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# Recovery and Characterization of a Serine Collagenolytic Extract from Snow Crab (*Chionoecetes opilio*) By-products

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**Abstract** Sequential acidic precipitation followed by a single chromatographic step (gel filtration) allowed the recovery of a collagenolytic fraction containing several proteases from by-products of snow crab (*Chionoecetes opilio*). The partial purification was particularly efficient to recover tryptic (purification fold=1,352.5; yield=110%) but also chymotryptic, elastolytic, and collagenolytic activities. A temperature of 40 °C and pH 8.0–8.5 were optimal for enzyme activity, which was stable for 2 h under these conditions. Calcium was not required for stability and thus activity. The isoelectric points of the protein components ranged from 3.7 to 4.6. Zymography revealed 29 and 48 kDa major components and others from 22 to 56 kDa. Enzymes were inhibited by PMSF and TLCK but were insensitive to TPCK. In view of these properties, the proteases likely belong to the serine collagenase group. Inhibition by EDTA could be due to a mechanism other than $Ca^{2+}$ chelation. Using a food system (ground fish), the fraction was more proteolytic than a commercial bacterial protease, suggesting potential applications in enzymatic hydrolysis processes.

**Keywords** *Chionoecetes opilio* · By-products · Serine collagenases · Acidic precipitation · Purification · Activity

## Introduction

In Eastern Québec (Canada), approximately 11,000 metric tons of snow crab (*Chionoecetes opilio*) are harvested yearly, providing crabmeat for human consumption and generating about 4,000 tons of processing wastes [1]. These wastes or by-products include the

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cephalothorax shell, hepatopancreas, viscera, and physiological liquid (hemolymph). They are typically discarded, which means regulation concerns due to high disposal costs. They are also composted as fertilizers, but this approach adds little value. Snow crab by-products nevertheless constitute a cheap, abundant, and valuable source of various biochemical components (lipids, proteins, and chitin) that can be exploited and converted into more profitable and marketable products such as enzymes, bioactive peptides, oils and biopolymers for food, nutraceutical, biotechnological, or pharmaceutical applications [2, 3].

Collagenases are digestive enzymes in many species of crab (Decapoda). They have been categorized as a special subgroup of chymotrypsin-like serine endopeptidases (EC 3.4.21.32) [4, 5]. Those isolated from the fiddler crab *Uca pugilator* [6–8], king crab *Paralithodes camtschatica* [9–11], greenshore crab *Carcinus maenas* [12], snow crab *C. opilio* [13] and many other crustaceans and fish [14, 15] resemble mammalian collagenases in splitting telopeptide fragments of native collagen and then slowly digesting alpha chains. This activity differs from that of bacterial collagenases, which attack over 200 positions of the collagen triple helix [10]. Unlike vertebrate and bacterial collagenases, which are metalloproteinases, marine serine collagenases do not appear to have any requirement for metal cofactors. Moreover, they are maximally active at alkaline pH, have unusually low protein isoelectric point (pI) and exhibit mixed substrate specificity (trypsin-like, chymoptrypsin-like, elastase-like, and collagenase-like) [7, 16, 17]. Their molecular weight range is 24–36 kDa [8, 12]. Some collagenases from Decapoda find medicinal and cosmetological uses (wound healing and peeling treatments) because they are relatively harmless to humans in comparison with the collagenase from *C. histolyticum*, the bacterium that causes gas gangrene [4, 18, 19]. Serine collagenases also have interesting applications in enzymatic bioprocesses, especially in the food industry, in which enzymatic hydrolysis is preferred to harsh acid or alkali treatments [20, 21]. Collagenases break down tissues and free oil from the protein matrix, enhancing lipid extraction [22, 23]. Those from cold water marine organisms have been shown to be more active than others at low temperatures [24, 25]. This unique property can be exploited advantageously in bioprocesses to prevent the loss of important labile nutrients.

The purification of marine serine collagenases typically involves an initial fractionation step with ammonium sulfate and (or) acetone, followed by chromatographic steps [8, 10, 26–31]. To the authors' knowledge, only one study has involved direct acidic precipitation at a specific pH [12]. For this paper, we evaluated the efficiency of sequential acidic precipitation as a first purification step in the recovery of collagenolytic proteases from snow crab by-products, characterized the activity of the fraction thus obtained and compared it with a commercial protease used for fish protein hydrolysis. We contemplated the feasibility of using this raw material for the production of proteolytic enzymes for bioprocesses.

