Extraction and purification of a highly thermostable alkaline caseinolytic protease from wastes *Litopenaeus vanamei* suitable for food and detergent industries

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*Running title: Purification of a highly thermostable protease from *Litopenaeus vanamei*

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Abstract

A novel thermostable protease was purified from *Litopenaeus vanamei* from Persian Gulf to homogeneity level by using ammonium sulfate precipitation and anion-exchange chromatography. The purified protease showed a single band on native and SDS-PAGE with a molecular weight of 24 kDa on SDS-PAGE. The enzyme showed the broad highest catalytic activity for hydrolysis of the substrate with maximal activity at pH 7 and 80 °C. Activity of the enzyme was inhibited by Hg^{2+} , Zn^{2+} , Co^{2+} and Cu^{2+} , while protease activity was increased in the presence of Fe^{2+} and Mn^{2+} by factors of 173% and 102%, respectively. Enzyme shows a broad substrate specificity and hydrolyzes both natural and synthetic substrates. Based on the Lineweaver–Burk plot, the K_m with casein as substrate was 16.8 μM and $V_{\max}$ was 82.6 $\mu\text{M}/\text{min}$. The enzyme, derived from *Litopenaeus vanamei*, possesses unique characteristics and could be used in various industrial and biotechnological applications.

Keywords: Purification; *Litopenaeus vanamei* protease; Marine wastes, Thermostable; Persian Gulf

1. Introduction

The history of proteolytic enzymes is intimately interwoven with that of protein chemistry. In the very early days, proteolytic enzymes were considered an impediment that had to be removed in the isolation of proteins generally (Neurath, 1999). The basic definition of a protease is an enzyme that hydrolyzes peptide bonds in proteins. Approximately 700 enzymes, or roughly 2% of the human genome, have known or putative proteolytic activity (Rawlings, Barrett, & Bateman, 2012; Sanman & Bogyo, 2014). Proteases are a highly complex group of enzymes that differ in their substrate specificity, catalytic mechanism and active site (Sumantha, Larroche, & Pandey, 2006; Vishwanatha, Rao, & Singh, 2009). There are five protease families: aspartyl, cysteine, metallo, serine, and threonine proteases. Cysteine, serine, and threonine proteases use nucleophilic active site residues to hydrolyze peptide bonds, whereas aspartyl and metallo proteases use active site residues to activate water molecules for nucleophilic attack (Sanman & Bogyo, 2014; Turk, Turk, & Turk, 2012). Proteolytic activity is central to many necessary biological processes, including development, differentiation, apoptosis, ripening, regulatory mechanisms, wound healing, germination, senescence and necrosis and the immune response (Sanman & Bogyo, 2014; Turk, Turk, & Turk, 2012). Proteases constitute one of the commercially important groups of enzymes, accounting for nearly 60% of the whole enzyme market and are frequently used in detergent, leather, pharmaceuticals, food and biotechnology industries (de Castro, Ohara, Nishide, Albernaz, Soares, & Sato, 2015; Vijayaraghavan, Lazarus, & Vincent, 2014). Proteases having also a variety of industrial applications. In the food industry, proteases are used to tenderize meat and related derivatives, to produce protein hydrolysate, to clarify juice and beer in the brewing industry, for cheese production in dairy industry, in baking industry and for the extraction of flavor and color compounds from plants. Proteases can also be

used in the forage industry to increase protein content and in recovering plant and animal protein to make health products (A. Homaei, 2015a, 2015b; A. Homaei, Barkheh, Sariri, & Stevanato, 2014; A. Homaei & Etemadipour, 2015; A. A. Homaei, Sajedi, Sariri, Seyfzadeh, & Stevanato, 2010). In this research, the sources of protease enzyme of *Litopenaeus vanamei* been studied for the first time and a novel thermostable protease was purified and characterized. Considering the fact that *Litopenaeus vanamei* exclusively found in Persian Gulf and the protease responsible for its unique proteolytic behavior it not studied so far, we aimed to extract, purify, identify and characterize this novel protease. The specific information obtained from this research has now opened the way to further characterize the enzyme and design for its various industrial and medical applications.

