CHARACTERIZATION OF ADENOSINE DEAMINASE (ADA) IN HEMOLYMPH OF *Biomphalaria glabrata*

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Received June 23, 2003 – Accepted October 24, 2003 – Distributed May 31, 2005

(With 2 figures)

ABSTRACT

Adenosine is an important signaling molecule for many cellular events. Adenosine deaminase (ADA) is a key enzyme for the control of extra- and intra-cellular levels of adenosine. Activity of ADA was detected in hemolymph of *B. glabrata* and its optimum assay conditions were determined experimentally. The pH variation from 6.2 to 7.8 caused no significant change in ADA activity. Using adenosine as a substrate, the apparent Km at pH 6.8 was 734 μmols.L^{-1}. Highest activity was found at 37°C. Standard assay conditions were established as being 15 minutes of incubation time, 0.4 μL of pure hemolymph per assay, pH 6.8, and 37°C. This enzyme showed activities of 834 ± 67 μmol.min^{-1}.L^{-1} (25°C) and 2029 ± 74 μmol.min^{-1}.L^{-1} (37°C), exceeding those in healthy human serum by 40 and 100 times, respectively. Higher incubation temperature caused a decrease in activity of 20% at 43°C or 70% at 50°C for 15 minutes. The ADA lost from 26% to 78% of its activity when hemolymph was pre-incubated at 50°C for 2 or 15 minutes, respectively. Since the ADA from hemolymph presented high levels, it can be concluded that in healthy and fed animals, adenosine is maintained at low concentrations. In addition, the small variation in activity over the 6.2 to 7.8 range of pH suggests that adenosine is maintained at low levels in hemolymph even under adverse conditions, in which the pH is altered.

Key words: adenosine deaminase, *Biomphalaria glabrata*, hemolymph.

RESUMO

Caracterização da adenosina desaminase (ADA) em Hemolinfa de *Biomphalaria Glabrata*

A adenosina é uma molécula sinalizadora de muitos eventos celulares. A adenosina desaminase (ADA) é enzima-chave para o controle dos níveis intra e extracelulares de adenosina. A atividade da ADA foi detectada em hemolinfa de *B. glabrata* e suas condições ótimas de ensaio foram determinadas experimentalmente. A variação do pH de 6,2 a 7,8 não causou mudança significativa na atividade. O Km aparente foi de 734 μmoles.L^{-1}, usando adenosina como substrato. A maior atividade foi encontrada usando 37°C como temperatura de incubação. As condições de ensaio-padrão foram então estabelecidas como sendo 15 minutos de tempo de incubação, 0,4 μL de hemolinfa por ensaio, pH 6,8 e 37°C de temperatura de incubação. A enzima apresentou atividades de 834 ± 67 μmols.min^{-1}.L^{-1} (25°C) e 2029 + 74 μmols.min^{-1}.L^{-1} (37°C), valores em torno de 40 e 100 vezes maiores que os níveis encontrados em soro de humanos sadios. Em temperaturas superiores, essa atividade cai 20% a 43°C e 70% a 50°C, em 15 minutos. A ADA perde de 26% a 78% de sua atividade quando a hemolinfa é pré-incubada a 50°C, de 2 a 15 minutos, respectivamente. Considerando os altos níveis de ADA

encontrados pode-se inferir que, em animais sadios e alimentados, a adenosina é mantida em baixas concentrações na hemolinfa. Tendo a atividade da enzima permanecido constante ante a larga faixa de pH testada, sugere-se que a ADA possa atuar com eficiência mesmo em situações adversas que determinem variações no pH da hemolinfa.

Palavras-chave: adenosina desaminase, *Biomphalaria glabrata*, hemolinfa.

INTRODUCTION

The nucleoside adenosine is both a metabolic precursor of nucleic acids and an important signaling molecule involved in regulating various physiological processes, mainly due to its inhibitory action on the immune system (Cohen *et al.*, 2002; Okusa *et al.*, 2000; Bouma *et al.*, 1996; Firestein *et al.*, 1995). Adenosine deaminase (ADA) participates in purine metabolism where it decomposes either adenosine or 2'-deoxy-adenosine, producing inosine or 2'-deoxy-inosine, respectively. The physiological roles of ADA are directly related to control of adenosine concentrations in intra- and extra-cellular space. More recently, ADA was found to be implicated in an extra-enzymatic activity (ecto-ADA) by binding to CD26 in the surface of lymphocytes, apparently functioning as an extra-cellular signaling molecule (Franco *et al.*, 1998). This enzyme has become an object of great interest also because ADA deficiency causes severe combined immunodeficiency disease (SCID) (Hirschhorn, 1995); in addition, it is an important marker for tuberculosis (Burgess *et al.*, 2001; Valdes *et al.*, 1995; Valdes *et al.*, 1996; Chalhoub *et al.*, 1996).