## Materials and Methods

### Chemicals

Synthetic chromogenic peptides Z-D-Ala-Arg-pNa (ZAANA) and Bz-L-Tyr-pNa (BTNA) were purchased from Peptides International, Inc. (Louisville, KY, USA). N-Succinyl-$(Ala)_3$-pNa (STANA), collagen type I from bovine achilles tendon, ninhydrin, L-leucine,

hemoglobin, phenylmethylsulphonyl fluoride (PMSF), p-tosyl-L-lysyl-chloromethylketone (TLCK), p-tosyl-L-phenylalanyl-chloromethylketone (TPCK), and ethylenediamine-tetra-acetic acid (EDTA) were from Sigma Chemical Co. (St. Louis, USA). All other chemicals were of the highest analytical grade.

## Preparation of Crude Extract

Snow crab by-products were obtained from E. Gagnon & Fils Ltée. (Ste. Thérèse de Gaspé, QC, Canada). They were shipped on ice to the pilot plant of the Aquatic Products Technology Centre (CTPA, MAPAQ, Gaspé, QC, Canada) where they were ground, decanted and centrifuged (11,000×*g*) at 0–4 °C. The supernatant (aqueous phase) was collected and stored at −40 °C in polypropylene bags until use.

Unless otherwise noted, all subsequent procedures were performed at 4 °C. One volume of supernatant was thawed, stirred for 30 min with 2 volumes of 50 mM tricine (pH 8.0) and then centrifuged at 10,000×*g* for 15 min. This supernatant was microfiltered through a 0.45 μm membrane filter and used as the crude extract.

## Sequential Precipitation of Proteins Using Acid, Ammonium Sulfate, or Acetone

Three types of precipitation were compared for the recovery of tryptic activity from snow crab by-products. The crude extract was first brought to pH 5.0 by stirring in glacial acetic acid, followed by centrifugation at 10,000×*g* for 10 min. The pellet was saved and the supernatant was adjusted sequentially to pH 4.5, 4.0, and 3.7, followed each time by centrifuging as above. The precipitates thus obtained were immediately dissolved in a small volume of 20 mM tricine (pH 8.0), readjusted to pH 8.0 using 2 M NaOH if required, microfiltered and stored at −40 °C until analysis.

Precipitation with ammonium sulfate was performed in a similar manner using a 100% saturated ammonium sulfate solution at 0 °C. The crude extract was first brought to 50% saturation and centrifuged, then to 60% saturation and 70% saturation by further addition of ammonium sulfate and centrifuged each time as above. The pellets thus obtained were dissolved in 20 mM tricine (pH 8.0), microfiltered, desalted by diafiltration using a stirred cell ultrafiltration membrane system (10 kDa molecular weight cut-off [MWCO], Millipore Inc., Bedford, USA) and stored at −40 °C.

Precipitation with acetone followed the same scheme at concentrations of 50%, 60%, and 70%. All precipitations were done in duplicate.

## Partial Purification of Collagenases

The acid precipitate found most active was concentrated using a stirred cell ultrafiltration membrane system (5 kDa MWCO). The concentrate was applied to a Superdex 75 pg Hiload 16/60 column (GE Healthcare, Baie d'Urfé, QC, Canada) using an HPLC system (Prep Star 218, Varian, Palo Alto, CA, USA). The column was equilibrated with 50 mM tricine containing 150 mM NaCl (pH 8.0) at a flow rate of 0.5 ml/min. The eluate was monitored at 280 nm using a photodiode array detector (Varian). Eight fractions of 10 ml were collected and stored at −40 °C until analysis. Protein molecular mass was estimated using a gel filtration calibration kit (Ribonuclease A, 13.7 kDa; Chymotrypsinogen A, 25 kDa; Ovalbumin, 43 kDa; Albumin, 67 kDa; Blue dextran, 2,000 kDa, GE Healthcare).

## Protein Determination

Protein content was determined using the Better Bradford assay kit (Pierce, Rockford, IL, USA), which is a modification of the Bradford method [32]. Bovine serum albumin was used as the standard. The assay was performed in triplicate.

