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Proteolytic and milk clotting activities in extracts obtained from the crustaceans *Munida*

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Abstract

In the present study, individuals of the crustaceans *Munida* have been investigated as a possible source of enzymes to be used in cheese making as an alternative to calf rennet. The crustaceans were blended and the extracts were filtered and tested for their proteolytic activity and milk clotting capability. The optimal values of pH and temperature of the total proteolytic activity determined on azocasein were in the ranges pH 6.5–7.5 and 55–60 °C, respectively. The extracts showed a higher capability to cleave α_{S1}-casein than β- and k-casein. As determined with specific inhibitors, using azocasein or zymography, the extracts appeared to contain serine proteases as well as cysteine and aspartic proteases. Hydrolysis of β-casein gave peptides which were different from those obtained with trypsin or a commercial calf rennet. Moderate milk clotting activity was also present.

The ability to cleave casein, in particular α_{S1}-casein, and the moderate clotting activity, indicate that the extracts of the crustaceans *Munida* could be useful in the dairy industry both for milk clotting, as an alternative or in addition to calf rennet, and for the acceleration of cheese ripening in order to reduce time and costs of storage and maturation of cheese.

Keywords: Crustaceans; Enzymes; Proteases; Milk clotting activity; Cheese making

1. Introduction

The use of enzymes in the food industry is becoming more and more interesting both for the production and for the conservation of food as an alternative or in addition to physico-chemical processes. However, only some of the numerous enzymes proposed for commercial exploitation are currently used in the food industry. This is mainly due to the instability of the enzymes during processing and the prohibitive costs of their production in large amounts. Among the enzymes employed in food processing, proteases are extensively used in food biotechnology, especially in cheese making. Calf rennet, the conventional milk clotting enzyme extracted from the calf's fourth stomach, is widely used in cheese making around the world. However, in many countries, the short life and the high price of calf rennet have encouraged the search for substitutes from sources such as micro-organisms [1], plants [2] and fish [3,4].

The first aim of our study was the detection of alternative sources, readily available at low cost, for the extraction of enzymes of potential interest in cheese making as an alternative to calf rennet. In the present report, individuals of the crustaceans *Munida* (family

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Galatheideae, order Decapodi) have been investigated. They live mainly in the Atlantic and in the Mediterranean Sea. These crustaceans have a relatively high content of proteolytic enzymes, whose applicability in biotechnology is completely unexplored. The crustaceans *Munida* currently have no commercial value.

2. Experimental

2.1. Biological materials

The crustaceans were fished in the southern Adriatic Sea and stored at −20 °C until usage.

2.2. Enzyme extraction procedure

In order to extract the enzymatic activity, the crustaceans were mixed with deionized water at a ratio of 1:2 (w/v) and homogenized in a cold Waring Blendor. The homogenates obtained were centrifuged at 20,000 × *g* at 4 °C for 10 min and the supernatants were filtered on Whatman n.3 filters.

2.3. Protein assay

Protein content was determined according to Bradford [5] using the Bio-Rad reagent in the micro-assay mode and bovine serum albumin as a standard.

2.4. Total proteolytic activity

Total proteolytic activity was assessed by using the spectrophotometric method based on azocasein [6]. Twenty microliters of the enzymatic extracts were added to 0.5 ml of 1.5% azocasein in 50 mM Tris–HCl buffer, pH 7.5 at 25 °C. The reaction was stopped after 15 min by the addition of 0.5 ml of 20% trichloracetic acid (TCA) and centrifugation for 6 min at 6500 rpm (Amicon microcentrifuge MC-13). The supernatants were separated from the undigested substrate and the absorbance at 340 nm of the released dye was recorded. One unit of total proteolytic activity (U) was $\Delta A_{340\,\mathrm{nm}}$/min/ml of extract. The assay included an appropriate blank, in which TCA was added before the substrate. The proteolytic specific activity was reported as U/mg of protein.

2.5. Effect of pH and temperature on proteolytic activity

To find the optimal pH and temperature of the enzymes in the extracts, a curve of activity on azocasein versus pH 3.0–9.0 was obtained at 25 °C. A 50 mM sodium citrate buffer was used in the pH range 3.0–6.0, 50 mM Tris–HCl in the pH range 7.0–9.0. Then a curve of activity as a function of temperature in the range 20–70 °C was obtained at pH 7.5.