This enzyme has been studied in a variety of animals like rats (Blackburn *et al.*, 2000), mice (Singh & Sharma, 1998), dogs (Altug & Agaoglu, 2000), mosquitoes (Ribeiro *et al.*, 2001), flies (Charlab *et al.*, 2000), and marine mollusks (Chen *et al.*, 2000). Recently, our laboratory has characterized ADA iso-forms from caprine tissues (Rodrigues *et al.*, 2000).

Since ADA plays significant roles in the immune systems of several animals, we have directed our study to this enzyme in infection models. One of these is infection of the mollusk *Biomphalaria glabrata* by *Schistosoma mansoni*, which can also infect man, and in some areas causes a significant public health problem (for a review, see Morgan *et al.*, 2001).

Although a large number of papers have focused on *B. glabrata*, nothing has yet been published about ADA in its tissues. This work describes some characteristics of ADA activity in this mollusk's hemolymph, and was undertaken as the first step in investigating the possible role of ADA during infection of *B. glabrata* by *S. mansoni*.

MATERIAL AND METHODS

Collection of B. glabrata *hemolymph*

Animals were obtained from the Biological Sciences Institute, Federal University of Minas Gerais, Brazil, and maintained at 25°C in the Parasitology Laboratory of Faculty of Medicine, Federal University of Ceará, Brazil.

Fed animals with shell diameters of between 15 and 18 mm were used. The hemolymph was obtained by perforating the shells with a collector constructed in our laboratory. This device consists of an eppendorf tube with a rubber stopper through which were two injection needles, one linked by tubing to another needle (to perforate the shell), which in turn is connected to a 10 ml syringe (to create negative pressure in the system).

After collection, the hemolymph was centrifuged at 10,000 x g (4°C). The supernatant was diluted 50 times in cold phosphate buffer (50 mmol.L^{-1}, pH 6.8) and used for ADA determination.

ADA assay

The ADA assay was based on the quantification of ammonium formed by deamination of adenosine catalysed by this enzyme (Giusti, 1974). Due to high levels of ADA activity in the hemolymph, the latter was diluted 50 times in assay buffer (see below) in order to maintain the volumes by the method. Thus, 20 µL of diluted hemolymph corresponds to 0.4 µL of pure hemolymph. The sample assay contained 200 µL of adenosine (2.2 mmol.L^{-1}) and 20 µL of

hemolymph (1:50, v:v), both diluted in phosphate buffer, 50 mmol.L^{-1}, pH 6.8 (assay buffer). This was incubated at 37°C for 15 minutes. The reaction was interrupted by the addition of 600 µL of phenol/sodium nitroprussiate (106/0.17 mmol.L^{-1}), followed by 600 µL of alcaline sodium hypochlorite (11 mmol.L^{-1} NaOCl in 125 mmol.L^{-1} NaOH), and incubated for 30 minutes at 37°C. A blue color developed due to formation of indophenol blue; absorbance at 268 nm was proportional to the concentration of ammonium released from adenosine. In order to discount spontaneous, non-enzymatic adenosine deamination, a control tube containing only adenosine was run in parallel, but the diluted hemolymph (20 µL) was added after addition of phenol reagent. For quantification of ammonium, a standard tube containing a known concentration of ammonium sulphate (200 µL – 75 µmol.L^{-1}) and a blank tube containing only buffer to discount background ammonium in the buffer (200 µL), were submitted to the same conditions as above, including the addition of hemolymph (20 µL) after the phenol/nitroprussiate reagent.

The ADA thermostability was studied in two ways. First, the effect of assay temperature was analyzed by incubating the hemolymph (20 µL – 1:50 v:v) in the presence of adenosine (2.2 mmols.L^{-1}) for 15 minutes from 25 to 50°C. Second, the same effect was analyzed by pre-incubating the hemolymph alone (1:50, v:v) from 0 to 15 minutes at 50°C, after which the ADA activity was measured.

The apparent Km value of ADA in hemolymph was determined based on the curve of the substrate calculated by a Lineweaver-Burk plot. The assay tubes containing 200 µL of adenosine in assay buffer (concentrations from 0 to 1 mmol.L^{-1}) and 20 µl of hemolymph (1:50, v:v, in assay buffer) were incubated at 37°C for 15 minutes. The quantification of ammonium was performed as described above.

The pH variation effect on ADA activity was tested under standard conditions, i.e., adenosine (200 µL – 2.2 mmol.L^{-1}) and hemolymph (20 µL – 1:50, v:v), both diluted in an assay buffer having different pH (from 6 to 8), at 15 minutes of incubation time.

