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THE EFFECT OF METAL IONS, OXALATE AND AMINOACIDS ON THE ACTITIVITY OF PYRUVATE KINASE FROM CURIMBATÁ (*Prochilodus lineatus)* EPAXIAL MUSCLE

(O efeito de ions metálicos, do oxalato e de aminoácidos sobre a atividade da piruvatoquinase do músculo epaxial do curimbatá (Prochilodus lineatus))

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RESUMO – Foi realizada pesquisa sobre o efeito de ions divalentes, do oxalato e de aminoácidos sobre a atividade da piruvatoquinase (PK) de purificada do músculo epaxial do peixe tropical, curimbatá (*Prochilodus lineatus).* Essa preparação e PK é ativada por Mg^{2+} e Mn^{2+} sendo sensível à ação de Cu^{2+}, Co^{2+}, Ni^{2+} e Zn^{2+}. Ni^{2+} e Zn^{2+} inibem a atividade da enzima em cerca de 70% e 80% respectivamente. A atividade da presente preparação de PK é inibida em cerca de 85% por oxalato na concentração de 2 mM. A piruvatoquinase do músculo epaxial do curimbatá mostra diversas características de isoenzima M2 mas não é inibida pela L-alanina.

Palavras chave: Piruvatoquinase; Curimbatá; Músculo epaxial.

ABSTRACT – A research has been carried out on the effect of divalent ions, oxalate and aminoacids on the activity of pyruvatekinase (PK) purified from the epaxial muscle of the tropical fish curimbatá *(Prochilodus lineatus).* This preparation of PK is activated by $Mg^{2+}$and Mn^{2+} being sensitive to Cu^{2+}, Co^{2+}, Ni^{2+} and Zn^{2+}. Ni^{2+} and Zn^{2+} inhibit PK activity in about 70% and 90%, respectively. The activity of this PK preparation is inhibited in about 85% by 2mM oxalate. In regard to aminoacids, PK from curimbata's epaxial muscle displays several characteristics of M2 isoenzymes, but is not inhibited by L-alanine.

Key words: Pyruvate kinase; Curimbatá; *Prochilodus lineatus*; Epaxial muscle.

Introduction

Piruvate kinase (E.C. 2.7.1.40. - Adenosine-5'-triphosphate: pyruvate phosphotransferase) controls the outflow of the glycolytic pathway and catalyzes the transfer of a phosphoryl group from PEP to ADP. Pyruvate is formed and ATP is generated concomitantly.

PK has been purified and studied in different tissues of vertebrates and invertebrates. Important studies were done by STAAL *et al*. (1971) with PK from human erythrocytes; PLAXTON and STOREY (1985) with molusc hepatopancreas; ROBERTS and ANDERSON (1985), OCAMPOS *et al*. (1986) and ZAMORA *et al*. (1992) with fish muscle; LOISEAU *et al*. (1993) with helmints; LAZOU and FROSINIS (1994) with *Artemia* embrios and PONTE SUCRE *et al*. (1993) with *Leishmania mexicana amazonensis*.

Allosteric properties of PK are important on the control of glycolysis regulation. They are dependent of cooperative effects, and of the action of allosteric inhibitors.

All phosphotransferases nucleotide dependent need one divalent ion. Pyruvate kinase from several tissues need one monovalent and one divalent ion, almost always K^{+} and Mg^{++} (BOYER, 1962; MUNDAY *et al*., 1980).

Several authors (RANDALL and ANDERSON, 1975; OCAMPOS *et al*., 1987; ISANI *et al*., 1994) showed the inhibitory effect of divalent ions (Ca^{++}, Co^{++}, Cu^{++}, Be^{++}, Zn^{++} on PK from fishes.

Several authors show that the aminoacid effect on PK isozymes change with the species. Avian and mammalian PK of the L type are activated by Fru-1,6-P_2 and inhibited by MgATP or L-alanine. However, PK of the M2 type is less sensitive to activation by Fru-1,6-P_2, but is inhibited by MgATP, L-alanine, and L-phenylalanine. M1 isoenzyme is less sensitive to regulatory influences (MUNDAY *et al*., 1980; INAMURA and TANAKA, 1982; ROBERTS and ANDERSON, 1985; ENGSTRON *et al*., 1987).

OCAMPOS *et al*. (1987) showed that L - alanine activates PK from striated muscle of *Mugil lisa* and *Chaetoditerus faber*. L-alanine and L-phenylalanine inhibit PK of *Anguila*

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rostrata; Carassius carassius and Oncorhynchus kisutch (JOHNSTON, 1975; GUDERLEY and CARDENAS, 1981; ROBERTS and ANDERSON, 1985).