2.6. Enzymatic hydrolysis of α_{S1}-, β- and k-casein

The enzymatic preparations were added to at the enzyme–substrate ratio (*E*/*S*) of 1/15 on a protein content basis. Enzymatic extract, 0.5 ml, was added to 5.0 ml of 0.5% α_{S1}-, β- and k-casein (Sigma) in 50 mM Tris–HCl buffer, pH 7.5. Samples were incubated at 32 °C. Aliquots of 0.5 ml were taken at different times. The reaction was stopped by the addition of 0.5 ml of 10% TCA. The mixtures were centrifuged for 10 min at 10,000 rpm and the absorbance of the supernatants was measured at 280 nm. The degree of hydrolysis (DH%) was defined as the percentage of broken peptide bonds, with an assumption that aromatic amino acids were partitioned in an equivalent manner during hydrolysis [7].

2.7. Inhibition studies

To determine the class and specificity of the enzyme, the proteases present in the extracts were incubated for 1 h at 25 °C with specific inhibitors and then added to azocasein as described above. The inhibitors utilised were: 1.0 mM phenylmethylsulphonyl fluoride (PMSF), a specific inhibitor for serine proteases; 1.0 mM *n*-tosyl-*l*-phenylanine chloromethyl ketone (TPCK), a specific inhibitor for chymotrypsin; 1.0 mM *n*-*p*-tosyl-*l*-lysine chloromethyl ketone (TLCK), a specific inhibitor for trypsin; 0.01 mM pepstatine, a specific inhibitor for aspartic proteases; 0.01 mM E-64 (*trans*-epoxysuccinyl-leucylamide-4-guanidinil-butane), a specific inhibitor for cystein proteases; 10 mM 1,10–phenanthroline (1,10 PA) and 10 mM EDTA, specific inhibitors for metallo-proteases. The inhibitors were tested also by zymography on 15% polyacrylamide gels copolymerised with 0.2% casein.

2.8. Mass spectrometry

Each peptide sample of 2.0 μl was mixed with 2.0 μl of saturated matrix solution (α-cyano-4-hydroxycinnamic acid in 50% acetonitrile/0.5% TFA), and 0.5 μl of matrix–peptide mixture was applied on the sample slide and allowed to dry. Spectra were obtained using the Ettan MALDI-ToF Pro Spectrometer from Amersham Biosciences (Uppsala, Sweden).

2.9. Milk clotting activity

The method is based on the visual evaluation of the appearance of the first clotting flakes in 25 ml of substrate: reconstituted Christian Hansen milk powder 11% in $CaCl_2$ (Merck) 0.5 g/l in the presence of 0.5 ml of enzymatic solution. The total milk clotting activity expressed as International Milk Clotting units/g (IMCU/g) of crustaceans was determined in reference to the coagulation times of standard coagulant mixtures of chymosin and pepsin (Chr. Hansen) specially prepared according to norm FIL-IDF 157/92 [8].

2.10. Lactodynamographic analysis

To evaluate the clotting effect on goat's milk, the extracts obtained from the crustaceans were subjected to lactodynamographic analysis, to measure the coagulation time (r), the velocity of the formation of the clot (K_{20}) and the consistency (A_{30}) of the clot at

