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Comparative studies on the thermal properties of a trypsin-like protease in two hermit crabs*

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ABSTRACT: The thermal characteristics of a trypsin-like protease were surveyed comparatively in two hermit crabs, *Pagurus bernhardus* (Linné) 1758 from the German Bight, and *Clibanarius striolatus* Dana 1852 from the Western Indo-Pacific. In both enzymes, activity is maximal at a temperature around 50 °C. Compared with *Pagurus*, the protease in *Clibanarius* is characterized by a considerably higher stability at elevated temperatures. Furthermore, the latter is less inhibited by two specific trypsin inhibitors. On an energetical level, distinct differences between the species are displayed. In both species, K_m is strongly affected by temperature; lowest K_m values do not coincide with the mean environmental temperature. The affinity of *Pagurus* protease for substrate at 40 °C is about 17 times that at 0 °C; in *Clibanarius* this factor amounts only to 4.4. At temperatures > 10 °C, activation energy in the tropical species *Clibanarius* is distinctly higher (28.3 $kJ \cdot mol^{-1}$) than in the boreal species *Pagurus* (20.0 $kJ \cdot mol^{-1}$).

INTRODUCTION

The body temperature of ectothermic invertebrates is close to the environmental temperature, and this implies that all metabolic processes are directly regulated by the thermal conditions of the habitat.

Digestive enzymes act as mediators between food uptake and metabolic turn-over; their functioning is controlled by nutritional and environmental conditions. With regard to abiotic factors, temperature plays a decisive role as it regulates all levels of biochemical and physiological processes (cf. Tande, 1988).

Since the comprehensive studies of Somero & Hochachka (1968), Somero et al. (1968), Somero (1969), Hochachka & Lewis (1970, 1971) on the mechanisms of thermal adaptation, especially on cold acclimation, different enzyme systems of poikilothermic and homoiothermic organisms have been surveyed (e.g. Hazel & Prosser, 1970; Hazel, 1972; Smith, 1973a, b; Johnston et al., 1975; Wodtke, 1976; Shaklee et al., 1977; Dittrich, 1990, 1992), and the results cast some doubt on the generality of the mechanism of thermal acclimation.

In Antarctic crustacean proteases, two characteristics point to pronounced adaptation to long-term constantly low temperatures: (1) lowering of activation energies, and (2)

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comparatively high residual activities at temperatures near the freezing point of water (Dittrich, 1990, 1992). However, data on the corresponding counterparts from heat-exposed regions are sparse.

To what extent are tropical crustaceans adapted to constantly high temperatures and how far do they differ from closely related species from cold-temperate regions?

Did the evolutionary adaptation to long-term high temperatures evoke the development of enzymes which are – from the energetical point of view – better suited for life under these conditions?

The present survey is based on the comparison of the properties of the same enzyme in two related species living under distinctly different temperature regimes: (1) *Pagurus bernhardus* from the German Bight, which represents a temperate habitat with annual temperature variations between 0 and about 15 °C. This large species lives in the sublittoral up to greater depths. (2) *Clibanarius striolatus*, a comparatively small species from the Western coast of the Indo-Pacific. The species is very common on rocky shores of Eastern Africa, which represent one of the most heat-exposed marine habitats. During low water, the specimens aggregate in dense clusters on the dry coral ground in full sunlight, waiting in a resting phase for the next high tide before they become active again.

Apart from comparing enzymatic characters, ecophysiological implications are considered critically in order to evaluate the close correlation between environmental conditions and evolutionary adaptations on a biochemical level.

MATERIALS AND METHODS

Adult *Pagurus bernhardus* were dredged from approximately 30 m depth near the island of Helgoland (German Bight, North Sea) in April 1990. Specimens of *Clibanarius striolatus* were collected during low water at the rocky shore about 10 miles north of Mombasa (Kenya) in August 1990.

For analyses, the gastric fluids of freshly dissected digestive tracts of hermit crabs were used. Trypsin-like protease activity was measured according to a method described in detail earlier using BAPA (N-benzoyl-L-arginine-4-p-nitroanilide) as a substrate (Dittrich, 1992).