ZAMORA (1989) verified that PK from ice-fish muscle was inhibited by MgATP and sodium oxalate and that this inhibition was partially reversed by MgATP.

In this report we wish to present results to improve the understanding of the kinetic properties of PK from epaxial muscle of curimbata *(Prochilodus lineatus*).

Materials and Methods

Materials. Adenosine-5'-diphosphate; adenosine-5'-triphosphate; DL-dithiothreitol; ethylenediaminetetraacetic acid; fructose-1,6-bisphosphate; lactic dehydrogenase; nicotinamide adenine dinucleotide, reduced form; phospho(enol)pyruvate; pyruvate kinase, bovine serum albumin, L-alanine, L-phenylalanine, phosphocellulose coarse mesh, red agarose.

Biological Material. Pyruvate kinase (PK) was obtained from curimbata (*Prochilodus lineatus*) epaxial muscle reared in the Aquiculture Training Research Center/Brazilian Institute of Environment and Renewable Natural Resources (CEPTA/IBAMA), in Pirassununga, SP, Brazil.

The muscle was obtained by dissection, washed in physiological solution at 4°C, and frozen at -20°C.

Purification. Muscle was minced and homogenized in 33 mM phosphate buffer (pH 6.5) containing 1 mM $MgCl_2$, 0.1 mM DTT (1 g tissue: 9 ml of buffer). The homogenate was centrifuged at 12,000 x g for 30 min, at 4°C. The pellet was discarded.

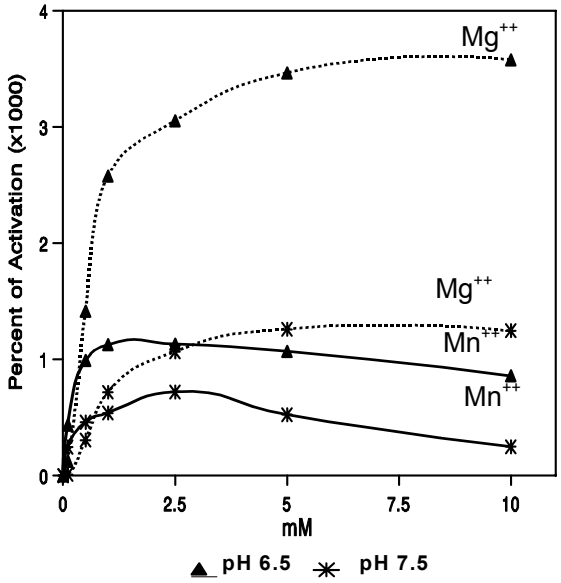

FIGURE 1 – EFFECT OF Mg^{+2} AND Mn^{+2} ON PK ACTIVITY OF CURIMBATA EPAXIAL MUSCLE BETWEEN pH 6.5 AND 7.5 AT 37°C. THE ASSAY MIXTURE (1,0 ml) CONTAINED 1.0 mM ADP, 1.0 mM PEP, 0.15 mM NADH, 3 IU/ml LDH, 70 mM KCL AND $MgCl_2$ OR $MnCl_2$ (0.1 to 10 mM) IN 25 mM IMIDAZOL-HCL (pH 6.5 AND 7.5). THE REACTION STARTED BY THE ADDITION OF 10 μL OF PK SAMPLE, CONTAINING 0.79 mg/ml PROTEIN.

Ammonium sulfate was added to the supernatant until reach 70% saturation. After centrifugation at 12,000 xg for 30 min (4°C) the pellet was resuspended in the buffer described above. The resuspended enzyme solution was dialyzed on the same buffer for 8 h at 4°C.

Ion exchange chromatography was done in one 1,4 x 20 cm phosphocellulose column equilibrated and washed on the above buffer. After the sample application the enzyme was eluted with a linear gradient of 0 to 0.5 M KCl in the same buffer, between tubes 29 and 32. The flow rate was 4 ml/10 min. Fractions containing PK activity were precipitated with ammonium sulfate (70%) and used for kinetic studies.

Pyruvate Kinase Assay. Pyruvate kinase activity was measured at 37°C spectrophotometrically, according to Bucher and Pfleiderer (1955) in a system containing 25 mM imidazol-HCl, pH 7.0, 1 mM PEP, 0.15 mM NADH, 1 mM ADP, 3 IU LDH, 70 mM KCl, 8 mM $MgCl_2$.

Protein Estimation. Protein was determined as described by LOWRY *et al*. (1951) with bovine serum albumin as a standard.

Results and Discussion

PK from curimbata muscle is dependent of Mg^{++} or Mn^{++}. The plots of velocity x concentration of substrate resulted in hyperbolic curves for both Mg^{++} or Mn^{++}.