2. Materials and methods

2.1. Materials

Casein Sigma (St. Louis, MO, USA), DEAE-cellulose, DEAE -Sephadex and Sephadex G-75 provided by Pharmacia (Uppsala, Sweden). All other chemicals were reagent grade and purchased from Merck (Darmstadt, Germany).

2.2. Specimen collection, crude enzyme extract and enzyme purification

Litopenaeus vannamei, caught from Persian Gulf, was immediately frozen and transported to our enzymology laboratory in Hormozgan university, Bandar Abbas. The frozen samples were then stored at -80 °C until used. Fresh shrimp pancreas was freed from the adhering meninges and blood and the grey matter was removed by gross dissection. Pancreas tissue was re-suspended in 10 ml of 50 mM phosphate buffer, pH 7.5. The suspension was subjected to sonic disruption, 10 s on, 45 s Off for 15 min, and cell debris were discarded by centrifugation at $15,000 \times g$ for 20 min. The supernatant was subjected to ammonium sulfate precipitation. The protein was then

dissolved in minimal amount of phosphate buffer and dialyzed against the same buffer for 24 h at 4 °C, changing dialysis buffer every 8 hours. The dialyzed sample was subjected to anion-exchange chromatography, using a DEAE-cellulose column, previously equilibrated with 50 mM phosphate buffer pH 7.5. The column was washed with the same buffer until no protein was detected in the eluent. Then it was eluted using a gradient of 0–1 M NaCl in the same buffer at a flow rate 1 ml/min and 2 ml fractions were collected. To determine the protein content of each fraction the absorbance at 280 nm was measured using UV–vis spectrophotometer. The active fractions from this and subsequent chromatographies were measured with casein as a substrate. Fractions with proteolytic activity were pooled and applied to a DEAE -Sephadex fast flow column, pre-equilibrated with 50 mM phosphate buffer at pH 7.5. The column was washed with the same buffer until no protein was detected in the eluent. Then it was eluted using a gradient of 0–1 M NaCl in the same buffer at a flow rate 1 ml/min. The enzyme from the previous step was placed on a column of Sephadex G-75 previously equilibrated with 50 mM phosphate buffer pH 7.5. A flow rate of 1 ml/min was maintained and fractions of 2 ml were collected. The enzyme appeared in the effluent, immediately after the void volume, and the active fractions from tubes were pooled. and concentrated in an ultrafiltration chamber with a 5 kDa membrane cut-off and analyzed for proteins and enzyme activity. The concentrated enzyme was dialysed against 50 mM phosphate buffer pH 7.5.

2.3. Electrophoresis, measurement of protease activity and protein concentration

The proteins were identified by sodium dodecyl sulfate polyacrylamide gel electrophoresis using a Mini-PROTEAN electrophoretic system (BioRad). Electrophoresis was carried out at a constant current of 85 mA (Laemmli, 1970) and the gels were stained with Comassie Brilliant Blue R-250 (Wilson, 1983). Protease activity was determined at room temperature, using casein as substrate,

by the method of Homaei (A. Homaei, Barkheh, Sariri, & Stevanato, 2014; A. Homaei & Etemadipour, 2015; A. A. Homaei, Sajedi, Sariri, Seyfzadeh, & Stevanato, 2010). One unit of protease is defined as the amount of enzyme that hydrolyzes casein to produce equivalent absorbance to 1 μ mol of tyrosine/min with tyrosine as standard. Protein concentration was estimated by the Bradford method (Bradford & Biachem, 1968) using bovine serum albumin as standard.

2.4. Characterization of purified protease from Litopenaeus vanamei

2.4.1. Optimum temperature and thermostability

Optimum temperature of pure protease activity was measured at different temperatures in the range 20–100 °C, in phosphate buffer of pH 7, as previously described (A. Homaei, 2015a; A. Homaei, Barkheh, Sariri, & Stevanato, 2014; A. Homaei & Etemadipour, 2015; A. A. Homaei, Sajedi, Sariri, Seyfzadeh, & Stevanato, 2010) The maximum activities obtained under the conditions tested were taken as 100%. Enzyme temperature stability was evaluated by incubating enzyme at 80 °C in 50 mM phosphate buffer pH 7, for different intervals of time, then cooled on ice and the residual activity determined under the assay conditions. Control measurements were carried out measuring the activity of the same enzyme solution kept on ice for the thermal stability.

