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Stability and effects of organic solvents on endopeptidases from the gastric fluid of the marine crab *Cancer pagurus*

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Abstract

Activities of proteolytic enzymes, represented by azocasein digestion (total protease), trypsin, and chymotrypsin from the gastric fluid of the marine crab, *Cancer pagurus*, were evaluated for operational parameters like pH-optimum, thermal stability, and effectors such as metal ions and organic solvents. Total protease activity was $98.5 \pm 4.3\ \Delta E_{366}\ \text{min}^{-1}\ \text{ml}^{-1}$. Trypsin and chymotrypsin activities amounted to $14.7 \pm 1.5\ \text{U ml}^{-1}$ and $197 \pm 59\ \text{U ml}^{-1}$, respectively. The maximum activity of total protease appeared at pH 5–7, that of trypsin at pH 7–9, and that of chymotrypsin at pH 6.5–7. Trypsin and chymotrypsin activities were slightly activated by Ca^{2+}, but were drastically reduced by Zn^{2+}. In the presence of 2-propanol, trypsin activity was enhanced 8-fold at 30 °C. Highest amplification of activity of up to 30-fold, however, appeared below 12 °C. The effects of ethanol and acetone were less distinct. These caused a 2.5-fold increase of activity. Methanol was the least activating solvent.

Activities of all enzymes remained stable for up to 127 days at 5 °C, keeping up to 70% of the initial activity. When stored at 25 °C for 120 days, total protease decreased to about 40% and trypsin and chymotrypsin activities kept more than 60% of initial activity. Zymograms showed five major and several minor proteolytic bands with apparent molecular weight of 20–45 kDa. After long-term storage at room temperature, the major bands remained active, while some bands with minor activity disappeared. This proteolytic enzyme preparation from the gastric fluid of crab seems suitable for biotechnological application at neutral pH and in the presence of organic solvents.

Keywords: Endopeptidases; Gastric fluid; Stability; Organic solvents; *Cancer pagurus*

1. Introduction

Enzyme catalyzed reactions play important roles in various industrial processes such as food production, beverages and brewing, animal feed processing, health care, and cosmetics. Furthermore, the production and processing of pulp and paper, textiles, and leather depend on enzymatic catalysis [1]. The demand of industrial enzymes progressively increased during the last decades. This includes the quantity of enzyme protein as well as the quality and purity of enzyme preparations.

Marine organisms and particularly crustaceans have a significant potential of delivering valuable enzymes. Crustaceans occupy a huge variety of ecological niches. In response to environmental factors they have developed highly specialized biochemical adaptations which are particularly reflected by the immeasurable diversity of digestive enzymes [2–6]. The detailed function of digestive enzymes in crustaceans and particularly their synergetic interplay is still poorly understood. It can be expected that enzymes with hitherto unknown properties and specificities are frequent within crustaceans.

Some proteolytic enzyme preparations from crustaceans already raised attention for dermatological applications. Preparations of collagenolytic enzymes from the Antarctic krill and King Crab species have been successfully evaluated in wound debridement [7–14].

Commercial fishing is the most common source of crustaceans and other marine organisms. However, a major disadvantage of enzyme preparation from fisheries products or bycatches is the lack of quality due to inappropriate handling and storage during transfer. Several crustacean

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species, however, are captured in coastal areas or are farmed as delicatessen. Using these species, transfer to appropriate processing locations could be optimized and highest quality as well as quantity of enzyme preparations could be maintained.

We have chosen for our study the edible crab, *Cancer pagurus* (Decapoda, Brachyura). It is a major target of coastal fisheries on the Island of Helgoland, Germany. Due to its size of up to 1 kg, it can provide high amounts of enzymes. We studied the catalytic properties and the stability of proteinases of the gastric fluid which may be of interest in industrial or biotechnological applications. The parameters tested were pH, temperature, and influence of effectors such as metal ions and organic solvents. Furthermore, the long-term stability of the proteolytic enzymes was studied over a period of several months.

2. Material and methods

2.1. Samples

Adult crabs, *C. pagurus*, were captured around the Island of Helgoland, North Sea (54°11N 7°53E) and were maintained unfed in aquaria. After 2 days, the gastric fluid was drawn from the stomach of individuals through the esophagus with a syringe and a flexible Teflon tube. The fluid was immediately transferred into reaction cups and centrifuged at 15,000 × *g* for 10 min. Aliquots of the supernatant were frozen at −80 °C until further analysis or were lyophilized to determine the water content of the fluid.

2.2. Protein and enzyme assays

Protein was determined with the dye-binding method [15] using a commercial protein assay (BioRad 500-0006). Gastric fluid was diluted 1:100 with de-mineralized water (a. dem.). Fifty microliters of the sample were applied in triplicate into 96-well microplates. Bovine serum albumin (BSA, BioRad 500-0007) of 0.1 mg ml^{-1} was used as a standard.

Total protease activity was determined with azocasein as substrate. The samples (20 μl of a 1:100 dilution in a. dem.) were incubated with 200 μl of 0.1 M Tris/HCl-buffer in a 1.5 ml reaction tube for 5 min at 30 °C. The reaction was started with 50 μl of azocasein solution (1% (w/v) in buffer) and incubated for 30 min at the same temperature under permanent shaking. The reaction was terminated with 500 μl 8% trichloroacetic acid (TCA) and cooling on ice. The caps were centrifuged for 15 min, 15,000 × *g*, and 4 °C, and the absorbance of the supernatant was read at 366 nm in a spectrophotometer. Tests and controls were run in triplicate. Controls received the samples after the addition of TCA. The activity (U = ΔE_{366} min^{-1}) was normalized to 1 ml of gastric fluid (U ml^{-1}) or 1 mg of protein (U mg^{-1}).