The highest stimulation seen with Mg^{++} was observed with concentrations of 10 mM $MgCl_2$ (pH 6.5) and 5 mM (pH 7.5). The maximal rates in the presence of Mn^{+2} were seen with 1,0 mM (pH 6.5) and 2,5 mM (pH 7.5). Concentrations higher than 2.5 mM are inhibitory, this effect being stronger at pH 7.5.

Mn^{++} is a substitute of Mg^{++}, but the activation seen with Mn^{++} is 33% at pH 6.5 and 60% at pH 7.5 of the activation seen in the presence of Mg^{++} (FIGURE 1). ZAMORA (1989) observed 40% activation in ice-fish. PLAXTON and STOREY (1985) and BLAIR and WALKER (1984) observed 40-60% in molusc hepatopancreas and rat liver, respectively.

Kinetic studies revealed that the cation forms one complex metal-nucleotide, which is the active form of the substrate. PK needs one divalent cation to perform its catalytic activity (BOYER, 1962), because the nucleotide substrate is the complex MgADP.

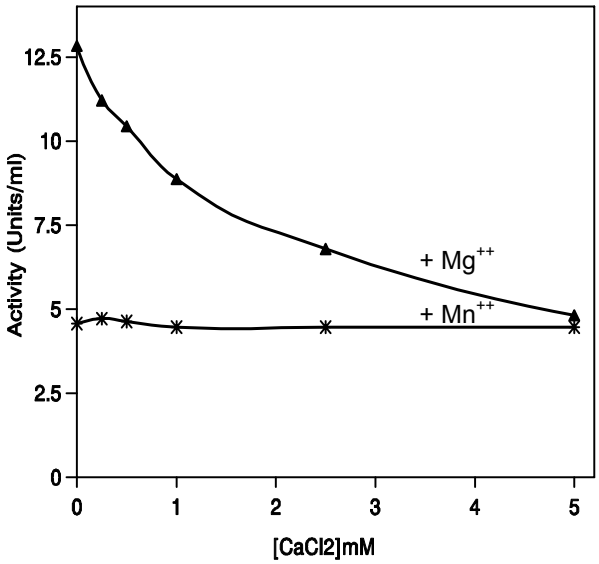

FIGURE 2 – EFFECT OF Ca^{++} ON PK ACTIVITY OF CURIMBATA EPAXIAL MUSCLE AT pH 7.0 AND 37ºC. THE ASSAY MIXTURE (1.0 ml) Contained 1.0 mM ADP, 1.0 mM PEP, 0.15 mM NADH, 3 IU/ml LDH, 70 mM KCl, 8,0 mM $MgCl_2$ Or 7.0 mM $MnCl_2$ AND $CaCl_2$ (0-5 mM) IN 25 mM IMIDAZOL-HCL, pH 7.0. THE REACTION STARTED BY THE ADDITION OF 10 μL OF PK SAMPLE, CONTAINING 0.79 mg/ml PROTEIN.

Ca^{++} is a strong inhibitor of PK from curimbata epaxial muscle in the presence of Mg^{++} (FIGURE 2). The inhibition was of more or less 50% in the presence of 2,5 mM $CaCl_2$. In the presence of Mn^{++}, no inhibition was seen until concentrations of 5 mM were used. The same results were seen in *Mytilus edulis* (DE ZWAAN *et al*., 1975), *Chaenocephalus aceratus* (ZAMORA, 1989) and *Dicentrarchus labrax (ISAN et al.*, 1994).

Generally Ca^{++} competes with Mg^{++} or Mn^{++}. The lower inhibition seen with Mn^{++} is due the fact that Mn^{++} forms more stable complexes with ADP (ISANI *et al*., 1994).

PK from curimbata is sensitive to ions Cu^{++}, Zn^{++}, Ni^{++} and Co^{++} (decreased order of inhibition) (FIGURE 3). The inhibitory effect of Ni^{+2} on curimbata epaxial muscle PK is of approximately 70% in the presence of 4 mM $NiCl_2$. Fru-1,6-P_2 0.5 mM reverted this effect in more or less 30 %. $ZnCl_2$ 0.05 mM inhibited PK from curimbata in 90%. Fru-1,6-P_2 did not revert this effect. The inhibitory effect of Zn^{+2} was seen in PK from muscle and liver of *Dicentrarchus labrax* (ISANI *et al*., 1994). Cu^{++} inhibited strongly the enzyme (85% in the presence of 0,1 mM $CuCl_2$). Fru-1,6-P_2 reverted this effect in 40%. The reversal was of 100% in the presence of 0,01 mM $CuCl_2$. PK from sturgeon muscle (*Acipenser fulvescens*) was 75% inhibited in the presence of 0,15 mM $CuCl_2$. Fru-1,6-P_2 reduced this effect to 5% (RANDALL and ANDERSON, 1975).

