PRELIMINARY EVIDENCE OF AN OXIDATIVE STRESS SYSTEM IN FRESHWATER CRAYFISH *AUSTROPOTAMOBIUS PALLIPES ITALICUS*

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ABSTRACT

Oxidative damage reflects an imbalance between the production of oxidants and removal or scavenging of those oxidants. The antioxidants neutralize via enzymatic and non-enzymatic mechanisms the toxic effects of the free radicals, acting at different levels both within the cell and in the extra cellular fluids. A study on the oxidative defenses under conditions of stress temperature was carried out in the freshwater crayfish *Austropotamobius pallipes italicus*, an endangered species now distributed in scattered areas in Italy and a few Europeans countries. Glutatione peroxidase (GPX) and Glutatione reductase (GR) activity have been measured in the hemolymph, hepatopancreas and muscle tissue by an enzymatic assay in male individuals exposed, for seven days, to three different temperature: 4 ˚C, 15 ˚C and 25 ˚C. Antioxidative enzyme activity was found in the hemolymph, but not in the hepatopancreas and in the muscle tissue. The enzyme activity varied in the hemolymph according to the temperature the animals were exposed to. As far as the GPX is concerned we found the activity only in the hemolymph of animals exposed to the temperature of 15 ˚C. On the contrary, GR activity was detected in the hemolymph at the three considered temperatures. Although the highest level of GR activity was found at 25 ˚C, followed by 4 ˚C and 15 ˚C, it was nonetheless very low and much lower than the level of GPX activity. Very little is known on oxidative stress in crustaceans and virtually nothing in the freshwater crayfish *A. pallipes italicus*. Our data, although preliminary, indicate that the antioxidant defenses provide a useful criterion for the thermal tolerance in studies on natural distribution and suitability of aquacultural environments.

Key-words: *Austropotamobius pallipes italicus*; oxidative stress; glutatione peroxidase; glutatione reductase.

LE STRESS D'OXYDATION DE L'ÉCREVISSE *AUSTROPOTAMOBIUS PALLIPES ITALICUS*

RÉSUMÉ

Les lésions dues à l'oxydation reflètent un déséquilibre entre la production des oxydants et leur élimination. Les antioxydants neutralisent les effets toxiques des radicaux libres par réactions enzymatiques et non-enzymatiques, et agissent soit à un niveau cellulaire soit à un niveau extra-cellulaire. Une étude a été réalisée sur les défenses

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contre l'oxydation de l'écrevisse *Austropotamobius pallipes italicus* soumise à un stress thermique. *A. pallipes italicus* est une espèce menacée, qui vie actuellement dans des zones limitées de l'Italie et d'Europe. L'activité des enzymes – glutatione peroxidase (GPX) et glutatione reductase (GR) – a été mesurée dans l'hémolymphe, dans l'hépatopancréas et dans le tissu musculaire des écrevisses mâles qui ont été stockés à 4 ˚C, 15 ˚C et 25 ˚C pendant 7 jours. On a relevé une activité enzymatique dans l'hémolymphe, mais pas dans l'hépatopancréas ni dans le tissu musculaire. L'activité enzymatique varie dans l'hémolymphe en fonction des températures auxquelles les animaux étaient soumis. L'activité GPX n'a été trouvée que dans l'hémolymphe d'animaux soumis à une température de 15 ˚C. Par contre, l'activité GR a été trouvée dans l'hémolymphe d'animaux soumis aux trois différentes températures (4 ˚C, 15 ˚C et 25 ˚C). Le niveau le plus élevé d'activité GR a été relevé à 25 ˚C, suivi de 4 ˚C et 15 ˚C. Dans tous les cas, le niveau du GR était de beaucoup inférieur au niveau du GPX. On connaît très peu de chose sur le stress d'oxydation des crustacés et de l'écrevisse *A. pallipes italicus.* Nos données, bien qu'elles soient préliminaires, dénotent que les défenses contre l'oxydation fournissent un critère utile pour les études de la tolérance thermique associée à la distribution des espèces dans le milieu naturel et à l'importance des habitats aquatiques.