RESULTS

Standard conditions of hemolymph ADA were defined by studying ADA activity dependence on: time of incubation, pH, enzyme concentration, and substrate concentration. Figure 1A shows enzyme activity dependence on incubation time (0-60 minutes). Fifteen minutes was adopted as the standard incubation time throughout the work because it relates to a linear region of the curve and presents reliable absorbance values.

The effect of pH on ADA is presented in Fig. 1B. As can be seen, there were no statistically significant differences between ADA measured in the pH range of 6.2 to 7.8 (Tukey test). However, pH 6.8 was adopted as the standard condition because it approximates ADA optimum pH in other animals and presents reliable absorbance values (Valdes *et al.*, 1996; Rodrigues *et al.*, 2000; Altug *et al.*, 2000).

The gradual increase of hemolymph volume in the assay caused a linear increase in ADA activity, as demonstrated in Fig. 1C. The standard volume of 20 µL (1:50, equivalent to 0.4 µL pure hemolymph in the figure; see methods) was chosen because it lays in the linear region of the curve, affords reliable spectrophotometer readings, and allows enough room for growing volume of substrate in determining the apparent Km (see below).

The Km and maximum velocity of ADA were estimated using a Lineweaver-Burk plot by the variation of substrate concentration from 0 to 2.1 mmoles.L^{-1}, as shown in Fig. 1D. The curve equation ($y = 0.0002042.x + 0.0003723$) gave an apparent Km of 734 µmol.L^{-1} and Vmax of 2686 µmol NH_3.min^{-1}.L^{-1} (s.e.m., $n = 6$).

The increase of incubation temperature from 25 to 37°C caused an increase in enzyme activity of from 834 ± 67 to 2029 ± 74 µmol.min^{-1}.L^{-1} (s.e.m., $n = 8$). However, further increase produced activity decrease (Fig. 2A). Enzyme activity decay was observed when the incubation temperature was 43°C or 50°C (about 20% or 70%, respectively). The adopted incubation temperature was 37°C because it resulted in the highest activity.

The pre-incubation of hemolymph alone at 50°C for up to 15 minutes caused enzyme activity decay. Figure 2B shows that within 2 minutes of pre-incubation at 50°C, the ADA lost about 26% of its activity (from 1733 + 123 µmol.min^{-1}.L^{-1} – s.e.m., $n = 8$, at t = 0 to 1278 ± 119 µmol.min^{-1}.L^{-1}, – s.e.m., $n = 8$, at 2 min) and 78% when it was preincubated for 15 minutes (375 ± 66 µmol.min^{-1}.L^{-1} – s.e.m., $n = 8$).