RESULTS

The temperature optima of degradation activity at pH 8.3, displayed after an incubation time of 10 min, are very similar in both species (Fig. 1). Significant differences become evident in the low-temperature range. In *Clibanarius*, enzyme activity shows a rapid decrease when temperature is lowered and is less than 1 % of maximum at 0 °C. Contrarily, the trypsin-like protease in *Pagurus* is less affected by reduced temperatures; at 0 °C, it still shows about 5 % of maximum activity.

Both enzymes are characterized by a remarkable stability (Fig. 2). When incubated at 40 °C, a temperature which leads to denaturation of some kinds of enzymes, the proteases still display unimpaired activity after 120 min. Even at 50 °C, the protease of *Clibanarius* does not lose any activity after 120 min; by contrast, in *Pagurus* after 40 min of incubation, activity was reduced by more than 20 % and after 120 min, only 9 % residual activity was found. At 60 °C, the *Pagurus* protease loses its activity completely within 30 min, while in

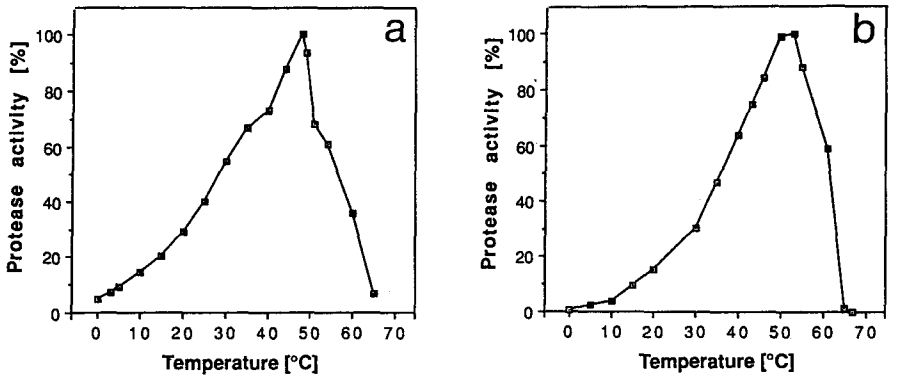

Fig. 1. Temperature dependence of hydrolysis rate of N-benzoyl-L-arginine-*p*-nitroanilide (L-BAPA) at pH 8.3 in (a) *Pagurus bernhardus* and (b) *Clibanarius striolatus*

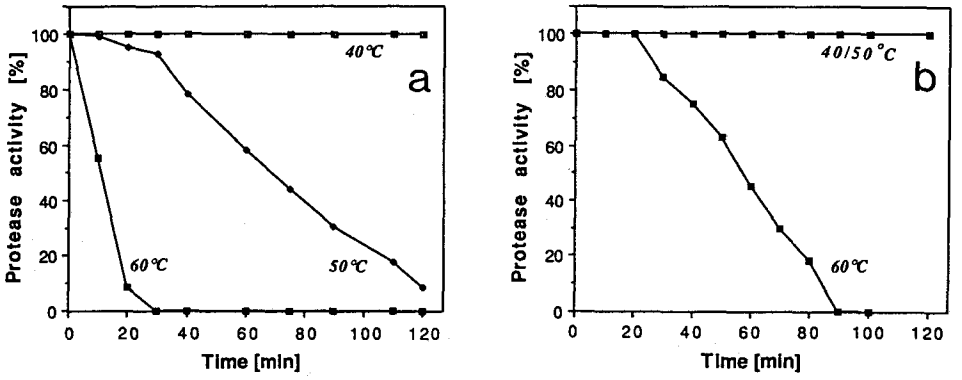

Fig. 2. Thermal stability of the trypsin-like protease in (a) *Pagurus bernhardus* and (b) *Clibanarius striolatus*

the *Clibanarius* protease after the same period of time, approximately 85 % of maximal activity is displayed.

Distinct differences are evident in the affinity of the enzyme for the substrate (Fig. 3), the measure of which is the reciprocal value of the Michaelis constant, K_m. The K_m-values were determined by double reciprocal plots (1/v versus 1/[S]), in which v represents the reaction velocity and [S] the substrate concentration. In *Pagurus*, the pronounced temperature dependence results in a sharp increase of the affinity when temperature is lowered; in contrast, in *Clibanarius*, affinity remains fairly low over the whole temperature range.