2.4.2. Determination of optimum pH and pH stability

The effect of pH on the activity was evaluated by measuring pure enzyme activity at pH 3–12, at room temperature in a mixed buffer containing 50 mM acetate, phosphate and glycine. Maximum activity obtained within the range was expressed as 100% and residual activities calculated. The effect of pH on enzyme stability was evaluated after incubation of the enzyme at pH 3 and 12 for 60 min at 25 °C, then pH value was adjusted to 7 and the residual activity determined

according to the assay conditions. Control measurements were carried out measuring the activity of the same enzyme solution kept in the buffer at pH 7 for the pH stability experiments.

2.4.3. Substrate specificity

The substrate specificity of the protease purified was assayed with different protein substrates such as Casein, Azocasein, Egg albumin, Gelatin, Hemoglobin and BSA (1%, w/v). The relative activity was estimated by standard assay.

2.4.4. Determination of kinetic parameters

Catalytic activity of enzyme was investigated at different substrate concentrations under assay conditions. K_m , V_{max} , K_{cat} and K_{cat}/K_m values were determined using Lineweaver–Burk plots.

3. Results and discussion

3.1. Purification of protease

The results of the enzyme purification procedure are presented in Table 1. Proteins were precipitated when the crude enzyme was saturated to 80% with ammonium sulfate. The proteolytic activities after this step was calculated as $1728 \mu\text{mol min}^{-1}$. The protein precipitate was then dissolved in minimal amount of 50 mM phosphate buffer pH 7.5 and dialyzed against the same buffer. The initial anion exchange chromatography with DEAE-cellulose column separated the protease from most of other extracellular proteins. Proteolytic active fractions showed proteolytic activities with $1327 \mu\text{M/min}$, checked using casein as a substrate, were pooled and applied to a DEAE -Sephadex fast flow column, pre-equilibrated with 50 M sodium phosphate buffer at pH 7.5. Although this purification step was not very effective, the proteolytic activity was $1031 \mu\text{mol min}^{-1}$. 18.5-fold purification was obtained after Sephadex G-75 gel

filtration column with a yield of 42%, and a specific activity of $871 \mu\text{mol min}^{-1} \text{mg}^{-1}$. It displayed homogeneity on SDS-PAGE and migrated with a molecular mass of about 24 kDa (Fig. 1). Finally, the proteolytic activity of this enzyme was determined as $871 \mu\text{mol min}^{-1}$.

The protein bands at molecular weight of around 55 and 60 KDa in Lane 1, DEAE-cellulose pool; lane 2, DEAE-Sephadex pool in SDS-PAGE analysis do not have proteolytic activity.

3.2. *Effect of pH on enzyme activity*

The hydrolysis of casein by purified enzyme at various pHs ranging from 3 to 12 was determined. The enzyme exhibited optimum activity in a pH range of 4–10 with the maximum activity at pH 7 (Fig. 2a). The pH profile of the purified *Litopenaeus vannamei* protease showed a broad pH activity profile and retains a high proteolytic activity. So that it has the 70% of its maximum activity in the range of pH 5–9. The broad range of pH activity profile and its optimum activity at lower and higher pH values make this enzyme highly attractive for both the basic research studies and the industrial processes. Currently, there are several industrial applications which take place at low and high pH and consequently there is a great deal of interest to improve pH activity for proteases. Achieving this goal is very difficult task at the present time (Anwar & Saleemuddin, 1998; Rao, Sathish, Ravichandra, & Prakasham, 2009; Sana, Ghosh, Saha, & Mukherjee, 2006).