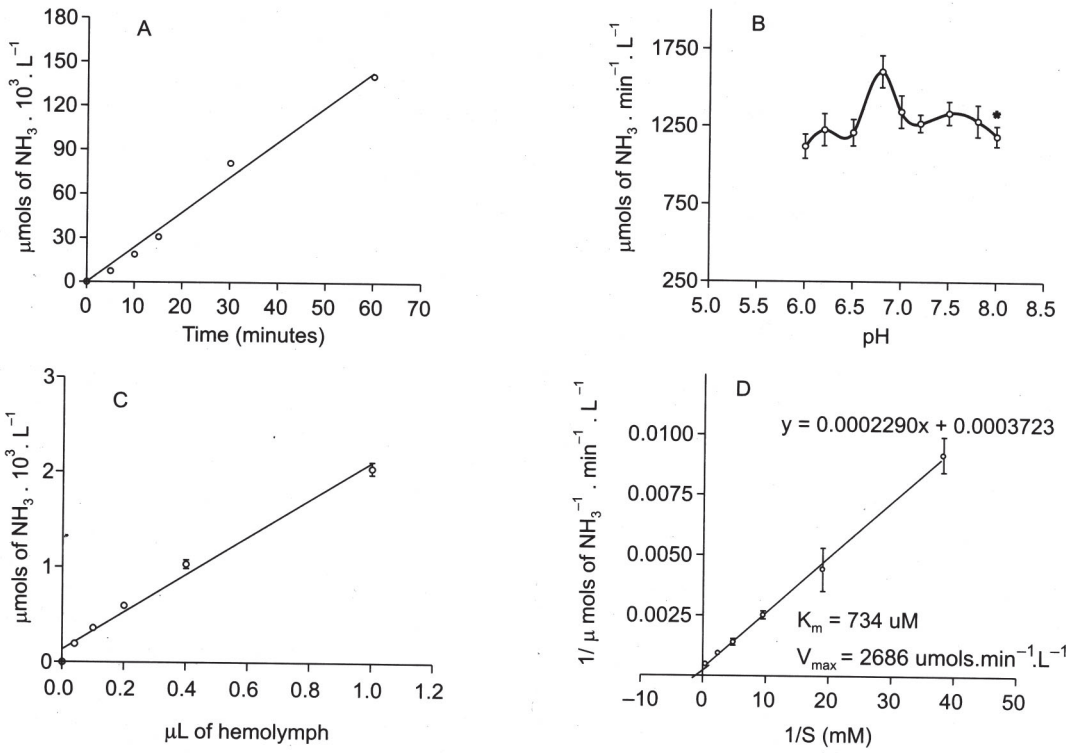

Fig. 1 — Basic parameters of ADA activity in hemolymph of *B. glabrata*. A: variation of ADA activity as a function of incubation time. B: variation of ADA activity as a function of pH. C: variation of ADA activity as a function of hemolymph volume in the assay. D: Lineweaver-Burk plot of ADA activity for the estimation of Km and Vmax. The equation found was: Y = 0.0002290x + 0.0003723. The standard conditions were as follows: pH 6.8, incubation temperature = 37°C, volume of pure hemolymph = 0.4 µL, adenosine = 1 mmol.L^{-1}, 15 minutes of incubation time. The values represent the mean ± s.e.m., n = 6-8.

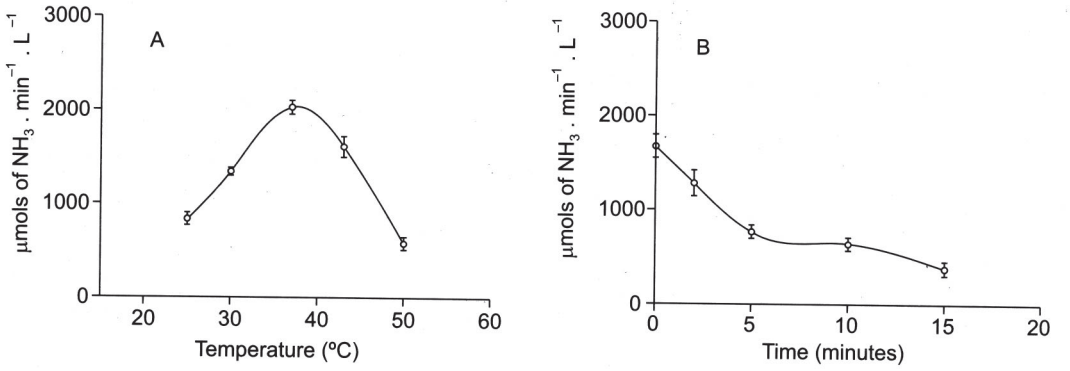

Fig. 2 — Effect of temperature on ADA activity in hemolymph. A: ADA activity as a function of temperature (incubation time – 15 min.). B: ADA activity in hemolymph pre-incubated at 50°C from 0 to 15 minutes. After pre-incubation, ADA activity was assayed under standard conditions. Standard conditions were as follows: pH 6.8, incubation temperature = 37°C, 0.4 µL hemolymph, adenosine = 1 mmol.L^{-1}, 15 minutes of incubation time. The values represent the mean ± s.e.m., n = 8.

DISCUSSION

Under the conditions in which this work was done, the highest activity for hemolymph ADA in *B. glabrata* was observed at 37°C. However, the ambient temperature of the laboratory where the animals were kept was around 25°C, which should be the working temperature of the enzyme in the animal. Using 25°C as the assay temperature, the ADA of the hemolymph presented activity about 40 times higher than ADA in human serum, which is 10-20 $\mu mols.min^{-1}.L^{-1}$ (Giusti, 1974), and other animal sera assayed at 37°C (Sathar *et al.*, 1999; Rodrigues *et al.*, 2000). This could mean that in *B. glabrata* adenosine concentrations in hemolymph are maintained at very low levels.

Even though little or nothing is known about the role of adenosine in *B. glabrata*, by taking into account the role of this nucleoside in man and most animals, one may suggest that even a small increase of adenosine level can trigger important events in this animal. Thus, this level has to be strongly controlled. In addition, the large working pH range of ADA in hemolymph could also be interpreted as a factor involved in effectively controlling adenosine levels, even under extreme metabolic conditions.

It should be added that preliminary results of our laboratory work have demonstrated the existence of ADA iso-forms in the hepatopancreas of *B. glabrata* (Nunes *et al.*, 2002).

The data obtained in this work represents a contribution to the study of ADA characteristics in *B. glabrata*, since it has determined some basic parameters of enzyme kinetics. These include incubation time, pH variation effects, apparent Km and Vmax, besides its thermostability.

The above data has encouraged us to go on with this investigation, and to continue the search for ADA iso-forms in other tissues of the mollusk.

Aknowledgements — The second author thanks the Fundação Cearense de Apoio ao Desenvolvimento Científico e Tecnológico (FUNCAP) for a scholarship. We are indebted to Dr. Vietla S. Rao for reviewing the manuscript.

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