trichloroacetic acid. The mixture was allowed to stand at room temperature for 15 min and then centrifuged at $10,000\times g$ for 15 min to remove the precipitate. The acid-soluble material was estimated spectrophotometrically at 280 nm. A standard curve was generated using solutions of 0–50 mg/l tyrosine. One unit of protease activity was defined as the amount of enzyme required to liberate 1 μg of tyrosine per min under the experimental conditions used. Values are the means of three independent experiments.

Properties of the enzyme

pH optimum and pH stability

The optimum pH of the crude protease was studied over a pH range of 5.0–12.0 using casein as a substrate at 60 °C. For the measurement of pH stability, the crude enzyme preparation was incubated for 1 h at 30 °C in different buffers, and then the residual proteolytic activity was determined under standard assay conditions. The following buffer systems were used: 100 mM acetate buffer, pH 5.0–6.0; 100 mM Tris-HCl buffer, pH 7.0–8.0; 100 mM glycine-NaOH buffer, pH 9.0–11.0; 100 mM Na_2HPO_4 -NaOH buffer pH 12.0.

Effect of temperature on activity and stability

To investigate the effect of temperature, the activity was tested using casein as a substrate at the temperature range from 30 to 70 °C in 100 mM Tris-HCl buffer, pH 8.0. Thermal stability was examined by incubating the enzyme preparation for 60 min at different temperatures from 30 to 60 °C. Aliquots were withdrawn at desired time intervals to test the remaining activity at pH 8.0 and 60 °C. The non-heated crude enzyme was taken as 100 %.

Effects of metal ions on the alkaline proteolytic activity

The influence of various metal ions at a concentration of 5 mM, on enzyme activity was investigated by adding the monovalent (Na^+ or K^+) or divalent (Mg^{2+} , Hg^{2+} , Ca^{2+} , Zn^{2+} , Cu^{2+} , Ba^{2+} or Mn^{2+}) metal ions to the reaction mixture. The activity of the crude enzyme without any metallic ions was considered as 100 %.

Effect of NaCl concentration

Enzyme activity was assayed in the presence of NaCl at various concentrations (0–15 % (w/v)). The relative enzyme activity was determined at 60 °C for 15 min, using casein as a substrate.

Effects of enzyme inhibitors on protease activity

The effects of enzyme inhibitors (5 mM), on protease activity were studied using phenylmethylsulfonyl fluoride (PMSF), soybean trypsin inhibitor (SBTI), β -mercaptoethanol, pepstatin A and ethylene-diaminetetraacetic acid (EDTA). The alkaline crude enzyme extract was preincubated with each inhibitor for 30 min at 25 °C, and then the remaining protease activity was tested using casein as a substrate. The activity of the enzyme assayed in the absence of inhibitors was taken as control.

Effects of surfactants and oxidizing agents

The suitability of the crude protease as a detergent additive was determined by testing its stability towards some surfactants (SDS, Triton X-100, Tween 80) and oxidizing agents (sodium perborate). The enzyme preparation was incubated with different concentrations of additives for 1 h at 30 °C, and then the residual proteolytic activities were measured under standard assay conditions. The activity of the enzyme preparation, incubated under similar conditions without any additives, was taken as 100 %.

Detergent compatibility

The compatibility of the crude alkaline protease extract with liquid laundry detergents was studied using commercially available detergents: Dixan (Henkel-Spain), Nadhif (Henkel-Alki-Tunisia), Lav+ (STID-Tunisia), Carrefour (U.E/Geproduceerd-France) and Tex'til (U.E/Vervaardigd-Belgium). Commercial detergents were diluted 100-fold in tap water to simulate wash solution. The endogenous enzymes contained in these detergents were inactivated by heating the diluted detergents for 1 h at 65 °C prior to the addition of the enzyme preparation. The crude protease at a concentration of 12,000 U/ml was incubated with different detergents for 1 h at 30 and 40 °C, and then the remaining activities were determined under the standard assay conditions. The enzyme activity of a control, without detergent, incubated under the similar conditions, was taken as 100 %.