Mots-clés : *Austropotamobius pallipes italicus ;* stress d'oxydation ; glutatione peroxidase ; glutatione reductase.

INTRODUCTION

In the cellular metabolism of aerobic organisms some enzymatic and non-enzymatic reactions can produce oxyradicals, called reactive oxygen species (ROS). These are formed due to the incomplete reduction of oxygen, which may generate the superoxide anion (O_2-), hydrogen peroxide (H_2O_2) or the hydroxyl radical (OH), as well as other partially reduced molecules. ROS are capable of damaging biological macromolecules such as DNA, carbohydrates, or proteins, thus compromising an organism (HALLIWELL and GUTTERIDGE, 1989; PACKER, 1995). All cells possess enzymatic and non-enzymatic antioxidant defenses to inactivate these damaging molecules. The most important of the nonenzymatic antioxidants is the tripeptide γ-glutamyl-cystein-1-glycin or reduced-glutathione (GSH), seemingly ubiquitous in animal cells. When GSH is present, any ROS will be instantly quenched with immediate formation of oxidized glutathione (GSSG) that is subsequently enzymatically reduced by glutathione reductase (GR) to restore GSH (GS SG + NADPH + H + → 2GSH + NADP +). The reduced form of glutathione (GSH) may be oxidized by H_2O_2 or organic peroxides to oxidized glutathione (GSSG) either spontaneously or via glutathione peroxidase (GPX) catalysis (2GSH + ROOH → GSSG + ROH + H_2O). In vertebrates, GSH originates from different organs; particularly from the liver that releases it into the blood stream. It has been proposed that the higher concentrations of plasma GSH are an indicator of high tolerance to environmental stress (SIEMS *et al.*, 1999).

Recently, GSH concentrations have been detected in *Procambarus clarkii* exposed to toxic stress and in *P. clarkii* and *P. digueti* after variations of photoperiod and light-irradiance (ALMAR *et al.*, 1987; PRIETO-SAGREDO *et al.*, 2000). There are not reports on the GR and GPX activity neither in crayfish nor in crustaceans exposed to environmental stress. However, the dynamic of this system could be a good indicator of the mechanisms allowing the different species to cope with the changing environmental stresses. In general, temperature is a major limiting factor for aquatic poikilothermic animals. Temperature influences the geographic and local distribution, growth, metabolism, reproduction and life history, and has indirect effects through its influence on water chemistry. Freshwater crayfish show different resistance to temperature stress, depending on dissimilar adaptive abilities that imply a certain metabolic strategies. *A. pallipes italicus* survive in low water temperature at near freezing point. Furthermore, the range of the water temperature

of habitats of *A. pallipes italicus* is relatively narrow, up to only 20 °C in summer. Thus, knowledge of the capacity of this species to deal with the changing of the environmental temperature is important for the survival of *A. pallipes italicus* in relation to restocking areas that have been depopulated and affecting its possible distribution range. It has been recently observed that one of the biochemical mechanisms that supports freeze tolerance and anoxia tolerance in vertebrates is an increase in the activities of antioxidant enzymes (STOREY and STOREY, 1990; HERMES-LIMA and STOREY, 1993). Hence, the aim of the present work was to investigate whether both GR and GPX are present in the hemolymph, hepatopancreas and muscle tissue of the freshwater crayfish A. *pallipes italicus* kept at different temperatures.

MATERIALS AND METHODS

Nine adult males intermolt of *A. pallipes italicus* were collected in the river Sassinoro, which belongs to the Morcone municipality, situated in the district of Benevento (Campania region, South of Italy).