Trypsin-activity was determined with the substrate N^{α}-benzoyl-L-arginine 4-nitroanilide hydrochloride (L-BAPA, Merck 1.10754) [16]: 960 μl of buffer (0.1 M Tris/HCl) and 20 μl of sample (1:100 dilution in a. dem.) were incubated for 5 min at 30 °C in a temperature-controlled cuvette holder. The reaction was started with 20 μl of L-BAPA (50 mM in dimethylsulfoxide) and continuously monitored at 405 nm for another 5 min. The substrate concentration in the cuvette was 1 mM. The activity was expressed as U ml^{-1} (=μmol min^{-1} ml^{-1}) using the extinction coefficient ε_{405} = 10.2 l min^{-1} cm^{-1}.

Chymotrypsin activity was measured as described above for trypsin. However, the gastric fluid had to be diluted 1:1000 in a. dem. The substrate used was *N*-succinyl-ala-ala-pro-phe *p*-nitroanilide (SAAPPNA, Sigma S-7388). The activity was also expressed as U ml^{-1} (ε_{405} = 10.2 l min^{-1} cm^{-1}).

2.3. Stability

The thermal profile of enzyme activity was measured at temperatures between 0 and 72 °C. The incubation was performed in a temperature-controlled cuvette holder. Buffer and samples were incubated for 5 min before the reaction was initiated.

In order to determine the thermal stability of proteolytic enzymes, aliquots of the enzyme solution were incubated successively for 5, 10, 20, 40, and 60 min at 0, 20, 30, 40, 50, and 60 °C. Immediately after incubation, enzyme activity was measured as described above.

The long-term stability was determined over a period of 120 days. Samples were diluted 1:100 with dem. water and stored at 5 °C (refrigerator) and at room temperature, respectively. Activity in subsamples was measured in intervals.

2.4. Effects of pH, metals, and organic solvents

Tris/HCl-buffer (0.1 M) was supplemented with 0.01 M NaCl, KCl, $CaCl_2$, $MgCl_2$, $ZnCl_2$, respectively, and adjusted to pH 7.0. The activities of total protease, trypsin, and chymotrypsin were measured in supplemented and, for control, in unsupplemented buffer.

The effect of different pH-values on the enzyme assays was measured in 0.1 M Tris/HCl-buffer + (0.01 M Ca^{2+}) in the range of pH 5–9.

The effect of organic solvents was studied on enzyme assays for total protease, trypsin, and chymotrypsin. The assays were performed in 0.1 M Tris/HCl-buffer pH 7.0 supplemented with 0.01 M Ca^{2+} and increasing concentrations (0–25%) of the following organic solvents: 2-propanol, ethanol, methanol, and acetone (all analytical grade).

2.5. Substrate–gel-electrophoresis

The proteolytic activity of the samples was also monitored by substrate–gel-electrophoresis [17]. Samples were separated by SDS–PAGE (12% T) in a water cooled Hoefer-SE 250 device at 1–3 °C. Running conditions were 15 mA per gel at maximum 300 V. After the run, the gel was incubated

Table 1
Specific activity of total protease, trypsin, and chymotrypsin

	U ml^{-1}	U mg$^{-1}_{prot}$
Total protease[a]	98.5 ± 4.3	5.43 ± 0.61
Trypsin	14.7 ± 1.5	0.76 ± 0.09
Chymotrypsin	196.9 ± 59.3	10.80 ± 2.4

All enzymes were assayed in 0.1 M Tris/HCl. Average and S.D. (n = 5–6).
[a] The activity of total protease was defined as U = ΔE_{366} min^{-1} while the activities of trypsin and chymotrypsin were defined as U = μmol min^{-1} using an extinction coefficient of 10.2 l mmol^{-1} cm^{-1}.

in 100 ml of ice-cold casein-solution (3% in 0. 1 M Tris/HCl, pH 8.0) on an orbital shaker. The casein was allowed to penetrate into the gel for 30 min. Thereafter, the incubation was continued at room temperature for another 60 min. Finally, the gel was thoroughly washed with a. dem. and stained for protein with coomassie-brilliant blue. Bands of activity appeared pale on an otherwise blue-dyed background caused by undigested casein.

3. Results and discussion

3.1. The gastric fluid

The activity of total protease in the gastric fluid amounted to 98.5 ΔE_{366} min^{-1} ml^{-1} while that of trypsin and chymotrypsin amounted to 14.7 and 196.9 U ml^{-1} (Table 1). The volume of gastric fluid that we could obtain from individual crabs depended on their size and amounted to up to 5 ml. The color of the fluid varied from dark olive green to brown and brownish-red. No obvious relation was evident between the color and the investigated characteristics of neither the gastric fluid nor the proteolytic activities.

The average pH of the gastric fluid was slightly acidic at 5.8. Some of the individuals had higher pH values around 6.2. The specific weight amounted to 1033 mg ml^{-1} and thus was similar to the specific weight of seawater (Table 2). The amount of dry matter within the gastric fluid was almost 10% and the protein amounted to 2%. Besides proteins, the gastric fluid contains inorganic salts and emulsifiers [18,19].

3.2. Effect of buffers and metal ions

In general, all buffers that we used were suitable for the measurement of total protease, trypsin, and chymotrypsin activities from the gastric fluid of crab (Table 3). However, Tris/HCl seemed to be most favorable for assays of total protease and trypsin. In contrast, chymotrypsin showed highest activity when assayed in citrate/phosphate buffer. Phosphate containing buffers, however, cannot be supplemented with Ca^{2+} and other metal ions due to the precipitation of phosphate–metal complexes. Therefore, the effect of Ca^{2+} on enzyme activity could only be studied with Tris and imidazole.