Organic solvent-stability assay

The organic solvent stability of the enzyme was studied by incubating the crude enzyme with various organic solvents (50 %; v/v; methanol, N,N-dimethylformamide (DMF), DMSO, diethyl ether, hexane, acetone, and isopropanol) at 30 °C with shaking (150 rpm) for 30 days. Aliquots were withdrawn at desired time intervals to test the remaining protease activity. Crude enzyme diluted using buffer was considered as control (Manni et al. 2010).

Deproteinization of shrimp wastes by *S. basilisca* proteases

Shrimp shell wastes (15 g) were mixed with 100 mM Tris-HCl buffer, (pH 8.0) at a ratio of 1:3 (w/v), minced and then cooked for 20 min at 90 °C to inactivate endogenous enzymes. The cooked sample was then homogenized in a Moulinex blender for about 2 min. The pH of the mixture was adjusted to 8.0, and then the shrimp waste proteins were digested with the crude protease extract of *S. basilisca* using different enzyme/substrate (E/S) ratio (unit of enzyme per milligram of protein). After incubation for 3 h at 30, 40 or 50 °C the reaction was stopped by heating the solution at 90 °C during 20 min to inactivate enzymes. The shrimp waste protein hydrolysates were then centrifuged at $5,000 \times g$ for 20 min to separate insoluble and soluble fractions. The solid phase was washed and then dried for 1 h at 60 °C. The protein content was analysed for calculation of the protein removal. Deproteinization (DP) was expressed as percentages and computed by the following equation as described by Rao et al. 2000.

$$\%DP = \frac{[(PO \times O) - (PR \times R)] \times 100}{Po \times O}$$

where *PO* and *PR* are the protein concentrations (percent) before and after hydrolysis, while *O* and *R* represent the mass (grams) of original sample and hydrolyzed residue in dry weight basis, respectively.

Statistical analysis

Statistical analyses were performed with SPSS ver. 2.0, professional edition using ANOVA analysis. Differences were considered significant at $p < 0.05$. All tests were carried out in triplicate.

Results and discussion

Characterization of the viscera protease extract

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and zymography of crude alkaline protease extract

In order to estimate the number of proteases in the alkaline crude enzyme extract, sample was separated by SDS-PAGE, and then proteolytic activity was revealed by casein zymogram activity staining. As can be observed in Fig. 1, the crude enzyme extract showed at least five major clear bands of protease activity with different molecular weights. This result suggests that at least five major proteinases were present in *S. basilisca* viscera.

Fig. 1 SDS-PAGE (a) and zymogram (b) of the crude alkaline protease extract from the viscera of *S. basilisca*

Effects of enzyme inhibitors on protease activity

The effects of various enzyme inhibitors, such as chelating agents and group-specific reagents, on enzyme activity were studied and reported in Table 1. PMSF, a serine enzyme inhibitor, strongly affect the proteolytic activity of the crude enzyme extract and 97 % of its original activity was lost. This indicates the presence of serine proteases, in particular trypsin and chymotrypsin. On the other hand, β -mercaptoethanol was without influence on the enzyme activity. However, metalloprotease inhibitor EDTA inhibited the protease activity by 56 %, indicating the importance of some metal ions in enzyme stabilization.

The effect of specific enzyme inhibitors on protease activity was also analysed by zymogram activity staining (Fig. 2). The different classes of proteases could be identified after incubation with specific inhibitors by the disappearance or reduced intensity of the bands compared to the control without inhibitors. PMSF, a serine inhibitor, inhibited all proteases as revealed by the complete disappearance of P3 and P4 bands, and by the reduction of intensity of the other bands (P1, P2 and P5). Besides, the addition of EDTA caused the disappearance of P1 and P2 bands. This suggest that these high molecular weight enzymes are metalloproteases or serine-metalloproteases, since these enzymes were also affected by PMSF. β -mercaptoethanol and pepstatine A were without influence on proteolytic activity. These finding shows that P3, P4 and P5 are of the serine protease class.