## Enzyme Activity Assays

### *Tryptic, Chymotryptic, and Elastolytic Activities*

Synthetic peptides ZAANA, BTNA, and STANA were used to measure tryptic, chymotryptic, and elastolytic activities, respectively [14, 18, 31]. Stock solutions of ZAANA (10 mM) and BTNA (12.3 mM) in N,N-dimethylformamide, and STANA (11 mM) in dimethyl sulfoxide were prepared. Peptide stock solutions (50 μl) were mixed with 2.5 ml of 50 mM Tris–HCl buffer (pH 8.0), pre-incubated at 37 °C for 5 min and 50 μl of sample (enzyme solution) were added, giving a final peptide concentration of approximately 0.2 mM. The reaction was stopped after 60 min of incubation by adding 0.2 ml of 3 M HCl. For blanks, the sample was added after the HCl. Hydrolysis was monitored as the increase of absorbance by liberated p-nitroanilide at 410 nm, using the molar extinction coefficient $\varepsilon_{410}$=9,800 $M^{-1}cm^{-1}$ [31]. Enzyme activity was calculated as (sample absorbance—blank absorbance)$\times 10^6/(9{,}800\times 0.05)$ where $10^6$ is the mole-to-micromole conversion factor and 0.05 is the volume of sample in ml. One unit of activity (U) was defined as the amount of enzyme that cleaves 1 μmol of peptide per hour under the above conditions. Each assay was performed in triplicate.

### *Collagenolytic Activity*

This procedure was based on the methods of Moore and Stein [33] and Park et al. [29] with slight modifications. A reaction mixture containing 5 mg of collagen type I, 1 ml of 50 mM Tris–HCl (pH 8.0) and 0.1 ml of sample was incubated at 37 °C for 1 h. The reaction was stopped by adding 0.2 ml of 50% trichloroacetic acid (TCA). For blanks, the sample was added after the TCA. After 10 min at room temperature, the solution was centrifuged at 5,000×*g* for 15 min. The supernatant (0.1 ml) was mixed with 0.5 ml of ninhydrin solution (2%), incubated at 100 °C for 20 min, cooled to room temperature, and diluted in 2.5 ml of 50% 1-propanol for measurement of absorbance at 570 nm. The concentration of hydrolyzed amino acids was determined from a standard curve of L-leucine. One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol of leucine per hour under the above conditions. The assay was performed in triplicate.

### *Proteolytic Activity on Hemoglobin and Minced Herring*

The procedure using hemoglobin was based on the method of Stocknes [34] with slight modifications. The reaction mixture contained 0.3 ml of 0.1 M tricine (pH 8.0), 0.1 ml of substrate solution containing 1 mg of hemoglobin in tricine buffer and 0.1 ml of sample and was incubated at 40 °C for 1 h. The reaction was stopped by adding 0.5 ml of 5% TCA. For blanks, the sample was added after the TCA. The mixture was centrifuged at 5,000×*g* for 15 min and 0.1 ml of supernatant was assayed for protein content using the BCA protein assay (Pierce), which is based on the method of Smith et al. [35].

For the other procedure, cooked herring minced to a paste (6.3 mg containing approximately 1 mg of protein) was homogenized in 0.4 ml of 0.1 M tricine (pH 8.0) and 0.1 ml of sample was

then added. After incubation at 37 °C for 1 h and addition of TCA, the reaction mixture was centrifuged at 15,000×*g* for 15 min to remove non-hydrolyzed material and 0.1 ml of supernatant was assayed for protein content using the BCA method.

A commercial *Bacillus* protease complex (Protamex® from Novozymes, Bagsvaerd, Denmark) commonly used for industrial proteolytic processes was compared with the studied collagenolytic fractions. For this purpose, an aqueous solution of 0.1 mg/ml of Protamex® was prepared and the reaction was carried out at pH 6.0, which is optimal for this product. Its optimal temperature range is 35–50 °C. For each assay, one unit of activity was defined as the amount of enzyme that hydrolyzed 1 mg of protein per hour under the defined conditions. Each assay was performed in quadruplicate.

### *Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis*

The protein molecular mass profile of the collagenolytic fractions was determined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with the NuPAGE® gel System (Invitrogen, Carlsbad, CA, USA). Electrophoresis was run on a pre-cast Bis-Tris, 4–12% gradient gel (Invitrogen) under denaturing and reducing conditions. Protein bands were stained with Bio-Safe Coomassie Blue G-250 (Bio-Rad Laboratories, Hercules, CA, USA). Standard protein molecular mass markers (EZ-Run™ Pre-Stained Rec Protein Ladder, Fisher Scientific, Waltham, MA, USA) were used.