Table 2
Overall properties of gastric fluid from the marine crab *C. pagurus* (n = 5–9)

pH	5.8 ± 0.3 (8)
Specific weight (mg ml^{-1})	1033.0 ± 13.7 (6)
Dry matter (%, w/w)	9.7 ± 1.4 (5)
Protein (mg ml^{-1})	21.5 ± 5.1 (9)
Protein (mg g^{-1})	21.1 ± 4.9 (9)

Table 3
Effects of different buffers on the activities of total protease, trypsin, and chymotrypsin of *C. pagurus*

	Total protease	Trypsin	Chymotrypsin
Phosphate	88.8 ± 1.1	102.2 ± 2.2	104.7 ± 12.1
Citrate/phosphate	89.6 ± 0.6	86.2 ± 2.6	128.6 ± 10.2
Tris	104.3 ± 2.6	104.3 ± 1.2	100.8 ± 1.7
Imidazole	98.3 ± 2.0	93.6 ± 0.9	91.3 ± 5.6
Tris + Ca^{2+}	110.2 ± 4.1	117.2 ± 1.5	110.9 ± 4.4
Imidazole + Ca^{2+}	108.8 ± 2.2	93.2 ± 2.7	73.7 ± 10.0

Buffer concentration was 0.01 M, pH 7.0. Activities were calculated in relation to the averaged activity (n = 3).

The effect of calcium was not consistent in our assays (Table 3). It enhanced total protease, trypsin, and chymotrypsin by 6–13% when applied with Tris/HCl. However, when applied with imidazole, an increase of activity was only observed for total protease (10%) while trypsin activity remained unaffected and chymotrypsin activity even decreased for 20%.

Calcium is essential for vertebrate proteases. However, its relevance for the activity and stability of crustacean serine-proteases is still controversial. On one hand it was shown that crustacean trypsins do not require Ca^{2+} and also no effect of Ca^{2+} was observed on purified trypsin-like enzymes [2,20]. On the other hand activating and stabilizing effects of calcium were found on various proteases including serine proteases [21].

Other metals such as Na^+ and Mg^{2+} had no significant effect on enzyme activities in our study. Only K^+ increased the activity of total protease, but not trypsin and chymotrypsin. Zn^{2+}, in contrast, significantly inhibited trypsin and chymotrypsin activity (Table 4).

Data on the effects of Na^+ on serine proteinase are scarce. Na^+ had an activating effect on carboxypeptidases from King Crab [14]. Different to our study, Mg^{2+} inhibited trypsin like activity in shrimp but the negative effects of Zn^{2+} was confirmed [20].

Table 4
Effects of different metal ions on the activities of total protease, trypsin, and chymotrypsin of *C. pagurus*

	Total protease	Trypsin	Chymotrypsin
NaCl	96.8 ± 7.3	99.7 ± 1.5	101.4 ± 11.4
KCl	109.5 ± 2.7	98.8 ± 1.7	95.0 ± 11.5
$CaCl_2$	101.4 ± 3.8	105.0 ± 1.2	116.3 ± 19.2
$MgCl_2$	101.9 ± 9.2	97.0 ± 1.0	105.6 ± 10.1
$ZnCl_2$	Not determined	44.0 ± 5.9	19.1 ± 2.3

Metal concentrations were 0.01 M in 0.1 M Tris/HCl-buffer, pH 7.0. The activities were calculated in relation to the activity of unsupplemented Tris/HCl buffer average and S.D. (n = 3).

The effects of metals on the activities of endopeptidases show a broad variety and demand separate detailed investigations. In our study, the absence of Ca^{2+} was not fatal on enzyme activity as it was influenced to the same degree by the choice of the buffer system. Nevertheless, for standard assays we supplemented the buffer (Tris/HCl) with 0.01 M $CaCl_2$.

3.3. pH-profiles

The pH-profile of total protease as measured with azocasein showed high activity over the range between pH 5 and 7 (Fig. 1a) and thus matching the native conditions. Towards pH 8 the activity decreased slightly. In contrast, trypsin had maximum activity at pH 8 but decreased drastically at pHs below 7 (Fig. 1b). Chymotrypsin showed maximum activity within a narrow range around pH 7. Acid as well as alkaline conditions caused a rapid decrease of activity (Fig. 1c).

It seems surprising that the pH-profiles of activity of both, trypsin as well as chymotrypsin do not exactly match with the pH of the gastric fluid. However, literature data support our results. The gastric pH for some crustacean species were reported to range between pH 5.3 and 6.2 [5,22], the maximum activity of total proteinase appeared in a broad range between pH 5 and 8 [21,23–25] and the maximum of trypsin activity was found at pH 7–8.2 [21,24]. However, also the maximum activity of chymotrypsin ranged between pH 5 and 8 [26]. The latter result confirms, that the lack of chy-