Effect of pH on protease activity and stability

The relative activity values at various pHs from 5.0 to 12.0 are shown in Fig. 3a. The enzyme preparation was highly active between pH 7.0 and 9.0 with an optimum at pH 8.0 when incubated for 15 min at 60 °C. The relative activities

Table 1 Effects of various enzyme inhibitors and metal ions on the activity of the crude alkaline enzyme extract from *S. basilisca*

Chemicals	Concentration	Activity (%)
None	—	100
PMSF	5 mM	2.86±0.36
EDTA	5 mM	44.26±1.04
SBTI	1 mg/ml	22±1.41
β-mercaptoethanol	5 mM	100
Pepstatine	5 mM	100
Ca ²⁺	5 mM	119±2.82
Ba ²⁺	5 mM	96.26±2.46
Zn ²⁺	5 mM	4±0.97
Cu ²⁺	5 mM	4.22±0.67
Mg ²⁺	5 mM	95.41±2.24
Mn ²⁺	5 mM	37.51±0.692
K ⁺	5 mM	101.08±1.27
Na ⁺	5 mM	97.22±0.39
Hg ²⁺	5 mM	9.16±0.48

The crude enzyme was pre-incubated with various enzyme inhibitors for 30 min at 25 °C and the remaining activity was determined at pH 8.0 and 60 °C. Enzyme activity measured in the absence of any inhibitor was taken as 100 %. The effect of metal ions on the activity of the crude enzyme was determined by incubating the enzyme in the presence of various metal ions for 15 min at 60 °C and pH 8.0. *PMSF* Phenylmethylsulfonyl fluoride; *EDTA* Ethylene-diaminetetraacetic acid; *SBTI* Soybean trypsin inhibitor

at pH 9.0 and 10.0 were about 79 % and 55 %, respectively. This characteristic likely contributes to its physiological role in intestinal tissue, where pH is high (Male et al. 1995), and is a relevant aspect that enables its use in liquid detergent formulations, as the pH of laundry detergents is commonly alkaline (Maurer 2004). The optimum pH for zebra blenny proteases was similar to that reported by Bougateg et al. (2008) for proteases extracted from the viscera of Sardinella (*Sardinella aurita*).

Fig. 2 Zymogram of alkaline proteases from the viscera of *S. basilisca* treated with several enzyme inhibitors (5 mM). Lane 1: crude enzyme extract without inhibitor; lane 2, 3, 4 and 5: crude enzyme extract preincubated for 30 min at 25 °C with PMSF, EDTA, β-mercaptoethanol and pepstatine **Fig. 3** pH profile (a) and pH stability (b) of the crude alkaline protease from viscera of the zebra blenny (*S. basilisca*). Protease activity was assayed in the pH range from 5.0 to 12.0 at 60 °C. The maximum activity obtained at pH 8.0 was considered as 100 % activity. The pH stability was determined by incubating the enzyme in different buffers for 60 min at 30 °C, and the residual enzyme activity was determined at pH 8.0 and 60 °C using casein as a substrate. The activity of the enzyme before incubation was taken as 100 %. Buffer solutions used for pH activity and stability are presented in “Material and Methods” section

The pH stability profile, reported in Fig. 3b, showed that the *S. basilisca* enzyme preparation is highly stable at a pH range between 6.0 and 11.0, maintaining 100 % of its original activity after 1 h incubation at 30 °C between pH 6.0 and 9.0. The residual activities at pH 10.0 and 11.0 were about 93 % and 92 %, respectively. These results suggest that the viscera of *S. basilisca* would be a potential source of proteases for certain industrial applications that require high alkaline conditions.

Effect of temperature on protease activity and stability

The effect of temperature on protease activity was determined by assaying enzyme activity at different temperatures (Fig. 4a). The enzyme extract from zebra blenny viscera was active at temperatures from 30 to 70 °C with an optimum between 55 and 60 °C. The relative activities at 40 and 50 °C were about 36 % and 92 %, respectively. However, an appreciable decrease in enzyme activity was observed above 60 °C, due to thermal denaturation. At 70 °C, the relative activity was only 24 % of that of 60 °C. The optimum temperature for *S. basilisca* proteases was similar to that from *Colossoma*

macropomum (Espósito et al. 2009a) and higher than those of proteases from common carp (*Cyprinus carpio* L.) (Espósito et al. 2009b) and striped seabream (*lithognathus mormyrus*) (El-Hadj Ali et al. 2011) which exhibited an optimum temperature at 50 °C.