### *Isoelectric Focusing*

Protein isoelectric point determination was done using Novex® pre-cast vertical pH 3–7 isoelectric focusing (IEF) 5% gels (Invitrogen). After electrophoresis, the gel was fixed in 12% TCA for 30 min and protein bands were stained with Bio-Safe Coomassie Blue G-250 (Bio-Rad Laboratories). IEF Markers 3–10 Serva Liquid Mix (Invitrogen) were used for pI determinations.

### *Gelatin Zymography*

Gelatinolytic activity was assessed using a Novex® 10% zymogram, 0.1% gelatin gel (Invitrogen), under reversibly denaturing and non-reducing conditions at 4 °C. The tested fraction (10 μl) was mixed with 10 μl of inhibitor solution (100 mM PMSF or 10 mM TLCK or 10 mM TPCK or 100 mM EDTA) and incubated at room temperature for 60 min. Controls incubated without inhibitor were prepared with 20 mM tricine buffer (pH 8.0). Each treatment was diluted 1:1 in sample buffer and 20 μl were loaded onto the gel. After electrophoresis, the gel was washed twice with Triton® X-100 (30 min each) to remove SDS and allow enzyme renaturation. The gel was then incubated at 37 °C overnight in an incubation buffer (100 mM Tris–HCl, 5 mM $CaCl_2$, 0.005% Brij-35, 0.001% sodium azide, pH 8.0) and stained with Bio-Safe Coomassie Blue G-250. Enzymes with gelatinolytic activity were detected as clear bands against a blue background of undegraded substrate. The same standard protein marker was used as in the SDS-PAGE procedure.

### *Effects of pH and Temperature on Proteolytic Activity*

Measurement of ZAANA-hydrolyzing activity, as described above, was chosen to evaluate the stability of the proteolytic activities found in the extract of crab by-products. For pH

studies, a Britton–Robinson buffer system [36] was used over the pH range 5–12, instead of Tris–HCl buffer. For temperature studies, the assay was run over a range of 5–70 °C. Enzyme thermal stability was evaluated by pre-incubating the fractions at 40 °C for 0, 30, 60, 90, and 120 min and measuring the residual activity.

*Statistical Analysis*

Data were analyzed by analysis of variance with the least significant difference test at $p=0.05$ using STATGRAPHICS 4.1 software (Rockville, MD, USA).

## Results and Discussion

### Comparison of Sequential Precipitations Using Acid, Ammonium Sulfate, or Acetone for the Recovery of Tryptic Activity

It is well known that serine collagenases from marine organisms display primarily trypsin-like activities measurable using specific synthetic substrates [7, 8, 16]. The ZAANA assay is known for its simplicity, reliability and sensitivity [18] and was therefore chosen as an indicator of the abundance of trypsin-like collagenolytic activity recovered from the crude extract at each fractionation step. Sequential acidic precipitation concentrated the proteases with greater efficiency, producing the highest purification factor and specific activity in the fraction obtained at pH 3.7 (Table 1). This indicates that the recovered enzymes are likely acidic proteins. Precipitates obtained below pH 3.7 contained almost no detectable activity (results not shown). Enzymes were not denatured irreversibly either at this pH when rapidly

**Table 1** Comparison of tryptic activity recovered from snow crab by-products extract by sequential acidic, ammonium sulfate, or acetone precipitations

| Fractions | Total protein mg | Specific activity U/mg | Purification (fold) |
|---|---|---|---|
| Crude extract | 1,024.5 | 13.3 | 1 |
| Acidic precipitation | | | |
| Fraction pH 5 | 276.3 | 17.2 | 1.3 |
| Fraction pH 4.5 | 81.9 | 67.2 | 5.0 |
| Fraction pH 4 | 32.9 | 231.2 | 17.3 |
| Fraction pH 3.7 | 7.4 | 1,212.3a | 91.1 |
| Ammonium sulfate precipitation | | | |
| Fraction 50% | 478.1 | 4.9 | 0.4 |
| Fraction 60% | 251.8 | 14.4 | 1.1 |
| Fraction 70% | 24.2 | 103.0c | 7.7 |
| Acetone precipitation | | | |
| Fraction 50% | 535.9 | 11.8 | 0.9 |
| Fraction 60% | 73.1 | 110.5 | 8.3 |
| Fraction 70% | 17.7 | 656.5b | 49.3 |

Data are expressed as mean values ($n=2$). Highest specific activities obtained for the three types of precipitation were compared using an ANOVA with a LSD test at $p=0.05$. Values with different letters are significantly different

re-dissolved in buffer at pH 8.0, but were more prone to denaturation when precipitated using both other methods. The fraction obtained at pH 3.7 by sequential acidic precipitation was therefore selected for the partial purification, done to enhance the recovery of the tryptic activity.