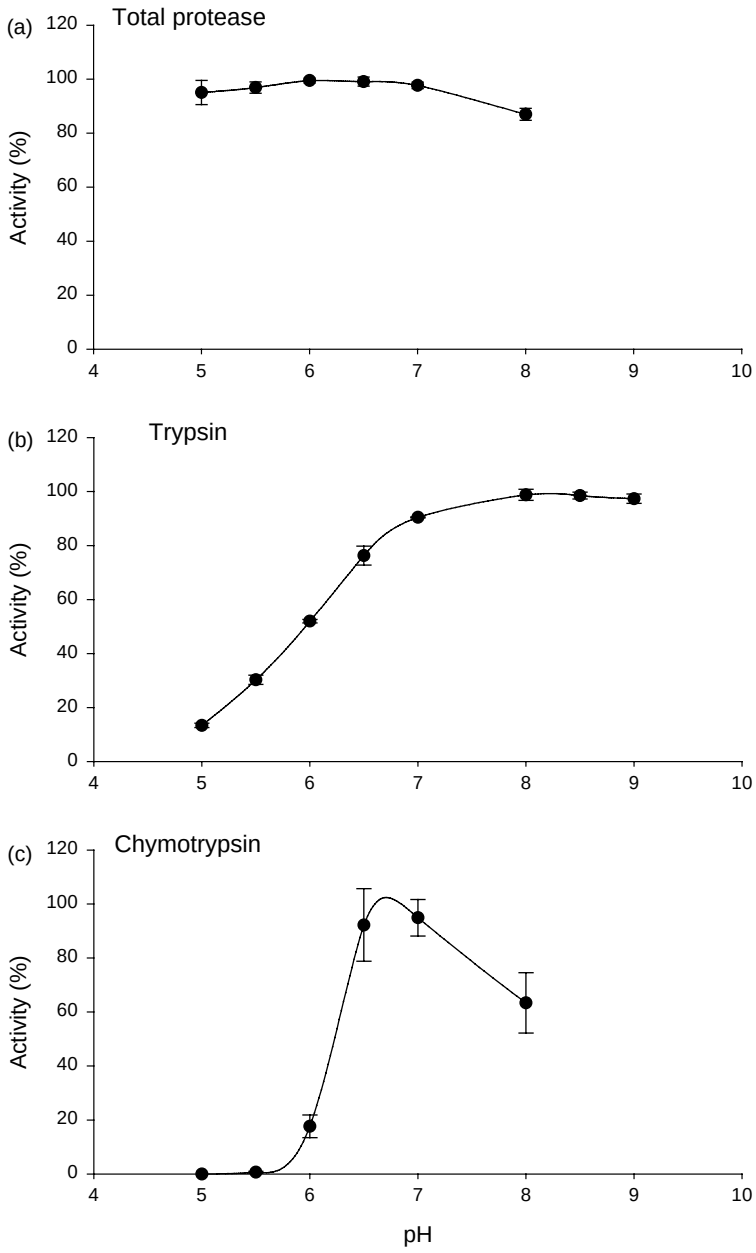

Fig. 1. Effects of pH on the activities of (a) total protease, (b) trypsin, and (c) chymotrypsin incubated with 0.1 mol l^{-1} Tris/HCl and 0.01 M Ca^{2+}.

motrypsin activity at pH lower than 6 in our study is not due to substrate insufficiency in this pH-range, since the author used the same substrate as we did in our study. As concluded earlier the pH of the circulating fluid may vary between sites within digestive organs or the pH in the foregut may rise during feeding towards neutral values allowing for sufficient catalytic turnover [2].

3.4. Effects of organic solvents

Organic solvents affected the performance of the enzymes in different ways. Total protease activity was slightly elevated at 10% concentration of acetone, methanol, and ethanol (Fig. 2a). Above 20% rising solvent concentration caused a continuous loss of activity. At 80% of solvent concentration only about 25% of initial protease activity remained. Different to the solvents mentioned above, 2-propanol caused a continuous decrease of total protease activity.

Trypsin activity, in contrast, was strongly elevated by organic solvents. The most enhancing effect was due to 2-propanol. The activity rose concomitantly towards 8-fold of initial value at a concentration of 40% of 2-propanol (Fig. 2b). Further increase of the 2-propanol concentration caused a continuous decrease of activity. The effects of