Thermal stability of protease extract from zebra blenny viscera is depicted in Fig. 4b. The crude protease extract remained thermally stable as it retained about 80 % and 73 % of activity after being incubated at 50 °C, for 30 and 60 min, respectively. Moreover, about 100 and 86 % of the maximum activity was retained after incubation for 60 min at 30 and 40 °C, respectively.

Effects of metal ions on protease activity

Table 1 displays the effects of various metal ions at a concentration of 5 mM on the activity of crude enzyme extract from zebra blenny viscera. The enzyme preparation proved sensitive to ions, mainly Zn^{2+} , Cu^{2+} , Mn^{2+} and Hg^{2+} . It is known that Hg^{2+} is among ions that act on sulfhydryl residues in proteins (Male et al. 1995). The inhibition caused by this ion suggests the relevance of sulfhydryl residues for the catalytic action of most proteases (Bezerra et al. 2005). The effects of Cu^{2+} and Mn^{2+} (respectively, 4 % and 37.5 %) proved to be stronger than those recorded for *L.*

mormyrus enzyme (respectively, 82 % and 69 %) (El-Hadj Ali et al. 2011). Only Ca^{2+} increased enzyme activity to 119 %. However, K^+ , Na^+ , Ba^{2+} and Mg^{2+} showed no influence on proteolytic activity. A similar result was observed by El-Hadj Ali et al. (2011) where *L. mormyrus* crude enzyme retained 100 % of its activity in the presence of K^+ , Na^+ , Ba^{2+} and Mg^{2+} when incubated under the same conditions.

Effect of NaCl concentration

The effect of NaCl concentration on the activity of zebra blenny crude enzyme was studied at pH 8.0 and 60 °C by the addition of NaCl to the reaction mixture. As reported in Fig. 5, a continuous decrease in proteolytic activity was observed with increasing NaCl concentration. The relative activity at 15 % NaCl was approximately 27 %. The decrease in activity could be explained by the denaturation of enzyme caused by the “salting out” (Klomklao et al. 2004). The activity in the presence of high salt may allow this crude enzyme extract to be used in accelerating protein hydrolysis under hypersaline conditions such as in high salt fish products.

Effects of oxidizing agent and surfactants

In addition to activity and stability at high pH range and various temperatures, enzymes incorporated into detergent formulations must be compatible and stable with all commonly used detergent compounds like surfactants, bleaches and other additives, which might be present in the formulation (stability during storage and washing) (Kumar and Takagi 1999). Important commercial detergent proteases like Subtilisin Carlsberg, Subtilisin BPN, Alcalase, Esperase and Savinase are stable in the presence of various detergent components; however, most of them are unstable in the presence of oxidant agents (Gupta et al. 1999). Thus it is desirable to search for new proteases with novel properties from many different sources as possible. The zebra blenny enzyme preparation was incubated 60 min at 30 °C in the presence of additives and then the residual protease

Fig. 4 Temperature profile (a) and thermal stability (b) of the alkaline crude enzyme from the viscera of the zebra blenny (*S. basilisca*). Enzyme activity was assayed at different temperatures ranging from 30 to 70 °C at pH 8.0, using casein as a substrate. The activity of the enzyme at 60 °C was taken as 100 %. For thermal stability, the enzyme was incubated at different temperatures for 60 min. The residual enzyme activity was assayed at pH 8.0 and 60 °C. The non-heated enzyme was considered as control (100 %)

Fig. 5 Effect of NaCl concentration on the activity of the purified trypsin

activity was assayed at pH 8.0 and at 60 °C (Table 2). The crude protease was highly stable in the presence of the non-ionic surfactants. It retained 100 % of its initial activity in the presence of 5 % Tween 80 and more than 94 % with 5 % Triton X-100. In addition, the crude enzyme was relatively stable in the presence of the strong anionic surfactant, retaining approximately 89 % and 58 % of its initial activity in the presence of 0.1 % and 0.5 % SDS, respectively. SDS, can bind to globular proteins and form small aggregates around its polypeptide backbone, thus denaturing the protein native structure (Ghosh 2008). Several works also reported the instability of fish proteases against SDS and other similar surfactants (Jellouli et al. 2009; Esposito et al. 2009a, b). In addition, we investigated the effects of oxidizing agents (sodium perborate) on crude enzyme extract. The activity of proteolytic enzymes was little influenced by sodium perborate, and retained about 81 % and 62 % of its activity in the presence of 0.1 % and 1 % sodium perborate, respectively. The high stability of the enzyme in the presence of oxidising agents is a very important characteristic for its eventual use in detergent formulations.