A correlation between the level of activation energy (E_a) and mean ambient temperatures is evident (Fig. 4). In *Clibanarius*, a clear discontinuity in the slope does not allow a calculation of the activation energy over the whole assayed temperature range; a distinct change of the incline was found at about 10 °C. At temperatures > 10 °C, the activation energy of the *Clibanarius* protease, (28 $kJ \cdot mol^{-1}$) exceeds the one found in the *Pagurus* protease (20 $kJ \cdot mol^{-1}$) by 42 %.

In the proteases of both species, strong inhibition is achieved after application of

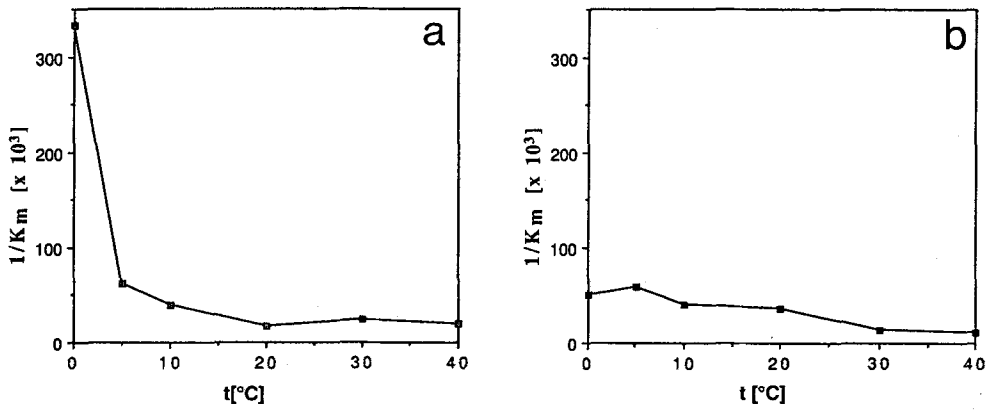

Fig. 3. Temperature dependence of the affinity for the substrate in (a) *Pagurus bernhardus* and (b) *Clibanarius striolatus*

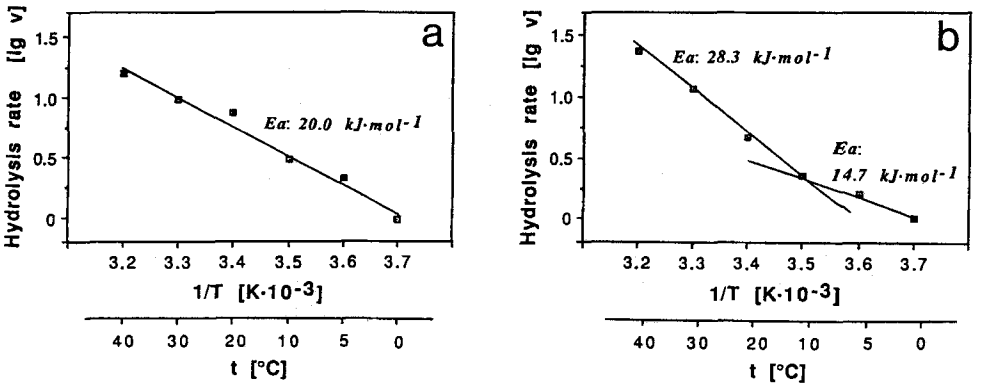

Fig. 4. Arrhenius plots and activation energy of the trypsin-like protease in (a) *Pagurus bernhardus* and (b) *Clibanarius striolatus*. lg v represents the logarithm of the relative hydrolysis rate

soybean trypsin inhibitor (SB) (Fig. 5). However, the degree of inhibition is different: In *Pagurus*, soybean trypsin inhibitor suppresses the activity to about 5 %, and N-tosyl-L-lysine-chloromethyl ketone (TLCK) to about 7 % of maximal activity, whereas in *Clibanarius* only to 30 % and 40 %, respectively. In both species, residual activity was calculated from similar absolute initial data. The final level of inhibition in both species is achieved immediately after application of soybean trypsin inhibitor and 5–10 min after application of TLCK.