Upon arrival animals were transferred to the laboratory, and acclimated in aquaria of approximately 100 l each (three animals for each aquarium), with a recirculating water system and natural circadian rhythm. Water quality parameters such as dissolved oxygen, pH, temperature, ammonia (NH_{4+}), nitrite (NO_{2-}) and nitrate (NO_{3-}), phosphates, total phosphorus, total chlorine, Ca hardness, conductibility, were monitored throughout the experiment. Animals were fed three times a week *ad libitum* and the excess of uneaten food promptly removed. The acclimation period lasted ten days at a temperature of 15 °C. This temperature was chosen as *A. pallipes italicus* grows well and has been shown to actively forage in natural conditions. After the acclimation period the temperature in the first aquarium was gently raised up to 25 °C, in the second was lowered at 4 °C, and in the third was kept at 15 °C. The trial lasted a week. After a week the animals were sacrificed after anesthesia on ice, always at the same time of the day (09:00), to avoid circadian variations. The hepatopancreas and the muscle tissue (from the tail) were removed. Samples were weighted separately, minced and diluted with homogenization buffer (50 mM Tris-HCl, 0.5 M NaCl, 20 mM $MgCl_{2,}$ pH 7.6) at a dilution of 1:2 (wt/vol), then homogenized in ice. Samples were then centrifuged at a 20 000 g, for 1 hr at 4 °C. The supernatant was collected and added with ammonium sulfate twice. The first time at a final concentration of 20%, the second time at a final concentration of 70%. The sample was dialyzed under stirring and with frequent changes. This sample is indicated as cytoplasmic extract.

Hemolymph was extracted from the dorsal region of the cephalothorax and immediately placed in 2.5% perchloric acid for protein precipitation in ice. Samples were centrifuged at 4 °C at 2 000 g for 10 min. Aliquots of the supernatant and cytoplasmic extracts were used for the measurement of glutathione reductase (GR) and glutathione peroxidase (GPX).

Assay of glutathione reductase activity (GR; EC 1.6.4.2)

The assay contained 2,0 mM NADPH in 10 nM Tris-HCl (pH 7.0), 2 mM EDTA, 20 mM GSSG, in 0.2 M potassium phosphate buffer (pH 7.0). The oxidation of NADPH was followed spectrophotometrically at 340 nm. Incubation temperature was consistent with the acclimation temperature.

Assay of glutathione peroxidase activity (GPX; EC 1.11.1.9)

The assay contained 1 mM EDTA, 10 mM GSH, 1,5 mM NADPH in 0.1% Na_2CO_3, GR 2.4 U/ml, 12 mM T-Butil-hydroperosside, 10 mM NaN_3, 1.5 mM H_2O_2 in 0.1 M potassium

phosphate buffer (pH 7.0). The oxidation of NADPH was followed spectrophotometrically at 340 nm. Incubation temperature was consistent with the acclimation temperature.

RESULTS

Antioxidative enzyme activity has been found in the hemolymph of the crayfish *A. pallipes italicus*, but not in the hepatopancreas and in the muscle tissue. The enzyme activity varied in the hemolymph according to the temperature the animals were exposed to (Table 1). As far as the GPX is concerned we found the activity only in the hemolymph of animals exposed at the temperature of 15 °C. On the other hand, GR activity was detected in the hemolymph at the three considered temperatures. Although the highest level of GR activity was found at the temperature of 25 °C, followed by 4 °C and 15 °C, it was nonetheless very low and much lower than the level of GPX activity.

Table 1

GPX and GR enzymatic activity in the hemolymph, hepatopancreas and muscle tissue.

Tableau 1

L'activité des enzymes – glutathione peroxidase (GPX) et glutathione reductase (GR) dans l'hémolymphe, dans l'hépatopancréas et dans le tissu musculaire.