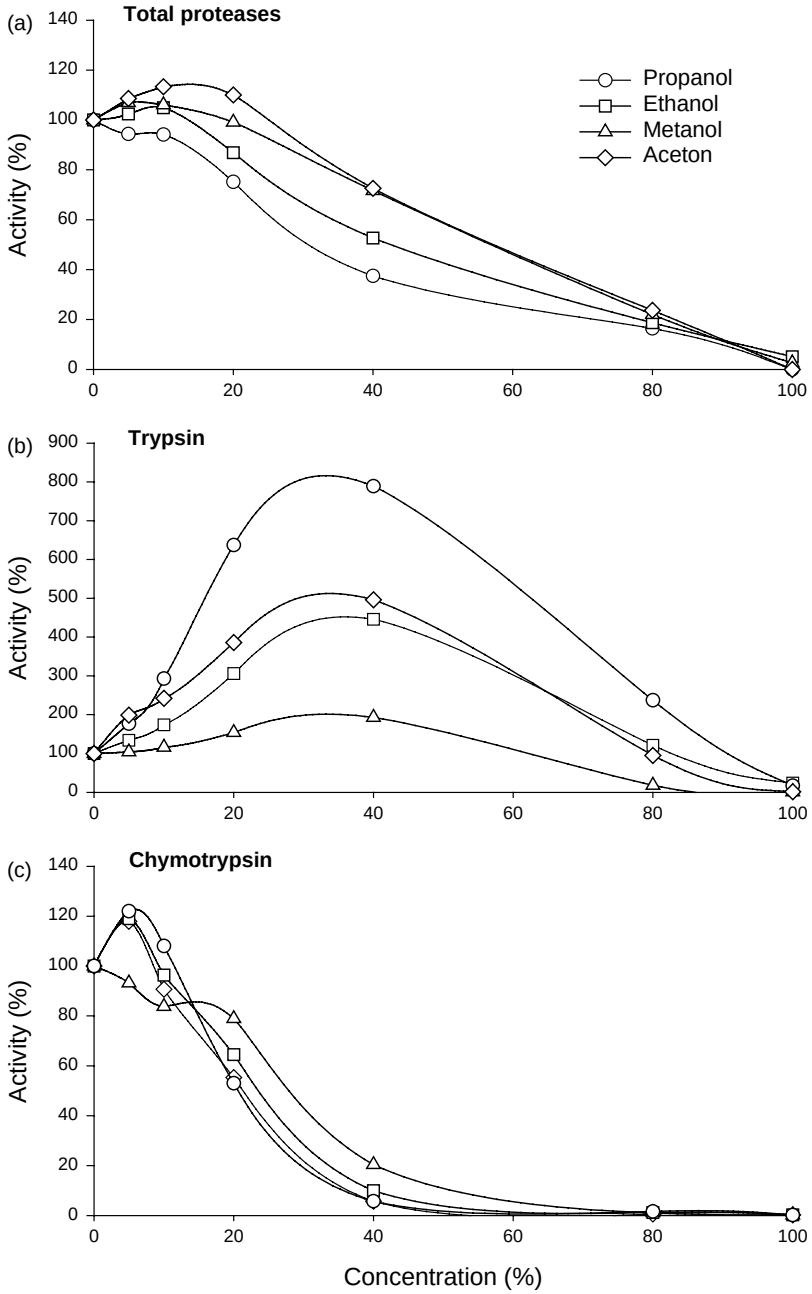

Fig. 2. Effects of organic solvents on the activity of (a) total protease, (b) trypsin, and (c) chymotrypsin at 30 °C incubation temperature.

acetone and ethanol were less conspicuous, although activities increased about 4-fold at 40% solvent saturation. The weakest effect was do to methanol, which only doubled trypsin activity at 40% of concentration. The enhancing effect of trypsin activity that we found with the substrate BAPA was confirmed by using TAME (N^{α}-*p*-tosyl-L-arginine methyl ester, Sigma T-4626) (results not shown).

Chymotrypsin was positively affected at low concentrations (5%) by 2-propanol, ethanol, and acetone, but not methanol (Fig. 2c). Higher solvent concentrations caused a rapid and continuous decrease of activity. At 40% of solvent concentration chymotrypsin activities were below 25% of values at zero concentration of solvents.

Investigations in fish showed that activities of trypsin as well as total proteolytic activity and, to a lesser extent, carboxypeptidase B were enhanced by 1-propanol [27]. However, other enzymes, such as elastase, collagenase, and carboxypeptidase A were inhibited. Since the propanol did not protect the trypsin from inhibition by specific trypsin inhibitors, the authors concluded, that the alcohol directly affect the reactivity of the enzyme rather than by blocking disturbing agents in the extract. The increase of trypsin activity may be due to a change in the enzyme conformation in the more hydrophobic medium. [20].

Organic solvents can be advantageous in various industrial enzymatic processes; e.g. the reaction media used in biocatalytic esterification and trans-esterification contains less than 1% water. The use of organic solvents can increase the solubility of non-polar substrates, increase the thermal stability of enzymes, decrease water-dependent side reactions, or eliminate microbial contamination [28].

Activation of trypsin-like enzymes by organic solvents, especially 2-propanol, seems to be a characteristic feature of crustacean enzymes. In comparison, porcine pancreatic trypsin showed only a 25% increase at 10% of 2-propanol concentration followed by a continuous decrease of activity at higher concentrations (Fig. 3). A propanol concentration of 5% caused a 49% amplification of activities of fish trypsin, a 21% amplification of bovine trypsin, and only a 5% amplification of hog trypsin [27].

3.5. Thermal profiles

We studied the thermal profiles of BAPA-hydrolysis by trypsin and the influence of 2-propanol on the activity pattern. Without 2-propanol, trypsin activity increased continuously from 0 to 50 °C. At 55–60 °C trypsin activity reached the maximum and decreased rapidly at higher temperatures (Fig. 4). The initial activity at 0 °C amounted to less than 1% of maximum activity. This thermal profile matches partly with previous work. In *C. pagurus* maximum trypsin activity appeared at 45 °C and thus 10–15 °C lower than we did in our study [29,30]. However, the residual activity at 0 °C was as low as in our study.

In the presence of 20% 2-propanol, activity increased with temperature more rapidly than without 2-propanol. The maximum activity was reached at 45–50 °C. The activity, however, was about four times higher than that without 2-propanol at 45–50 °C. Initial activity at 0 °C amounted to 3% of maximum activity. In the presence of 40% 2-propanol initial activity at 0 °C was distinctly elevated compared to profiles mentioned before. Activity increased with temperature towards a maximum at 40–45 °C. The activity at 0 °C amounted to more than 25% of maximum activity.

The shape of the thermal optimum curve of enzyme activities is a function of progressively rising turnover rates with temperature on one side and decreasing stability of the enzyme protein on the other side. At maximum activity,

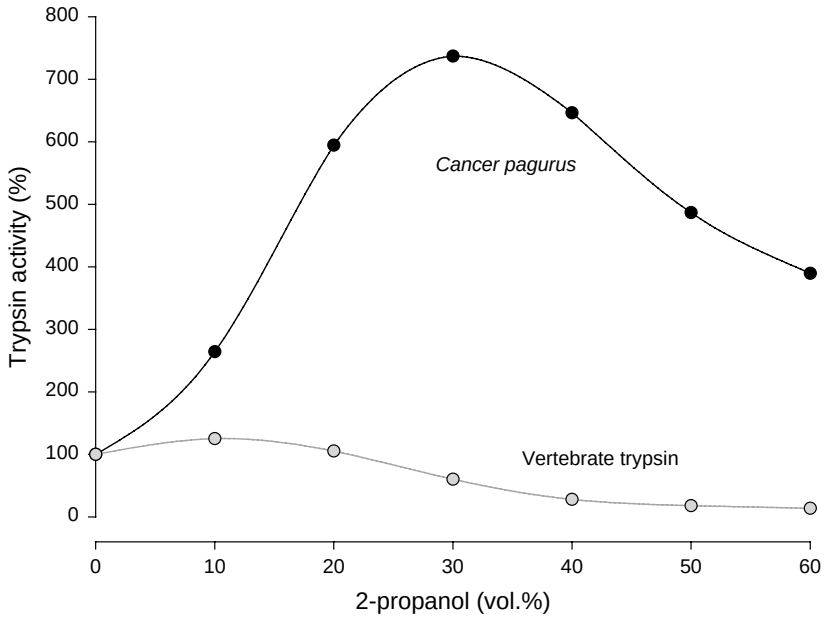

Fig. 3. Effects of different concentrations of 2-propanol on the activity of trypsin from *C. pagurus* and vertebrate trypsin (porcine pancreas).