3.3. *Effect of temperature on enzyme activity*

The influence of temperature on the enzyme activity is shown in Fig. 2b. The optimum activity temperature is between 50 and 100 °C and the maximum activity of the protease was observed at 80 °C. The activity of protease enzymes in broad temperature ranges is a desired characteristic for their application in detergent formulations (Asker, Mahmoud, El Shebwy, & el Aziz, 2013; Ay, Jensen, Kiil, Bach, & Simonsen, 2012; Mukherjee, Adhikari, & Rai, 2008; Sareen & Mishra,

2008). The enzyme shows 30% initial activity at 30 °C, but significantly increases at temperatures higher than 40 °C; In particular, at 80 and 90 °C, relative residual activity was about 100 and 96 %, respectively. The optimum temperature for protease of *B. alveayuensis* CAS 5 was found to be 50 °C. Thermostability studies concealed that the enzyme was 100% stable up to 70 °C; it retains 81% of its original activity at 80 °C and 35% even at 95 °C (Annamalai, Rajeswari, & Balasubramanian, 2014). Manachini et al. (Manachini, Fortina, & Parini, 1988) have reported an alkaline protease from *Bacillus thermoruber* that is active in a broad temperature range of 10–80 °C, with an optimum of 45 °C. An alkaline protease from *B. clausii* was observed to be stable in the temperature range of 30–80 °C, with an almost 100% activity in the temperature range of 30–50 °C (Joo, Kumar, Park, Paik, & Chang, 2003). These results indicated that the purified protease of *Litopenaeus vannamei* was more stable than other proteases reported to date (Deng, Wu, Zhang, Zhang, & Wen, 2010; Haddar, Agrebi, Bougatef, Hmidet, Sellami-Kamoun, & Nasri, 2009; Joo, Kumar, Park, Paik, & Chang, 2003; Manachini, Fortina, & Parini, 1988).

3.4. Thermostability and stability at extreme pHs of Enzyme

The time dependence of the enzyme stability with temperature, measured after incubating the enzyme at 80 °C, evidences a half life of more than 60 min (Fig. 2c). The enzyme retained more than 60% reactivity at 80 °C. Therefore, the purified protease from *Litopenaeus vannamei* showed the high thermostability. It should be mentioned that thermostability of the enzyme is one of the good characteristics of the protease. In addition, thermostable enzyme can decrease the risk of contaminants at high temperature in industries and also cost of external cooling and the increased substrate solubility, allowing for higher concentrations of low solubility materials and a lower viscosity of liquids and it may also be useful in mixing (Amid, ABD Manap, & Zohdi, 2014). They can also be used to study enzyme behavior under conditions that would denature

most enzymes, since enzymes resistant to heat are also, at room temperature, resistant to organic solvents, chaotropic agents, and proteolysis. Clearly, such stable enzymes also have a variety of applications in biotechnology, where protein stability may be an important practical and economic factor (Laidler & Peterman, 1979). Globular proteins exhibit marginal stabilities that are equivalent to only a small number of weak intermolecular interactions (Pfeil, 1998; P L. Privalov, 1992). In this respect, enzymes from thermophiles and hyperthermophiles do not differ significantly from their mesophilic counterparts. In most cases, thermophilic adaptation is accompanied by a small increase in the free energy of stabilization that is generated either by a flattening or lowering of the $\Delta G_{N \rightarrow U}$ vs temperature profiles, or by a shift to a higher stability maximum (Franks, 1994; A. A. Homaei, Mymandi, Sariri, Kamrani, Stevanato, Etezzad, et al., 2013; Jaenicke & Závodszky, 1990; Peter L Privalov & Gill, 1988).

On the other hand, the pH stability of this enzyme was measured at pH 3 and 12, as shown in Fig. 2d. These results reveal that our enzyme retained 3% its activity after 15 min and 0% of its original activity after 1 h at pH 3, while the residual activity of enzyme at pH 12 is nearly 40 and 15% after 15 and 60 min incubation, respectively (Fig. 2d). However, it exhibited good stability in the pH range of 7–9, with 100% stability at pH 7.5 (data not shown). Even at pH 10, the residual activity was 50%. The remarkable activity and stability over a wide pH range reveal the highly alkaline nature of this protease, which makes it suitable for applications in alkaline environments and with detergents.

3.5. Substrate specificity

The protease was active on a variety of modified (azocasein) or natural proteins. The protease exhibited the highest activity on casein (Fig. 3a). The substrate specificity studies revealed that the protease activity was higher when casein was used as substrate followed by pepton, BSA,

azocasein and hemoglobin respectively, but there were weak activity found with elastin and trypton. it can be suggested that this protease is able to digest different protein sources and converting them to small peptides and amino acids, indicating that this is a type of protease with broad range of substrates specificity and ability for unlimited proteolysis.