## Partial Purification of the Collagenolytic Extract

Figure 1 shows the distribution of protein plus tryptic and collagenolytic activities in eluted fractions from the gel filtration of the precipitate extract obtained at pH 3.7. The presence of salt (150 mM NaCl) in the mobile phase had no effect on the tryptic activity (result not shown). Fraction 3 from the eluate contained practically all of the tryptic activity and most of the collagenolytic activity. Chymotryptic and elastolytic activities were also found in this fraction but were undetectable in the others (results not shown). Based on the calibration markers, molecular masses in fraction 3 ranged between 19 and 40 kDa.

The performance of the partial purification procedure is presented in Table 2. It was especially efficient for recovering tryptic activity (purification factor of 1,352.5; yield factor of 110%) but also for chymotryptic, collagenolytic, and elastolytic activities (in decreasing order of catalytic efficiency, based on the substrates used). The yields of tryptic activity in excess of 100% could be explained by the elimination of inhibiting or interfering substances. Previous reports on marine collagenases typically indicate purification factors lower than 30 and yields lower than 10% [8, 14, 28, 29, 40]. In these studies, acetone and (or) ammonium sulfate precipitations were used as a first fractionation step, followed by one or two chromatographic techniques. The purification factor usually increases with the number of chromatographic steps. In the present work, sequential acidic precipitation followed by a single chromatographic step was sufficient to obtain high recovery of proteolytic activity (purification factor and yield). Superdex 75 gel filtration was a good selective technique for the separation of marine collagenases from other proteins because of their narrow range of molecular masses.

Tryptic activity remained predominant throughout the sequence of purification steps, indicating that it is likely the predominant proteolytic activity in snow crab by-products. Serine proteases from crustaceans have been reported to show mixed substrate specificity. In addition to collagenolytic activity, they display esterase and amidase activities towards

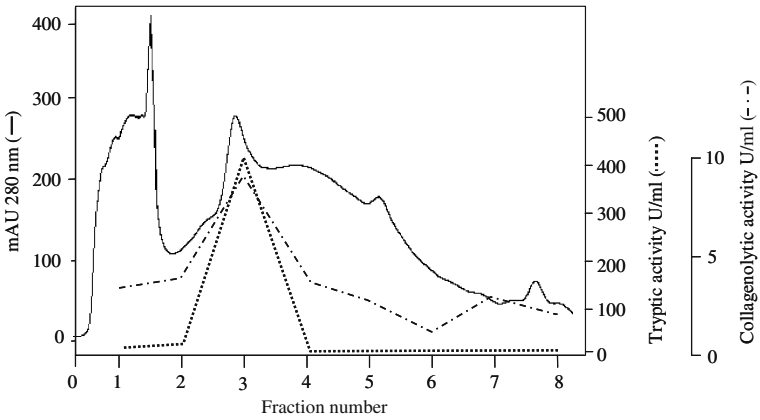


**Fig. 1** Superdex 75 gel filtration of the acidic precipitate (pH 3.7)

**Table 2** Partial purification of proteolytic enzymes from snow crab by-products

| Fractions | Tryptic activity | | | | | Chymotryptic activity | | | | Elastolytic activity | | | | Collagenolytic activity | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Total protein mg | Total U | Specific U/mg | Purification (fold) | Yield % | Total U | Specific U/mg | Purification (fold) | Yield % | Total U | Specific U/mg | Purification (fold) | Yield % | Total U | Specific U/mg | Purification (fold) | Yield % |
| Crude extract | 1,039.8 | 11,920 | 11.4 | 1 | 100 | 4,783.1 | 4.6 | 1 | 100 | 2,599.5 | 2.5 | 1 | 100 | 852.2 | 0.82 | 1 | 100 |
| Acidic precipitation | | | | | | | | | | | | | | | | | |
| Fraction pH 3.7 | 8.5 | 12,435 | 1,462.9 | 128.3 | 104.3 | 4,983 | 586.2 | 127.4 | 104 | 488.3 | 57.4 | 22.9 | 18.8 | 291.5 | 34.3 | 41.8 | 34 |
| Gel filtration | | | | | | | | | | | | | | | | | |
| Fraction 3 | 0.85 | 13,106 | 15,419.0 | 1,352.5 | 110 | 1,849 | 2,175.3 | 472.9 | 38.6 | 796.9 | 937.5 | 375 | 30.6 | 282.8 | 332.7 | 405.7 | 33 |

synthetic low-molecular weight peptides commonly used to assay tryptic and chymotryptic activities [4, 7, 38].