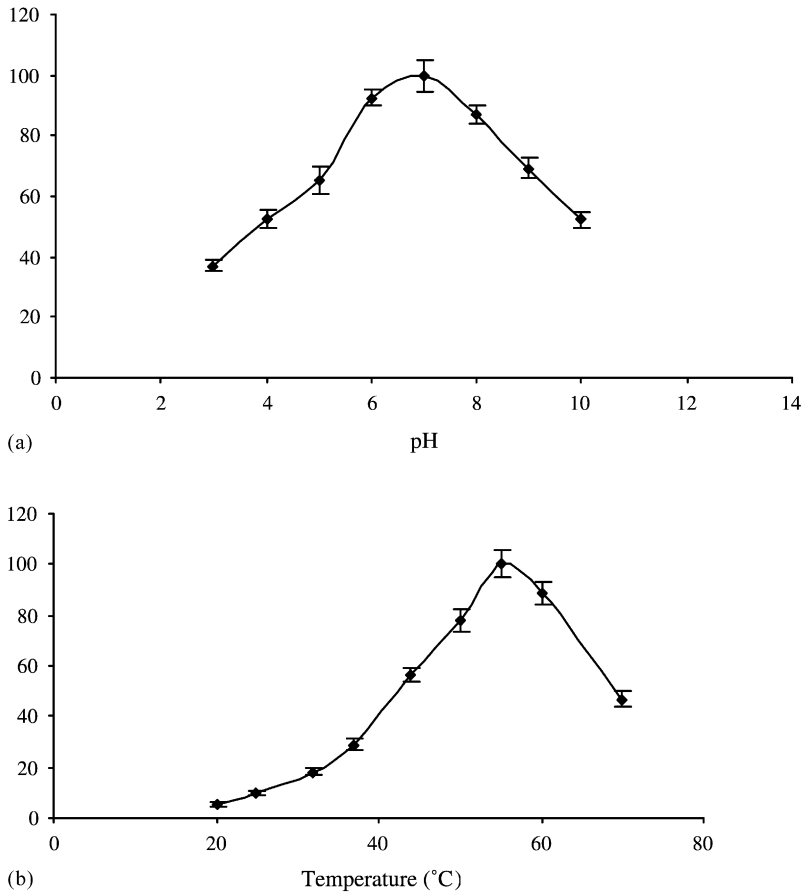

Fig. 1. Effect of pH (a) and temperature (b) on the hydrolysis of 1.5% azocasein in the presence of the extracts obtained from the crustaceans *Munida*.

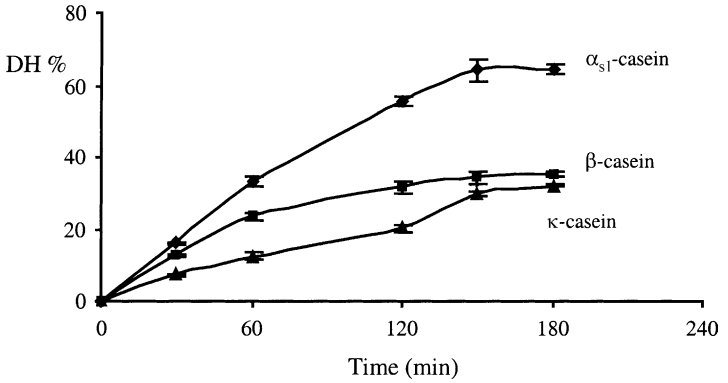

Fig. 2. Degree of hydrolysis (DH%) of α_{S1}-casein (◆), β-casein (●) and k-casein (▲) at 32 °C and pH 7.5 in the presence of the extracts obtained from the crustaceans *Munida*.

predetermined times, using a Foss Italia lactodynamograph with Formawin version 2.0 software. Goats were milked the evening prior to tests and the milk was stored at 4 °C and found to have the following characteristics: pH = 6.6; total nitrogen (Kjeldhal) = 3.5%, fats (Gerber) = 3.9%, acidity (Soxhlet) = 9.2 SH°.

3. Results and discussion

The crustacean extracts showed a protein content of 12.3 ± 1.3 mg/ml and a proteolytic specific activity of 0.08 ± 0.01 U/mg. The optimum of proteolytic activity was pH 6.5–7.5 (Fig. 1a) and 55–60 °C (Fig. 1b).

The degree of hydrolysis of α_{S1}-, β- and k-casein in the presence of extracts from the crustaceans is shown in Fig. 2. The degree of hydrolysis was determined by the analysis of variance ($P < 0.01$). Significant differences between DH% of α_{S1}-, β- and k-casein were found, and the extracts showed higher hydrolysis capability on α_{S1}-casein > β-casein > k-casein.

The results obtained from the studies with specific inhibitors of total proteolytic activity on azocasein are shown in Fig. 3. The inhibition by PMSF indicates the presence of serine proteases, possibly chymotrypsin

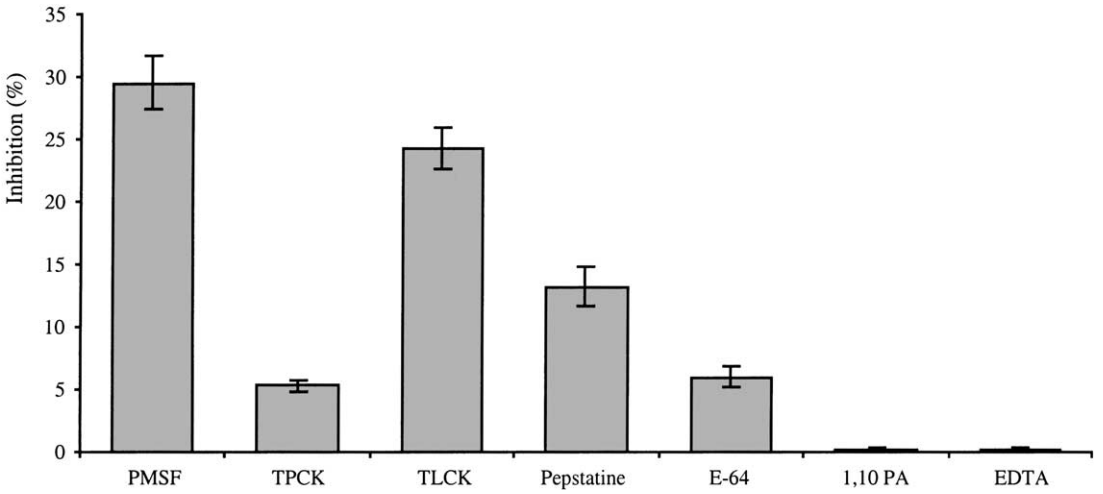

Fig. 3. Effect of specific inhibitors on total proteolytic activity of crustacean extracts.