DISCUSSION

Recent investigations on Antarctic and tropical crustaceans suggest the evolution of highly-adapted enzymes to cope with extreme – high as well as low – temperatures (Dittrich, 1990, 1992). The present comparative survey of the trypsin-like proteases in two

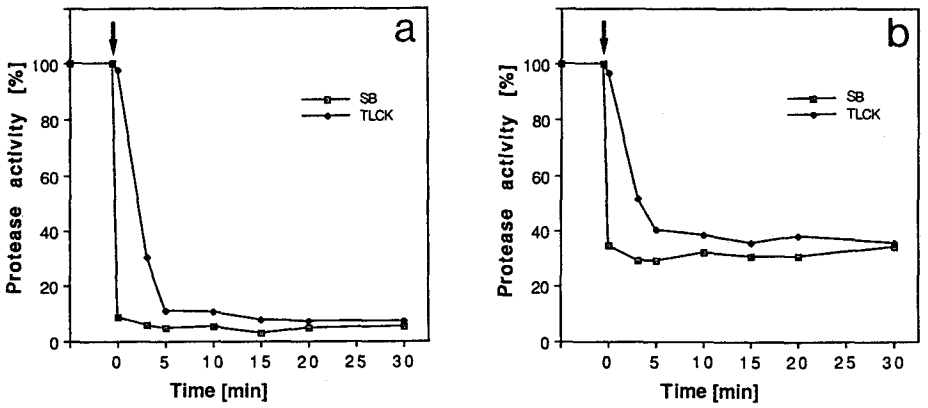

Fig. 5. Time-dependent influence of soybean trypsin inhibitor (SB) and N-tosyl-L-lysine-chloromethyl ketone (TLCK) at 25 °C on the trypsin-like protease activity in (a) *Pagurus bernhardus* and (b) *Clibanarius striolatus*. Arrows indicate addition of inhibitor

hermit crabs from quite different climatic regions serves to support the concept of thermal acclimation.

Distinct differences of the kinetic properties of the trypsin-like proteases are evident between the stenothermal tropical *Clibanarius*, living in a warm marine environment, and the eurythermal temperate *Pagurus* inhabiting a moderate environment.

In contrast to earlier reports (Scholander et al., 1953), temperature optima were found to be independent of environmental temperatures and are – at least in the trypsin-like proteases – at about 50 °C (Spindler & Buchholz, 1988; Dittrich, 1990).

In different enzymes of fish, K_m is lowest at a temperature that coincides closely to that of the habitat and which is therefore defined as "thermal optimum" (Somero & Hochachka, 1968; Baldwin & Hochachka, 1970; Hochachka & Lewis, 1970, 1971). In the present investigation, the proteases were found to display a distinct temperature dependence, increasing with rising temperatures, however, without passing through a minimum at temperatures near the mean ambient ones. The increase of K_m over the tested temperature range exceeds considerably those known e.g. for trout liver citrate synthases (Hochachka & Lewis, 1970).

The decrease of K_m at lowered temperatures is parallel with an increase of affinity for the substrate, which to a certain degree compensates for the decrease of the overall reaction at lowered temperatures. This effect is 3.8 times more pronounced in *Pagurus* than in *Clibanarius* protease. As a consequence, the distinctly higher activity of the *Pagurus* protease in the low-temperature range is probably due to the (relatively and absolutely) higher affinity for the substrate.