## Electrophoretic Characterization of Active Fractions

SDS-PAGE of fraction 3 from gel filtration revealed a major band at 29 kDa and two others at 33 and 48 kDa (Fig. 2). Molecular masses of the collagenolytic serine proteases from invertebrates have been estimated at similar values and within the range of 25–36 kDa [8, 11, 39, 40, 41]. Klimova et al. [13] found that the molecular mass of the serine collagenase from *C. opilio* was about 25 kDa. Electrophoretic patterns of the acidic precipitate fractions confirmed that the lower the pH, the higher the recovery of the above-mentioned bands, especially the 29 kDa. Since fraction 3 yielded three bands, no enzyme was purified. The measured proteolytic activity may therefore involve more than one enzyme.

The isoelectric points of proteins in fraction 3 were 3.6, 4.2, and 4.6 (Fig. 3). The two bands near pI 4.2 could of course correspond to a single protein, since small differences in the mobility of a single protein have been reported. This could be due to different degrees of phosphorylation, decarboxylation, or glycosylation [42]. The isoelectric focusing pattern confirmed that collagenolytic proteases have a low pI and thus are acidic proteins, in contrast to vertebrate trypsins and chymotrypsins [11, 12, 43, 44].

## Gelatin Zymography of Fraction 3

Fraction 3 displayed gelatinolytic activity in electrophoretic bands corresponding to molecular masses of 22, 29, 39, 48, and 56 kDa (Fig. 4). Among these, only 29 and 48 kDa were visualized on SDS-PAGE gel (Fig. 2). It should be mentioned that gelatin zymography is a very sensitive method that detects nanograms of proteins, in contrast with SDS-PAGE, which detects micrograms. Fraction 3 might thus contain several proteases at trace levels and major collagenases with masses of about 29 and 48 kDa. The white spot around 17 kDa could be the result of gelatin digestion by partially autolyzed but still active proteases. The SDS-PAGE band at 33 kDa (Fig. 2) was not seen on the zymogram, suggesting that the

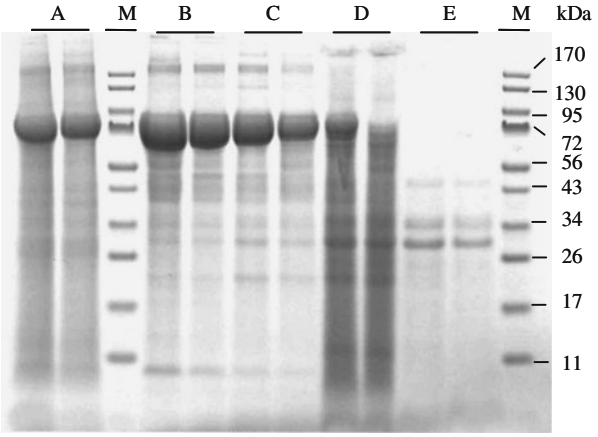


**Fig. 2** SDS-PAGE (4–12% gel gradient, with reducing agent) of the sequential acidic precipitates (pH 5–4.5–4–3.7) and fraction 3 from gel filtration. *A* pH 5, *B* pH 4.5, *C* pH 4, *D* pH 3.7, *E* fraction 3, *M* protein standard. All fractions were assayed at 30 and 20 μg protein, except for fraction 3 (5 and 2.5 μg)

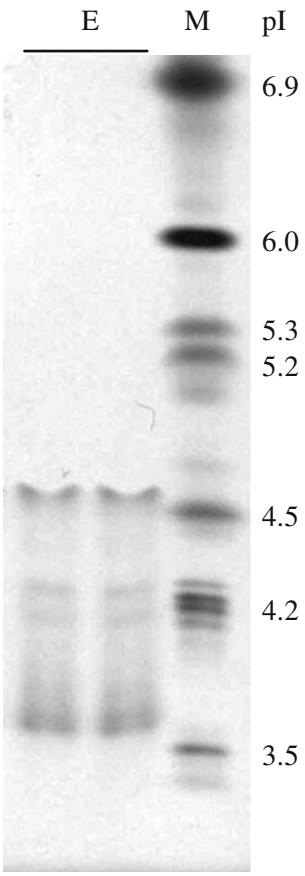


**Fig. 3** Isoelectric focusing (pH 3–7, 5% gel) of fraction 3 from gel filtration. *E* fraction 3 at 10 and 5 μg protein, *M* protein standard