Stability of the zebra blenny alkaline proteases in the presence of various commercial liquid detergents

An ideal detergent enzyme should be stable during storage and active during washing in the detergent solution for a long period of time. It should also maintain adequate temperature stability in order to be effective throughout a wide range of washing temperatures (Banik and Prakash 2004; Moreira et al.

2002). To check the compatibility of the crude protease with liquid detergents, enzyme preparation was preincubated in the presence of various commercial laundry detergents for 1 h at 30 and 40 °C. The data presented in Fig. 6 show that the alkaline proteases are more stable at 30 °C than 40 °C. Maximum stability was achieved with Carrefour since the enzyme retained 100 % and 98 % of its initial activity, after 1 h incubation, at 30 and 40 °C, respectively. The residual activities at 30 °C were about 99 %, 98 %, 96 % and 81 % when crude enzyme was incubated in presence of Dixan, Tex'til, Nadhif and LAV+, respectively ($p < 0.05$). Interestingly, *S. basilisca* proteases were more stable than Purafect® 2000E, a commercial alkaline protease used in detergent formulations. Since the proteolytic activity varied with each liquid detergent, the obtained results clearly indicated that the performance of enzymes in detergents depends on number of factors, including the detergents compounds.

Effects of organic solvents on enzyme stability

A previous study showed that peptide synthesis could be enhanced by the addition of organic solvents in the reaction mixture (Gupta 1992). To achieve a high yield in protease-catalyzed peptide synthesis, the stability of protease is very important because enzymes are usually denatured or inactivated in the presence of organic solvents before completing the reaction. Therefore, proteases, which are naturally stable in the presence of organic solvents, are very useful for peptide synthesis.

Organic solvent stability of the zebra blenny crude enzyme was evaluated by incubating the crude enzyme with 50 % of various organic solvents at 30 °C with constant shaking. In the absence of organic solvents, the half-life, time required to conserve 50 % of the initial activity, of *S. basilisca* proteases was approximately 4 days. In the presence of methanol, isopropanol, hexane and acetone the half-lives of *S. basilisca* proteases were about 3, 4, 4 and 6 days, respectively. Interestingly, the stability of the enzyme was highly enhanced by DMSO, DMF and diethyl ether. The half-lives of the enzyme in the presence of diethyl ether and DMF were 15 and 20 days, respectively, and that in the presence of DMSO was more than 30 days of incubation.

These results were similar to earlier reports showing increased protease stability in the presence of organic solvents. In fact, the half-life of the *B. cereus* SV1 protease was approximately 34 days in the absence of organic solvent. However, in the presence of ethyl acetate and DMF, the half-lives were 51 and 48 days, respectively (Manni et al. 2010). The half-lives of *L. mormyrus* proteases in the presence of DMSO, DMF, diethyl ether and hexane were more than 30 days while in the absence of organic solvents the half-life was 8 days (El-Hadj Ali et al. 2011).

Table 2 Stability of *S. basilisca* alkaline crude enzyme extract in the presence of various surfactants and oxidizing agents

Additifs	Concentration	Residual activity (%)
None	—	100
SDS	0.1 % (w/v)	89±1.41
	0.5 % (w/v)	58.1±1.20
	1 % (w/v)	39.4±1.83
Tween 80	1 % (v/v)	100
	5 % (v/v)	100
Triton X-100	1 % (v/v)	95±0.93
	5 % (v/v)	94.3±0.98
Perborate de sodium	0.1 % (w/v)	80.85±1.62
	1 % (w/v)	62.41±0.83

The alkaline crude enzyme extract was pre-incubated with surfactants and oxidizing agents for 1 h at 30 °C, and the remaining activity was measured at pH 8.0 and 60 °C. The activity is expressed as a percentage of the activity level in the absence of additives. SDS Sodium dodecyl sulfate