3.6. Effect of bivalent metal ions on protease activity

Relative activities of protease in the presence of different concentrations of various metal salts, known as strong inhibitors of proteases (A. Homaei, 2015a; A. Homaei, Barkheh, Sariri, & Stevanato, 2014; A. Homaei & Etemadipour, 2015), are depicted in Table 2. The enzyme activity was inhibited slightly by Na^+ , and K^+ , and strongly by Hg^{2+} , Zn^{2+} .and Co^{2+} . In particular, 15 mM Na^+ , and K^+ reduces residual activity of about 30–50 % in the case of enzyme. More enhanced is the difference when ions of the first transition series (Hg^{2+} , Cu^{2+} , Ni^{2+} , and Co^{2+}) at the same concentration were used; in this case, no residual activity for free enzyme incubated with Hg^{2+} , Cu^{2+} and Ni^{2+} was found, while values of ≤ 50 % for Mn^{2+} , Na^+ , and K^+ , were recorded.

3.5. Kinetic parameters and activation thermodynamic parameters

The proteolytic revealed a Michaelis-Menten type of kinetics when hydrolyzing casein (Fig. 3b). The values of K_m , $V_{\max}$, k_{cat} and k_{cat}/K_m measured from Lineweaver Burk plots (inset in Fig. 3b), as shown in Table 3a. As calculated from Lineweaver-Burke plots, the K_m and $V_{\max}$ were 16.8 μM and 82.6 $\mu\text{mol}/\text{min}$, respectively. The estimated K_m values indicated the affinity of enzyme toward the substrate. $V_{\max}$ is an indication of the catalytic activity of an enzyme which is usually desired to be as high as possible.

3.6. Thermodynamics of caseine hydrolysis

The Arrhenius plot was graphed utilizing the activity values in the temperature range of 20–80 °C for ficin. The activation energies of the enzymatic reaction, calculated by the slopes of the plots

of $6.5 \text{ kcal mol}^{-1} \text{K}^{-1}$ was obtained. In Table 3b, values of activation free energy ($\Delta G^\#$), activation enthalpy ($\Delta H^\#$) and activation entropy ($\Delta S^\#$) for the catalytic reaction at different temperature are reported. The negative value of activation entropy ($\Delta S^\#$) indicates that the structure of enzyme–substrate at transition state was more ordered (A. Homaei, 2015b). The lower enthalpy value of protease from *Litopenaeus vanamei* showed that the formation of transition state or activated complex between enzyme–substrate was very efficient. Moreover, lower $\Delta G^\#$ value suggested that the conversion of its transition complex into products was more spontaneous. The feasibility and extent of a chemical reaction is best determined by measuring change in Gibbs free energy ($\Delta G^\#$) for substrate hydrolysis, i.e. the conversion of ES complex into products. The lower is the free energy change the more feasible is the reaction, i.e. the conversion of the reactant to product will be spontaneous. The entropy was slightly lower, which explained that the transition complex had lesser disorder. The free energy for activation of substrate binding ($\Delta G^\#_{\text{E-S}}$) and the free energy for the formation of activation complex ($\Delta G^\#_{\text{E-T}}$) were 1.54 and -0.52 kcal mol^{-1} , respectively. The values of $\Delta G^\#_{\text{E-S}}$ and $\Delta G^\#_{\text{E-T}}$ again confirmed the greater affinity of enzyme towards substrate for hydrolysis and its spontaneous conversion into product.

4. Conclusion

Considering the production cost and reutilization of bioresources, protease production of marine wastes seems to be promising approach (A. Homaei, 2015b; A. Homaei, Ghanbarzadeh, & Monsef, 2016; Zeinali, Homaei, & Kamrani, 2015). The production of inexpensive proteinolytic enzymes not only solves environmental problems, but also promotes the economic value and utilization of marine wastes. This study fills a critical gap in the existing literature and provides new insights into the protease enzymes. Based on our results, the protease purified from wastes *Litopenaeus vanamei* has high stability towards temperature, pH, salts. Kinetic and

thermodynamic parameters of the enzyme suggested that it may be used commercially for the production of protein hydrolysate in protein processing and detergent industries.