corresponding protein was not a collagenase. After prior exposure to various enzyme inhibitors, proteases from fraction 3 were shown to be sensitive to serine protease and trypsin inhibitors PMSF and TLCK respectively, but insensitive to chymotrypsin inhibitor TPCK (Fig. 4). Several studies on decapod serine collagenases revealed that TPCK had no inhibitory effect on these enzymes [8, 11, 16, 45]. TPCK is an inhibitor of mammalian chymotrypsin. However, decapod serine collagenases, although having chymotryptic activity on the synthetic peptide BTNA, may show some differences from mammalian chymotrypsins in their active site structure, and thus may not be sensitive to TPCK. Meanwhile, EDTA inhibited all of the proteases in fraction 3. In spite of this, trypsin-like collagenolytic activity (based on the ZAANA assay) in each of the acidic precipitates was not influenced by the presence or absence of 10 mM $CaCl_2$ (data not shown). This is consistent with the presence of serine collagenases, which do not appear to have any metal requirement for stability, in contrast with vertebrate and bacterial collagenases, which are metalloproteinases [8, 37]. Since the enzymes did not require calcium for stability, inhibition by EDTA might result from some mechanism other than ion chelation. Moreover, the pH of the EDTA solution added to fraction 3 prior to zymography was 4.5. At this pH, the enzyme is insoluble and its residual activity is almost inexistent.

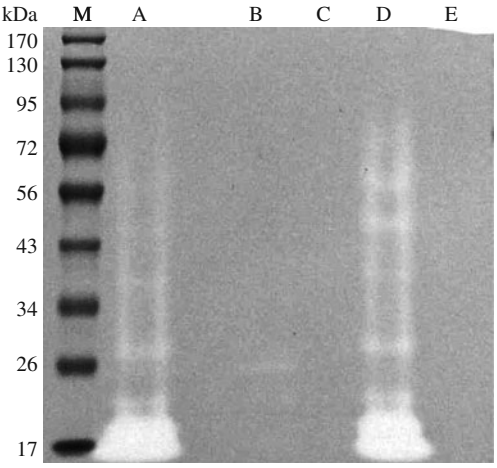


**Fig. 4** Zymogram (10% gel, 0.1% gelatin) of fraction 3 from gel filtration. *M* protein standard, *A* fraction 3 control, *B–E* fraction 3 with protease inhibitors PMSF, TLCK, TPCK, and EDTA

## Effect of pH and Temperature on Proteolytic Activity

The influence of pH and temperature on the tryptic activity of the active fractions (pH 3.7 precipitate and fraction 3 from gel filtration) is presented in Fig. 5. The activity profiles displayed the typical bell-curve shape. The optimal pH was slightly alkaline (8.5–9.0), while the optimal temperature was 40–45 °C. Thermal inactivation began around 50 °C. Similar values have been reported for serine collagenases from crab species *P. camtschatica*, *U. pugilator*, and *C. opilio* [13, 37, 46]. The relatively low temperature optima are due to metabolic and enzymatic adaptation of these species to cold water environments (0–5 °C). Many authors [25, 47–49] have shown that enzymes from cold water marine species were more catalytically active at low temperatures than their microbial, plant, or warm-blooded animal equivalents. The potential for cold water-adapted enzymes in industrial bioprocesses is vast, particularly in the food industry, where lower temperatures help prevent undesirable chemical reactions and preserve important nutrients as well as allowing energy cost reductions [16, 27].

## Thermal Stability of the Enzyme Activity

The thermal stability of the tryptic activity of pH 3.7 acidic precipitate and gel filtration fraction 3 was assayed at 40 °C (the optimal temperature for activity). After 1 and 2 h of incubation, the acidic precipitate retained 99.4% and 98.3% of its initial activity, while fraction 3 retained 91.7% and 82.5%, respectively. The studied collagenases were thus very stable at 40 °C for at least 2 h, corresponding to the duration of a typical enzymatic hydrolysis process. This property is a great advantage in view of industrial bioprocesses.