Fig. 6 Stability of the alkaline crude protease extract from zebra blenny (*S. basilisca*) in the presence of various commercial liquid detergents. The crude enzyme was incubated 1 h at 30 or 40 °C and pH 8.0 in the presence of solid detergents at a final concentration of 7 mg/ml and the remaining activities were determined at pH 8.0 and

60 °C using casein as a substrate. Enzyme activity of control sample without any detergent, incubated under the same conditions, was taken as 100 %. *a*, *b*, *c*, *d* and *e*: significance of detergents vs Nadhif, Dixan, LAV+, Carrefour and Tex'til, respectively ($p < 0.05$)

Enzymatic deproteinization of shrimp wastes by zebra blenny proteases

The application of microorganisms or proteolytic enzymes for deproteinization of marine crustacean wastes is a current research trend in conversion of wastes into useful bioactive substances. It is a simple and environment-friendly alternative to chemical methods employed in the preparation of chitin. Few studies on the use of proteolytic enzymes for the deproteinization of crustacean wastes have been reported. Many factors, such as the specificity of the enzyme used for the proteolysis, E/S ratio and the conditions used during hydrolysis (initial temperature value and hydrolysis time) have been reported to influence the enzymatic deproteinization process. In this study, the crude alkaline enzyme from *S. basilisca* was tested for the deproteinization of shrimp waste to produce chitin. Different temperatures and different E/S ratios between 0 and 15 were used to compare the deproteinization efficiency. As shown in Fig. 7a, deproteinizations at 40 °C and 50 °C were more efficient than that at 30 °C. The variation of E/S ratio at 40 °C was reported in Fig. 7b. The deproteinization rate with a ratio of 0.1 was about 49 %. The percentage of protein removal increased with increasing E/S ratio and reached about 77 % with an E/S ratio of 5 after incubation for 3 h at 40 °C. Beyond a ratio of 5, no significant increase in the deproteinization rate was observed.

The deproteinization activity of *S. basilisca* crude protease was similar to that of *L. mormyrus* (El-Hadj Ali et al. 2011) and *Zosterisessor ophiocephalus* (Sila et al. 2011). However, it was lower than that of solvent-stable metalloprotease from *B. cereus* SV1, which reached 88 % with E/S ratio of 20 (Manni et al. 2010).

The fact that deproteinization cannot reach 100 % may be explained by the non-accessibility of enzymes to some proteins protected by chitin. In addition, some portions of peptides are suggested to be linked covalently to a small

Fig. 7 Effect of temperature (a) and E/S ratio (b) on the deproteinization of shrimp waste. (a) *a*, *b* and *c*: significance of temperature vs 30 °C, 40 °C and 50 °C, respectively ($p < 0.05$). (b) *a*, *b*, *c*, *d* and *e*: significance of E/S values vs E/S=0.1, E/S=1, E/S=5, E/S=10 and E/S=15, respectively ($p < 0.05$)

number of the C-2 amino groups of chitins. Although the deproteinization percentage is lower than that of chemical treatment, enzymatic deproteinization avoids many problems of chemical treatments such as heavy metal residues, overhydrolysis, and breakdown of chitins, etc.

Conclusion

The present study reports the extraction, characterization and evaluation of digestive proteases from *S. basilisca* as detergent additive and in shrimp waste deproteinisation. The crude protease was active in a broad range of pH 7.0–10.0 and exhibited a high stability in the presence of oxidizing agent and various laundry liquid detergents, this makes it an ideal choice for applications in liquid detergent formulation. Additionally, the crude protease extract was found to be relatively effective in the deproteinization of shrimp waste powder. The protein removal with an E/S ratio of 5 was about 77 %. It is also stable in the presence of organic solvents.

Further research is needed to purify all alkaline proteases, and to determine their properties as a possible biotechnological tool in the fish processing and detergent processing industries.

Acknowledgments This work was funded by the Ministry of Higher Education and Scientific Research, Tunisia.