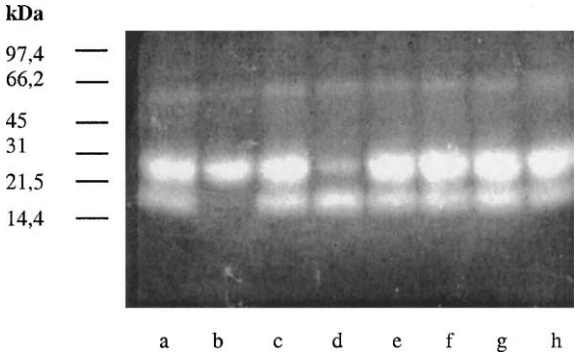

Fig. 4. Zymographic analysis of extracts obtained from crustaceans *Munida* in the absence (control) and in the presence of specific inhibitors (15% polyacrylamide/0.2% casein). Lanes: (a) control; (b) TLCK; (c) TPCK; (d) PMSF; (e) pepstatine; (f) E-64; (g) 1,10 PA; (h) EDTA. Molecular weights corresponding to the activity bands were determined using the low-range standard proteins of Bio-Rad.

and trypsin, since inhibition by TPCK and TLCK, respectively, was found. On the other hand, the inhibition by pepstatin and E-64 suggests the presence of aspartic and cystein proteases. There was no inhibition by 1,10 PA and EDTA, specific inhibitors for metallo-proteases.

Zymography revealed several bands of proteolytic activity in the range of 13–36 kDa (Fig. 4). A reduction in the intensity of some of the white bands was only found in the presence of TLCK (lane b) and PMSF (lane d).

We have compared the effect of the crustacean extracts, trypsin and the commercial liquid calf rennet of Clerici on 1% β-casein in 50 mM Tris–HCl/3 mM $CaCL_2$/pH 7.5 at 32 °C. The reaction was stopped with 10% TCA after 15/30/60 min, and the surnatants obtained after centrifugation were analysed by MALDI-ToF mass spectrometry.

The molecular mass of the peptides obtained by the hydrolytic treatment are shown in Table 1. The results indicate that the cleavage sites are different.

In order to determine the coagulant activity according to the norm FIL-IDF 157/92, the extracts obtained were concentrated five times by ultrafiltration with YM10 membrane. A moderate coagulant activity was found in all the samples tested. On average, the coagulant activity of the extracts obtained from the crustaceans was 150 times lower than the traditional commercial liquid calf rennet of Clerici, and 80 times lower than the common lamb rennet pastes [9]. When stored at −20 °C for 45 days, the extracts preserved about 70% of their milk clotting activity and 65% of their proteolytic activity.

Table 1
The molecular mass of the peptides obtained by the hydrolytic treatment

Crustacean extract	Trypsin	Rennet Clerici
1152.39	830.94	1669.54
1486.34	1138.13	1782.80
1614.56	1271.21	1881.87
1633.46	1384.31	2678.12
1746.62	2911.89	2878.22
2042.82		3027.24
3140.25		

The lactodynamographic analysis of concentrated extracts showed a moderate clotting effect on goat's milk.

4. Conclusions

In this study it is shown that the extracts obtained from the *Munida* crustacea contain proteolytic and milk clotting activity. On the basis of the effects of specific inhibitors on total proteolytic activity, the presence of serine proteases, aspartic and cystein proteases, were ascertained. The hydrolytic activity on β-casein was clearly different when compared with trypsin or calf rennet. The high degree of hydrolysis on casein, in particular on α_{S1}-casein, and the moderate clotting activity found in the extracts of the *Munida* crustacea could be useful in the dairy industry both for milk clotting, as an in alternative or in addition to calf rennet, and for the acceleration of cheese ripening to reduce the time and costs of storage and maturation of cheese. Moreover, understanding the influence of the crustacean extract specificity on casein degradation should facilitate efforts to develop procedures that could improve both the functional properties of cheese and the formation of functional peptides from casein to be used in different fields. In particular, since the peptides obtained from β-casein appear to be specific, it will be important to assess their impact on the organolectic and functional characteristics of cheese. Finally, from a technological point of view, it should be mentioned that these crustacean enzymes

could be very useful in processes carried out in the cold.

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