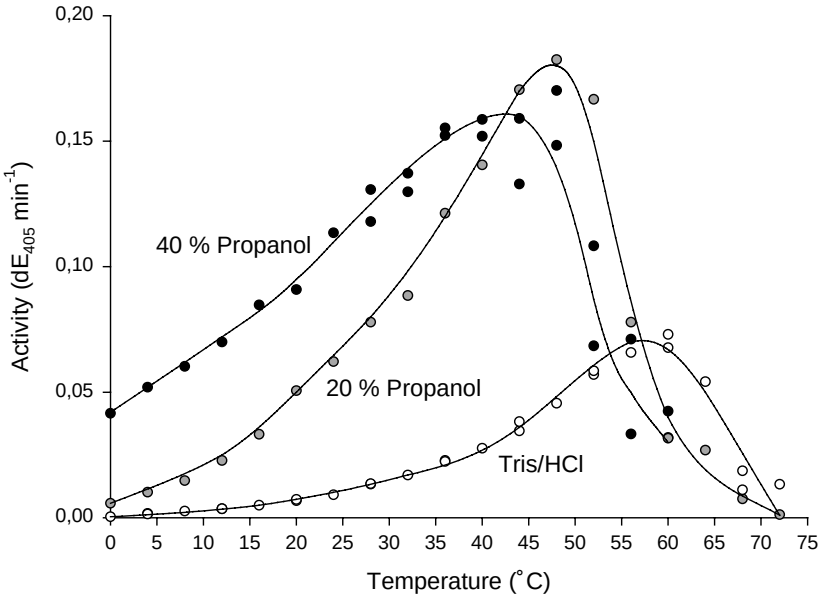

Fig. 4. Effects of different concentrations of 2-propanol on the thermal profile of trypsin-like activity from the gastric fluid of the crab *C. pagurus*.

the effect of thermal enzyme destruction already dominates above the thermodynamic increase of reaction velocity. Further increase of incubation temperature leads to a total loss of activity. 2-Propanol caused an increase of trypsin activity but also decreased the temperature of maximum from about 60 to 40 °C. Accordingly, 2-propanol must have a negative effect on the stability of trypsin at higher temperatures. At lower temperatures, however, 2-propanol significantly enhanced trypsin activity 20–30-fold (4–8 °C). The observed effects of 2-propanol on trypsin activity can be useful in biotechnological applications because it optimizes turnover rates at rate limiting thermal conditions. Accordingly, enzymes can develop higher efficiency at low temperatures.

3.6. Thermal stability

Total protease remained fully active at incubation temperatures of up to 40 °C for at least 60 min (Fig. 5a). At 50 °C, activity slightly decreased toward 70% if initial value. At 60 °C enzymes were almost entirely deactivated after 20 min of incubation. Trypsin activity remained stable at 30 °C (Fig. 5b). However, at 40 °C, activity decreased to 74% of the initial value. At 50 °C, the degradation of the enzyme proceded faster and approached 30% of initial activity after 60 min. At 60 °C, an almost total loss of trypsin activity appeared within 10 min. Chymotrypsin showed higher heat resistance than trypsin (Fig. 5c). No significant loss of activity appeared up to 40 °C. At 50 °C, the activity decreased towards 60% of initial value. Incubation at 60 °C extinguished chymotrypsin activity within 20 min.

Trypsin enzymes reflect the ecophysiological adaptation of *C. pagurus* to temperate environmental conditions [29,30]. Enzymes from tropical crabs which are exposed to elevated temperatures in rock pools or on the plain beach may show a higher heat resistance. Boreal species, in contrast, do not need heat resistant proteins, particularly when they live submerged at temperatures below 20 °C.

There are only few reports on the temperature resistance of chymotrypsin from crustaceans. In langostilla activity of chymotrypsin decreased below 60% of initial activity already after 1 h at 40 °C [26]. Thus chymotrypsin of langostilla is less heat resistant than chymotrypsin of. *C. pagurus.* Langostilla is a benthic–pelagic organism with a preference for temperatures below 16 °C.

3.7. Long-term stability

In the course of the long-term stability experiment the activity of total protease which was stored at room temperature decreased within the first day towards 95% of initial activity (Fig. 6a). During the first week activity did not drop below 90% of initial values. Later on, a continuous decrease was evident towards 40% of initial activity after 90 days. In contrast, the samples stored in the refrigerator maintained more than 70% of initial activity after 90 days. Trypsin, when stored at room temperature, dropped to 80% of initial activity within the first week and remained thereafter at about 70%. At the end of the experiment (127 days) trypsin activity still remained higher than 60% of initial value (Fig. 6b). Again, the samples which were stored in the refrigerator remained fully active for 2 weeks. Thereafter, activity slowly decreased but not below 80% of initial activity. Chymotrypsin activities were more variable than total protease and trypsin. Despite the scatter of the data points, no considerable decrease of activity appeared for more than 30 days in the samples which were stored at room temperature as well as in the refrigerated samples. During the experiment, chymotrypsin activities slowly decreased towards