References

- Anwar A, Saleemuddin M (1998) Alkaline proteases: a review. *Bioresour Technol* 64:175–183
- Banerjee UC, Sani RK, Azmi W, Soni R (1999) Thermostable alkaline protease from *Bacillus brevis* and its characterization as a laundry detergent additive. *Process Biochem* 35:213–219
- Bank RM, Prakash M (2004) Laundry detergent compatibility of the alkaline protease from *Bacillus cereus*. *Microbiol Res* 159:135–140
- Ben Khaled H, Ktari N, Ghorbel-Bellaaj O, Jridi M, Lassoued I, Nasri M (2011) Composition, functional properties and in vitro antioxidant activity of protein hydrolysates prepared from sardinelle (*Sardinella aurita*) muscle. *J Food Sci Technol*. doi:10.1007/s13197-011-0544-4
- Bezerra RS, Lins EJJ, Alencar RB, Paiva PMG, Chaves MEC, Coelho LCB, Jr LB C (2005) Alkaline proteinase from intestine of Nile tilapia (*Oreochromis niloticus*). *Process Biochem* 40:1829–1834
- Bhaskar N, Suresh PV, Sakhare PZ, Sachindra NM (2007) Shrimp biowaste fermentation with *Pediococcus acidolactici* CFR2182: optimization of fermentation conditions by response surface methodology and effect of optimized conditions on deproteinization/demineralization and carotenoid recovery. *Enzyme Microb Technol* 40:1427–1434
- Bougatef A, Haddar A, Ben Khaled H, Barkia A, Nasri M (2008) Digestive proteinases from Sardinelle (*S. aurita*): Characteristics, application in preparation of antioxidant protein hydrolysates and evaluation in liquid laundry commercial detergents. In Nasri M (ed) Recent Research Developments in Food by products Technology and biotechnology a special book in the area of Food Chemistry, (Research signpost. ISBN: 978-81-308-0259-6.), PP: 98–117
- Cowan D (1996) Industrial enzyme technology. *Trends Biotechnol* 4:177–178
- El-Hadj Ali N, Hmidet N, Ghorbel-Bellaaj O, Fakhfakh-Zouari N, Bougatef A, Nasri M (2011) Solvent-stable digestive alkaline proteinases from Striped Seabream (*Lithognathus mormyrus*) viscera: characteristics, application in the deproteinization of shrimp waste, and evaluation in laundry commercial detergents. *Appl Biochem Biotechnol* 164:1096–1110
- Espósito TS, Amaral IPG, Buarque DS, Oliveira GB, Carvalho LB Jr, Bezerra RS (2009a) Fish processing waste as a source of alkaline proteases for laundry detergent. *Food Chem* 112:125–130
- Espósito TS, Amaral IPG, Marcuschi M, Carvalho LB Jr, Bezerra RS (2009b) Surfactants-and oxidants-resistant alkaline proteases from common carp (*Cyprinus carpio* L.) processing waste. *J Food Biochem* 33:821–834
- Garcia-Carreno FL, Dimes LE, Haard NF (1993) Substrate-gel electrophoresis for composition and molecular weight of proteinases or proteinaceous proteinase inhibitors. *Anal Biochem* 214:65–69
- Ghorbel-Bellaaj O, Hmidet N, Jellouli K, Younes I, Maâlej H, Hachicha R, Nasri M (2011a) Shrimp waste fermentation with *Pseudomonas aeruginosa* A2: optimization of chitin extraction conditions through Plackett–Burman and response surface methodology approaches. *Int J Biol Macromol* 48:596–602
- Ghorbel-Bellaaj O, Jellouli K, Younes I, Manni L, Ouled Salem M, Nasri M (2011b) A solvent-stable metalloprotease produced by *Pseudomonas aeruginosa* A2 Grown on shrimp shell waste and its application in chitin extraction. *Appl Biochem Biotechnol* 164:410–425
- Ghosh S (2008) Interaction of trypsin with sodium dodecyl sulfate in aqueous medium: a conformational view. *Colloids Surf* 66:178–186
- Gildberg A (1992) Recovery of proteinases and protein hydrolysates from fish viscera. *Bioresour Technol* 39:271–276
- Gupta MN (1992) Enzyme function in organic solvents. *Eur J Biochem* 203:25–32
- Gupta R, Gupta K, Saxena RK, Khan S (1999) Bleach-stable alkaline protease from *Bacillus* sp. *Biotechnol Lett* 21:135–138
- Gupta R, Beg Q, Lorenz P (2002) Bacterial alkaline proteases: molecular approaches and industrial applications. *Appl Microbiol Biotechnol* 59:15–32
- Heymer A (1985) Morphologie, coloration, intersexualité et anatomie des organes reproducteurs chez *Blennius basiliscus* (Teleostei, Blenniidae). *Revue Française d'Aquariologie* 12:39–52
- Jellouli K, Bougatef A, Daassi D, Balti R, Barkia A, Nasri M (2009) New alkaline trypsin from the intestine of grey triggerfish (*Balistes caprisicus*) with high activity at low temperature: purification and characterisation. *Food Chem* 116:644–650
- Jo GH, Jung WJ, Kuk JH, Oh KT, Kim YJ, Park RD (2008) Screening of protease-producing *Serratia marcescens* FS-3 and its application to deproteinization of crab shell waste for chitin extraction. *Carbohydr Polym* 74:504–508
- Kembhavi AA, Kulkarni A, Pant A (1993) Salt-tolerant and thermostable alkaline protease from *Bacillus subtilis* NCIM No.64. *Appl Biochem Biotechnol* 38:83–92
- Klomklao S, Benjakul S, Visessanguan W (2004) Comparative studies on proteolytic activity of splenic extracts from three tuna species commonly used in Thailand. *J Food Biochem* 28:355–372
- Ktari N, Fakhfakh N, Balti R, Ben Khaled H, Nasri M, Bougatef A (2012) Effect of degree of hydrolysis and protease type on the