Enzyme	Temperature °C	Enzyme activity* Hemolymph	Enzyme activity* Epatopancreas	Enzyme activity* Muscle tissue
GPX	4	nd	nd	nd
	15	2.15 ± 0.42	nd	nd
	25	nd	nd	nd
GR	4	0.026 ± 0.05	nd	nd
	15	0.015 ± 0.04	nd	nd
	25	0.081 ± 0.09	nd	nd

* nmol/min./gr protein. nd = not detectable.
* nmol/min./gr protein. nd = indéterminable.

DISCUSSION

Research on oxidative stress and antioxidant activity has mainly been developed for vertebrates, especially for species possessing strategies to neutralize oxidative effects (GIL *et al.*, 1987; STOREY, 1996) and those usually exposed to polluted environments (VIDELA *et al.*, 1995; BAINY *et al.*, 1996). The levels of the antioxidant enzymes in a tissue or in the plasma or hemolymph of an organism may vary in response to multiple factors affecting the organism. We have studied the effect of different temperature acclimation on GPX and GR activity in the freshwater crayfish *A. pallipes italicus*. No GPX and GR activity was found in the hepatopancreas of *A. pallipes italicus*. This is in disagreement not only with freshwater fish, in which the liver is the main source of antioxidant enzymes, whose concentration increases following stressing events such as presence of pollutants in the water (AKSNES and NJAA, 1981; RADI *et al.*, 1985; AHMAD *et al.*, 2000), but also with studies carried out in the crayfish *P. clarkii* showing glutathione S-transferase activity fluctuation in the hepatopancreas (ALMAR *et al*., 1987; NIES *et al.*, 1991) and in the crayfish *P. clarkii* and *P. digueti* in which GR activity was found in both the hepatopancreas and the

hemolymph (PRIETO-SAGREDO *et al.*, 2000). We do not have an explanation for this at present, and we cannot exclude that the enzymatic activity could have been lost during the sample preparation procedures (partial purification with ammonium sulfate).

However, the results from the current study indicate temperature stress and a certain degree of a temperature-induced adaptive response in *A. pallipes italicus*. GR activity increased in the hemolymph of animals kept at 4° and 25 °C, suggesting a certain degree of mobilization of the antioxidant defenses in these animals. On the other hand, the value of GR activity in *A. pallipes* is quite low when compared to *P. clarkii* and *P. digueti* (PRIETO-SAGREDO *et al.*, 2000). The greater value of GR activity in the hemolymph of animals kept at 4 °C could be explained with the need of a high level of protection during the exposure to high oxygen concentrations, as occurs when the temperature of the water is low, that can possibly lead to a higher rate of radical production. On the other hand, the high GR activity registered at 25 °C could find an explanation in the fact that investigations in mammalian species show a clear correlation between oxygen radical production and metabolic rate (KU *et al.*, 1993). If this holds true for crayfish, as already demonstrated for cephalopods (ZIELINSKI and PORTNER, 2000), it appears conceivable that a high level of antioxidative response develops due to the high level of metabolic rate at 25 °C.

On the contrary, GPX activity was not detectable in the hemolymph of animals kept at 4° and 25 °C, while was present at 15 °C and showed a much higher value than GR activity. Although speculative, we can hypothesize that crayfish increase their GPX levels, as a preparation for oxidative stress, when under physiological temperature (15 °C). Since GPX is most likely synthesized in the hepatopancreas, as in the crab C*arcinus maenas* (ORBEA *et al.*, 2000), it is released into the hemolymph and therefore detectable.

CONCLUSION

Although, the low level of enzymatic antioxidant defense in *A. pallipes italicus* species is in line with its short life expectancy, it is very low when compared with other crayfish species. This could be a consequence of the habitat characteristics and the low temperature at which *A. pallipes italicus* is acclimated; therefore the very low metabolic rate does not require high levels of enzymatic activity. Above all, very little is known on oxidative stress in crustaceans and virtually nothing in the freshwater crayfish *A. pallipes italicus*. Our data, although preliminary, indicate that the antioxidant defenses provide a useful criterion for the thermal tolerance in studies on natural distribution and suitability of aquaculture environments.

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