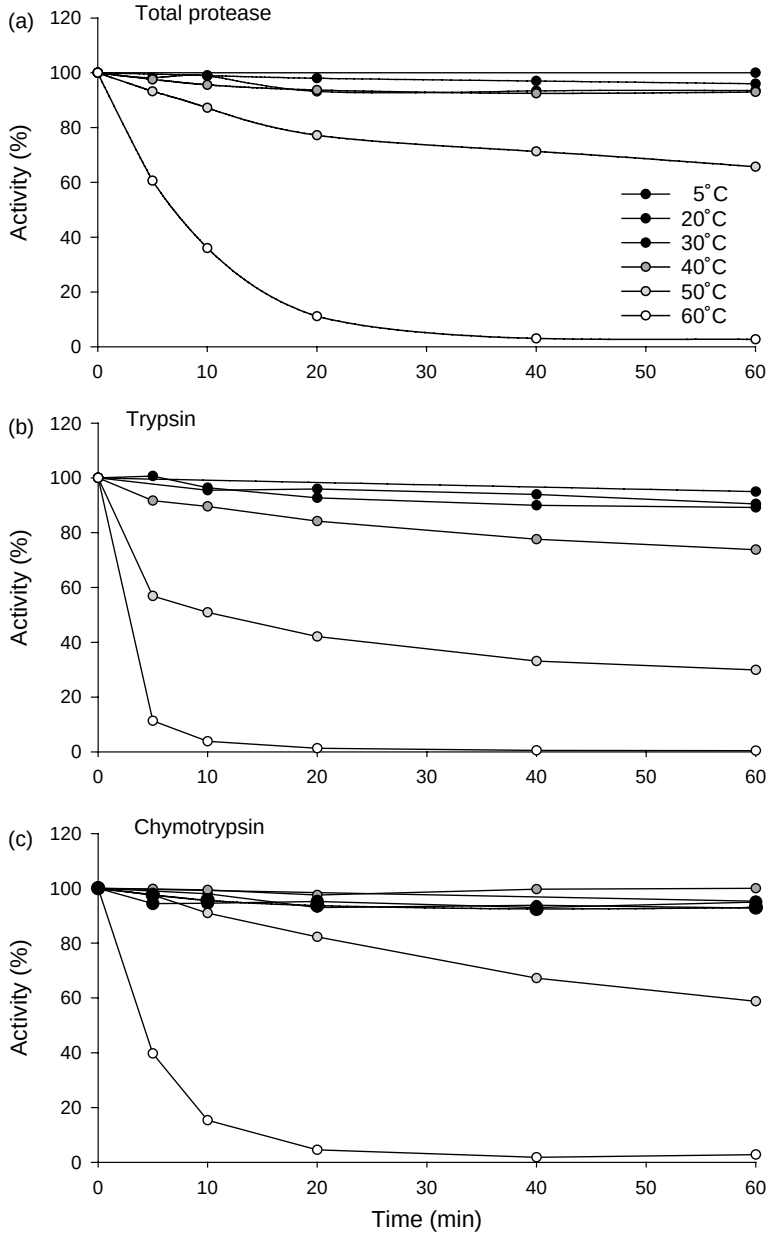

Fig. 5. Thermal stability of (a) total protease, (b) trypsin, and (c) chymotrypsin between 0 and 60 °C and incubation periods up to 60 min. After thermal exposure, enzyme activity was analyzed under standard conditions at 30 °C.

65% of initial value in the samples stored at room temperature. The refrigerated samples remained fully active even at the end of the experiment after 127 days (Fig. 6c).

Long-term stability has not been investigated previously on aqueous dilutions of gastric fluid from crabs. All enzyme activities remained surprisingly stable for up to 120 days although the enzymes were not maintained in a stability-enhancing medium, i.e. supplements of Ca^{2+}, glycerol, or ammonium sulfate, but just in plain de-mineralized water. The experiment had to be terminated after 114 and 127 days, respectively, because the amount of gastric fluid, which was provided for the experiments at the beginning, was almost consumed.

These results seem to be in contrast with our previous experiments on the thermal stability. However, also in the thermal stability experiment, activities remained stable at temperatures up to 30 °C. Rapid degradation first appeared at 40 °C. Apparently, these enzymes are vulnerable to thermal degradation but are highly resistant against autolysis. This property, however, make them suitable for biotechnological applications at low temperatures.

3.8. Zymograms

The substrate–SDS–PAGE showed five distinct activity bands with an apparent molecular weight of 20, 23, 32, 35,