- antioxidant activity of protein hydrolysates from cuttlefish (*Sepia officinalis*) by-products. J Aquat Food Prod Technol. doi:10.1080/1049850.2012.658961
- Kumar CG, Takagi H (1999) Microbial alkaline proteases from a bioindustrial viewpoint. Biotech Adv 17:561–594
- Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685
- Male R, Lorens LB, Smalas AO, Torrisen KR (1995) Molecular cloning and characterization of anionic and cationic variants of trypsin from Atlantic salmon. Eur J Biochem 232:677–685
- Manni L, Jellouli K, Ghorbel-Bellaaj O, Agrebi R, Haddar A, Sellami-Kamoun A, Nasri M (2010) An oxidant- and solvent-stable protease produced by *Bacillus cereus* SV1: application in the deproteinization of shrimp wastes and as a laundry detergent additive. Appl Biochem Biotechnol 160:2308–2321
- Maurer KH (2004) Detergent peptidases. Curr Opin Biotechnol 15:330–334
- Moreira KA, Albuquerque BF, Teixeira MFS, Porto ALF, Lima Filho JL (2002) Application of protease from *Nocardia* sp. as a laundry detergent additive. World J Microbiol Biotechnol 18:307–312
- Rao MS, Muñoz J, Stevens WF (2000) Critical factors in chitin production by fermentation of shrimp biowaste. Appl Microbiol Biotechnol 54:808–813
- Rinaudo M (2006) Chitin and chitosan: properties and applications. Prog Polym Sci 31:603–632
- Roberts GAF (1992) Chitin Chemistry. Macmillan, London
- Samal BB, Kara B, Stabinsky Y (1990) Stability of two novel serine proteinases in commercial laundry detergent formulations. Biotechnol Bioeng 35:650–652
- Shahidi F, Kamil YVAJ (2001) Enzymes from fish and aquatic invertebrates and their application in the food industry. Trends Food Sci Technol 12:435–464
- Shahidi F, Synowiecki J (1991) Isolation and characterization of nutrients and value-added products from snow crab (*Chionoecetes opilio*) and shrimp (*Pandalus borealis*) processing discards. J Agric Food Chem 39:1527–1532
- Sila A, Nasri R, Bougatef A, Nasri M (2011) Digestive alkaline proteases from the goby (*Zosterisessor ophiocephalus*) characterization and potential application as detergent additive and in the deproteinization of shrimp wastes. J Aquat Food Prod Technol 21:118–133
- Simpson BK (2000) Digestive proteases from marine animals. In: Haard NF, Simpson BK (eds) Seafood enzymes. Marcel Dekker, New York, pp 191–213
- Sini TK, Santhosh S, Mathew PT (2007) Study on the production of chitin and chitosan from shrimp shell by using *Bacillus subtilis* fermentation. Carbohydr Res 342:2423–2429