## Proteolytic Activities on Hemoglobin and Minced Herring

Fraction 3 from gel filtration was about eight times more active than the commercial *Bacillus* protease complex Protamex® at hydrolyzing hemoglobin (Table 3). In contrast, pH 3.7 acidic precipitate was about half as active as Protamex®. The enzymes were tested

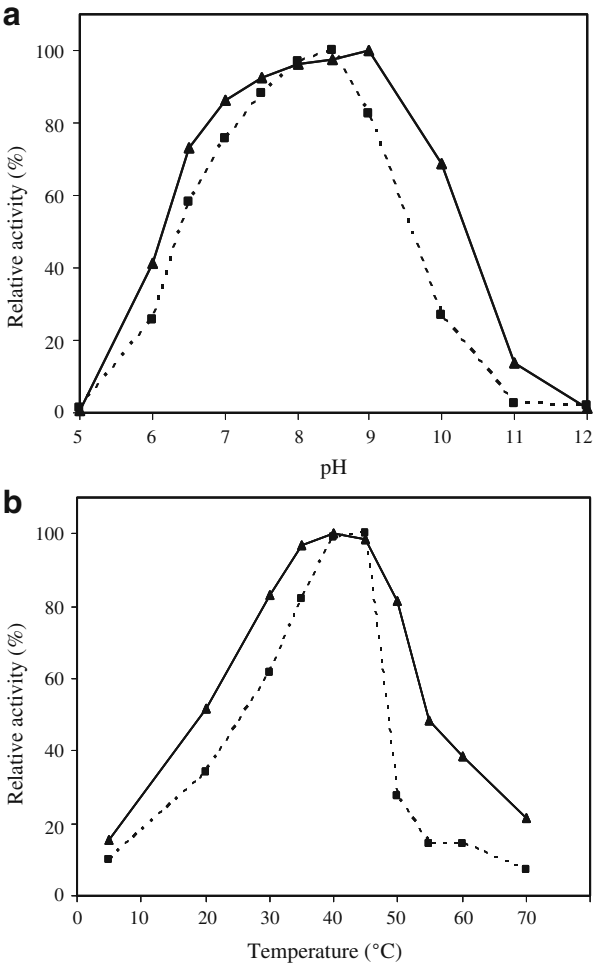


**Fig. 5** Effects of pH (**a**) and temperature (**b**) on the tryptic activity (expressed relative to maximal activity) of the acidic precipitate (pH 3.7) and fraction 3 from gel filtration. *Filled triangle* precipitate pH 3.7, *filled square* fraction 3

**Table 3** Proteolytic activities of the acidic precipitate (pH 3.7), fraction 3 from gel filtration and a commercial *Bacillus* protease complex (Protamex®) on hemoglobin and minced herring

| Fractions | Total protein mg | Specific activities U/mg | |
|---|---|---|---|
| | | Hemoglobin | Minced herring |
| Acidic precipitation | | | |
| Fraction pH 3.7 | 8.5 | 4.6a | 3.1a |
| Gel filtration | | | |
| Fraction 3 | 0.85 | 67.0c | 37.0c |
| Protamex® | 1.0 | 8.8b | 9.7b |

Data are expressed as means ($n$=4). For each substrate, specific activities were compared using an ANOVA with a LSD test at $p$=0.05. Values with different letters are significantly different

with minced herring as well, since this model substrate has significant potential for the development of high-value products (e.g., bioactive peptides, oil extracts) through enzymatic hydrolysis [50]. In this case, fraction 3 was found four times more efficient than Protamex®, while the acidic precipitate was about a third as efficient. This means that serine collagenases from *C. opilio* by-products could be more catalytically active than some commercial proteases under defined conditions. This greater catalytic efficiency could result from the synergism of non-identical specificities of the enzymes (broad range of substrate specificity).

## Conclusions

The simple and efficient partial purification procedure described in this paper allowed the recovery of an active fraction rich in serine collagenases from snow crab by-products. As a first fractionation step, sequential acidic precipitation is very appropriate in view of purification scale-up, because it is simple to manage and requires no organic solvents or subsequent salt removal.

The recovered fraction is a non-microbial source of collagenase with high catalytic activity and stability, and proteolytic efficiency comparable to or greater than that of a commercial protease in a food system. This represents potential advantages in bioprocesses. However, since these proteases come from a natural resource, their activity (specificity, temperature, and pH profile) is prone to variation and this must be considered if they are to be used for industrial applications.

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