The activation energy is an index characterizing the energetical barrier of an enzyme reaction and therefore serves to evaluate its efficiency. In vertebrates, a positive correlation between the body temperature of an organism and the activation energy of its enzymatic reactions has been found (Somero et al., 1968). In pyruvate kinase of different species (Somero & Hochachka, 1968), activation energy is lowest at temperatures approximate to the organism's body temperature. Remarkably, in *Clibanarius* the opposite effect

was observed; at temperatures $>10\,°C$ activation energy (28.3 $kJ \cdot mol^{-1}$) is about 1.9 times larger than at temperatures $<10\,°C$ (14.7 $kJ \cdot mol^{-1}$) and exceeds the activation energy of *Pagurus* by $>40\,\%$. Two possibilities may tentatively explain the discontinuity in the linearity of the regression line in the Arrhenius plot of *Clibanarius*: (1) the activation of different isoenzymes and/or (2) a temperature-dependent change of the conformation of the enzyme protein. Similar effects in Arrhenius plots of membrane bound enzymes are supposed to be due to phase changes in the lipid components of the membranes (Wodtke, 1976). The activation energy found in *Clibanarius* for temperatures $>10\,°C$ (28.3 $kJ \cdot mol^{-1}$) is one of the highest known so far in benthic crustaceans for the hydrolysis of L-BAPA by a trypsin-like protease; comparable values are known only in the euphausiids, *Meganyctiphanes norvegica* and *Euphausia superba* (Osnes & Mohr, 1985; Dittrich, 1992). In the latter, the planktonic mode of life obviously implies a higher metabolic turn-over rate (George, 1985). Compared with the activation energy found in the benthic Antarctic *Chorismus antarcticus* (11.9 $kJ \cdot mol^{-1}$ in the gastric fluid, 13.6 $kJ \cdot mol^{-1}$ in the midgut glands; Dittrich, 1992), in *Clibanarius*, this value is more than twice as high. Thus, a positive correlation between activation energy of the trypsin-like protease and environmental temperature is given.

Obviously, the extremely high environmental temperatures these hermit crabs are exposed to favoured the evolutionary development of enzymes which are characterized by fairly high activation energies. This implies that the enzymes are less easily stimulated to maximum activity. The remarkable reduction of activation energy at lower temperatures can be interpreted by two considerations: (1) As it is not very probable that the species is ever exposed to such low temperatures, a single enzyme which is still very active at low temperatures has no chance to disturb the equilibrium among the different metabolic pathways and it therefore does not exert a negative selection pressure. (2) The species derives from temperate- or cold-adapted ancestors, which – as a means of thermal adaptation – display a reduced activation energy at low temperatures. In the course of evolutionary adaptation to new temperature conditions, new enzymes with fairly high activation energies may have been developed.

Compared with other enzymes which suffer considerable losses of activity at temperatures of about 40 °C (Smith, 1973a, b), both proteases are characterized by a remarkable stability and remain unimpaired even after a 2 h incubation at 40 °C. Obviously, the salts and proteins in the crude extract enhance stability; in assays of purified enzymes which display less stability these compounds are absent (Osnes & Mohr, 1985). At 60 °C, the *Clibanarius* protease displays a remarkable stability and loses only 15 % of activity within the first 30 min; after the same period of time, the *Pagurus* protease is completely inactivated. Obviously, there exists a direct relationship between enzyme stability and environmental temperatures to which the species are acclimated. While the tropical *Clibanarius* hardly experiences temperatures below 20 °C, *Pagurus* may tolerate temperatures near 0 °C.

The different temperature dependence of the two enzymes as well as their activation energies suggest differences in the tertiary structures of their molecules. The present results which support those found in ATPase activity of tropical and Antarctic fishes (Johnston et al., 1975; Johnston & Walesby, 1979) are assumed to be due to an increase of weak bondings and – as a consequence – a tighter molecular structure in cold-adapted species. Rapid inactivation at higher temperatures would be caused by the opening of

just these weak interactions between intramolecular residues. The evolution of structure and function in proteolytic enzymes implies changes in the amino acid sequence; however, this should be subject to further analyses.

Changes in the enzyme structure are of decisive significance in evolution. Highly efficient enzymes, generally ascribed to truly cold-adapted forms (e.g. Somero et al., 1968) are also necessary for the survival of heat-exposed forms. However, while in heat-exposed species selection pressure favours the development of enzymes with a larger stability at higher temperatures, a reduction of activation energy is – besides changes in the patterns of isoenzymes (e.g. Baldwin & Hochachka, 1970; Shaklee et al., 1977) or the lipid composition of their membrane systems (Hazel, 1972) – one of the most effective means by which cold-exposed species cope in the long run with conditions in a low-energy-system.

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