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Legends

Fig. 1. SDS-PAGE analysis of samples obtained by the different purification steps. Proteins were detected by coomassie brilliant blue. Lane 1, DEAE-cellulose pool; lane 2, DEAE-Sephadex pool; lane 3, G-75 Sephadex pool. Lane M is the molecular mass marker.

Fig. 2. a) Effect of pH on protease activity. 100% relative activity refers to the percentage of the initial reaction rate obtained by the enzyme at the pH value of maximum activity. **b)** Effect of temperature, in the range 20–100 °C, on the enzyme activity. The activity at optimal temperature was taken as 100. **c)** Irreversible thermoinactivation at 80 °C. The activity of the same enzyme solution, kept on ice, was considered as the control (100%). **d)** pH stability of alkaline phosphatase at pH 3.0 (filled triangles) and 12.0 (filled squares) in mixed buffer. Control data were obtained measuring the activity of the same stock of enzyme solution kept for the same times at room temperature.

Fig. 3. a) Effect of different protease substrates on the protease activity. Enzyme activity in the presence of casein was considered 100%. **b)** Michaelis–Menten plot of the purified protease which reveals maximum velocity and Michaelis constant of the enzyme. In the *inset* are the Lineweaver–Burk plot for kinetics determinations of purified protease using casein as the substrate.

Figure 1

Figure 2

Figure 3

Table 1 Purification procedures of protease from *Litopenaeus vanamei*.

Steps of purification	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification fold	Yield (%)
Cell extract	154.1	2059.6	13.36	1	100
(NH ₄) ₂ SO ₄	56.1	1728.4	30.80	2.30	83.91
DEAE-cellulose	20.5	1327.7	64.76	4.84	64.46
DEAE -Sephadex	9.2	1031.8	112.15	8.39	50.10
Sephadex G-75	3.5	871.85	249.10	18.64	42.33

Table 2 Effect of various bivalent metal ions and their concentrations on *Litopenaeus vanamei* protease

Metals	Concentrations (mM)	Relative activity (%)
Control	No added	100
NaCl	1	93±0.1
	2	89±0.6
	5	85±0.1
	10	80±0.5
	15	75±0.8
KCl	1	98±0.8
	2	96±5
	5	79±3
	10	62±0.1
	15	51±0.7
CoCl ₂	1	37±0.8
	2	31±0.5
	5	23±0.4
	10	20±0.7
	15	9±0.1
ZnCl ₂	1	4±0.3
	2	4±0.6
	5	1±0.8
	10	0

	15	0
MnCl ₂	1	94±0.5
	2	94±0.8
	5	102±0.7
	10	95±0.5
	15	81±0.9
FeCl ₂	1	123±0.1
	2	123±3
	5	128±0.3
	10	171±0.2
	15	173±0.8
CuCl ₂	1	13±0.8
	2	13±0.5
	5	17±0.1
	10	21±0.4
	15	30±0.2
HgCl ₂	1	4±0.5
	2	4±0.2
	5	0
	10	0
	15	0

Table 3a The apparent kinetic constants of *Litopenaeus vanamei* protease. An enzyme concentration of 0.06 mg/mL for all kinetic measurements was used.

<i>Kinetics parameters</i>			
$K_m (\mu M)$	$V_{max} (\mu M.min^{-1})$	$k_{cat} (s^{-1})$	$k_{cat}/K_m (s^{-1} \mu M^{-1})$
16.8±0.5	82.6±0.4	38.2±0.7	2.27±0.8

Table 3b The kinetic and thermodynamic parameters for casein hydrolysis catalyzed by *Litopenaeus vanamei* protease.

E_a (kcal mol ⁻¹)	$\Delta H^\#$ (kcal mol ⁻¹)	$\Delta G^\#$ (kcal mol ⁻¹)	$\Delta S^\#$ (cal molK ⁻¹)	$\Delta G^\#_{E-S}$ (kcal mol ⁻¹)	$\Delta G^\#_{E-T}$ (kcal mol ⁻¹)
6.50±0.7	5.91±0.5	15.23±0.6	-9.32±0.8	1.66±0.5	-0.48±0.7