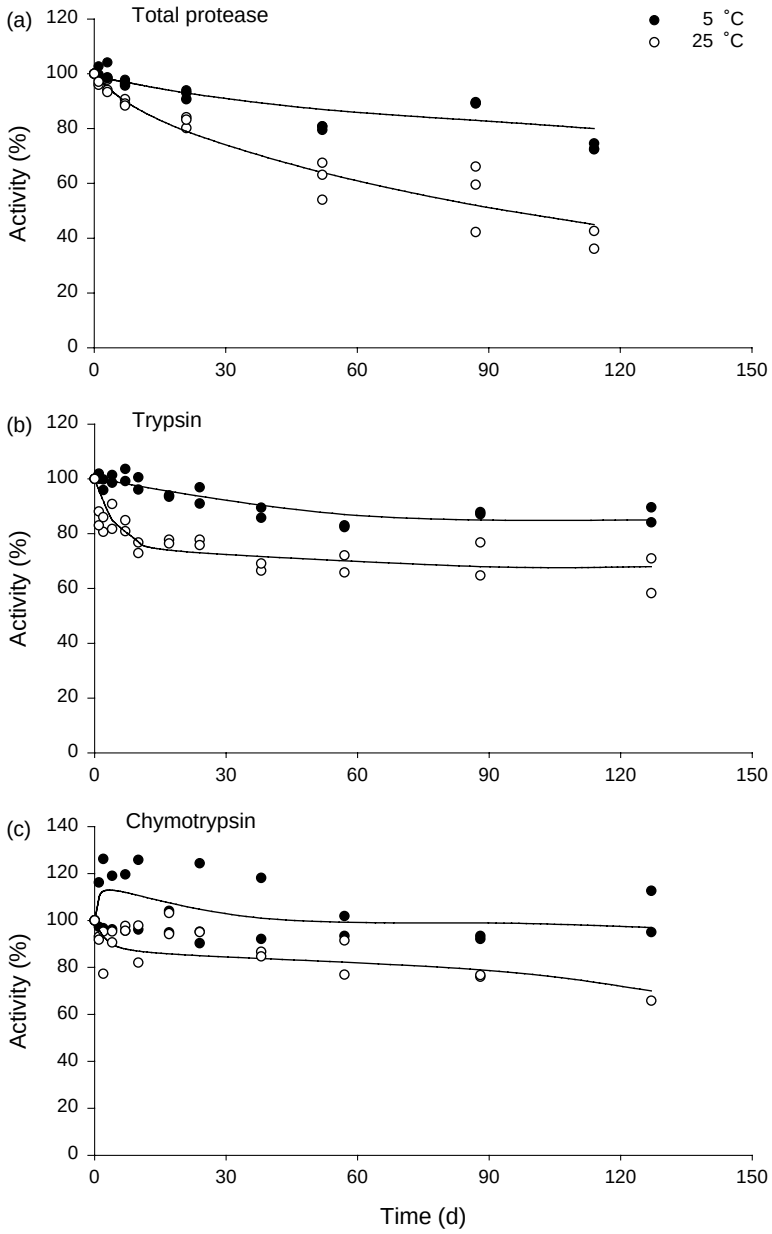

Fig. 6. Long-term stability of (a) total protease, (b) trypsin, and (c) chymotrypsin stored in the refrigerator (5 °C) and at room temperature (25 °C). Assays were carried out under standard conditions as described in the text.

and 45 kDa and several minor activity band (Fig. 7, lanes C_1–C_3). The comparison of zymograms from the beginning and the end of the long-term stability experiment showed that most of the major activity band remained active, when stored at 5 °C (Fig. 7, lanes 5°_1–5°_3). The most distinct loss of activity appeared merely in the 23 kDa band and in most of the bands with minor activity. In contrast, the samples stored at room temperature, showed a clear decrease of activity, particularly in the minor activity bands as well as in the 23 and in the 45 kDa band (Fig. 7, lanes RT_1 and RT_2). The remaining active bands were those with the apparent molecular masses of 20, 32, and 35 kDa.

Zymograms of proteases from gastric fluid of *C. pagurus* have not been published yet. However, in midgut gland extracts from langostilla and crayfish proteases were analyzed of which several could be identified as serine proteases [31]. In the midgut gland of shrimp nine major and several minor activity bands were distinguished [32]. At least two of the major band showed trypsin-like activity while another two exhibited chymotrypsin activity.

In our study, we did not seek to identify single activity bands; however we have evidence from chromatographic analysis that the gastric fluid contains three trypsin isoforms and one chymotrypsin isoform (Saborowski, pers. com.). Furthermore, first analysis shows, that the pattern of zymograms is similar between the midgut gland and the stomach content. This may indicate that all endopeptidases synthesized in the midgut gland are regurgitated into the stomach.

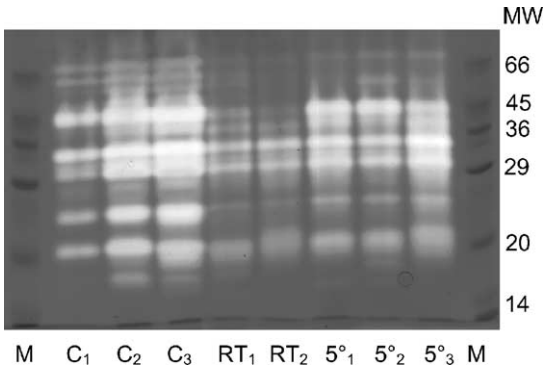

Fig. 7. Zymogram of endopeptidases of the gastric fluid stored at 5 °C and 25 °C for more than 90 days. M = marker, C_1–C_3 = controls, RT_1 and RT_2 = stored at room temperature, $5°_1$–$5°_3$ = stored at 5 °C in the refrigerator.

However, it also has to be considered that this similarity of pattern may be related to the limitation of the method, which is specific to detect only the casein-cleaving endopeptidases in the enzyme preparation. The detailed analysis of the activity bands will be subjected to upcoming investigations.

4. Conclusions

The pattern of proteolytic activity bands differs significantly between crustacean species [17,31]. Accordingly, species or superordinated taxonomic groups express individual enzyme patterns that contribute to a distinct species- or group-specific composition of the gastric digestive fluid and thus to individual catalytic properties. Depending on the size of the species, up to several milliliters of the highly concentrated enzyme mixture can be repeatedly withdrawn from the stomach without injuring the animal, thus exploiting sustained biological resources.

The enzymes that we tested here from the gastric fluid of edible crab *C. pagurus* proved to be highly stable in aqueous solutions and showed highest activities at neutral and slightly alkaline pH. Particularly trypsin was positively affected by low and medium concentrations of organic solvents and at low temperatures. Digestive enzymes can be obtained repeatedly from crustaceans. Therefore, they have the potential to at least partly substitute enzymes from genetically manipulated organisms. However, also waste that derives from shrimp cultures or from fisheries may be used as a source for enzyme extractions when processed adequately. The thoracic parts of shrimps and crabs, which usually are discarded, include the majority of the digestive tract.

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