In carp (C*arassius carassius*), L-phenylalanine effect was reverted by Fru-1,6-P_2. This effect was not observed in salmon (*Oncorhynchus kisutch*). In these fishes this inhibition is not so strong. M2 isoenzyme of mammals is sensitive to inhibition by L-alanine and L-phenylalanine. This effect is partially reverted by Fru-1,6-P_2. PK from curimbata muscle display many of the characteristics of M2 isoenzyme, but is not inhibited by L-alanine.

PK from fish and invertebrate tissues cannot be under the same classification as used for mammalian's PK. Each tissue has characteristic properties and biological functions which results in distinct isoenzymes and regulatory mechanisms.

Concentrations between 1 and 20 mM of MgATP result in inhibitions between 6 and 50% of PK from curimbata epaxial muscle.

Fru-1,6-P_2 had one smaller protector effect, more or less of 15%. Inhibition by MgATP has been reported in molusc and crustacean hepatopancreas (PLAXTON and STOREY, 1985; FIELDS, 1983; GUDERLEY, *et al.*, 1976).

Co^{++} had one smaller inhibitory effect than Cu^{++}, Zn^{++} and Ni^{++}. Five milimolar $CoCl_2$ inhibited 50% of the enzyme activity. Fru-1,6-P_2 protected PK (15 %) until the concentration of 2 mM. PK from *Mugil lisa* and paru (*Chaetodirus faber*) were highly sensitive to Be^{++} and Cu^{++}, and with lower intensity to Co^{++} (Ocampos *et al.*, 1987), in agreement with our results.

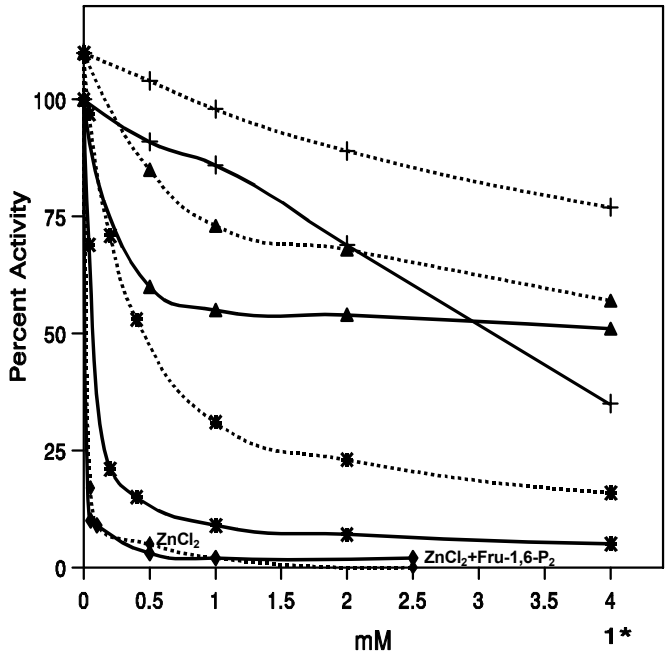

FIGURE 3 – EFFECT OF Ni^{++}, Co^{++}, Cu^{++} AND Zn^{++} ON PK ACTIVITY OF CURIMBATA EPAXIAL MUSCLE (pH 7.0) AT 37ºC. THE ASSAY MIXTURE CONTAINED (1.0 ml) 1.0 mM ADP, 1.0 mM PEP, 0.15 mM NADH, 3 IU/ml LDH, 70 mM KCl AND $NiCl_2$ OR $CoCl_2$ (0-4.0 mM) OR $CuCl_2$ (0-1.0 mM), OR ZnCl2 (0-2.5 mM) IN 25 mM IMIDAZOL-HCL BUFFER (pH 7.0). THE EXPERIMENT WAS PERFORMED WITH AND WITHOUT 0.5 mM FRU-1,6-P_2. THE REACTION STARTED BY THE ADDITION OF 10 μL OF PK SAMPLE, CONTAINING 0.79 mg/ml PROTEIN.

The inhibitory effect of Co^{++}, Cu^{++} and Zn^{++} was seen in PKs of fishes (ISANI *et al.*, 1994), moluscs (CORTESI *et al.*, 1985) and rats (IBSEN and TRIPPET, 1973). However, BAEK and NOVAK (1982) detected that PK from rabbit muscle was activated by Mg^{++}, Co^{++}, Mn^{++}, Ni^{++} and Zn^{++}, in decreased order of V_{max}.

Aminoacid and MgATP effects on PK activity are seen on FIGURE 4. Addition of 20 mM L-alanine in the assay mix did not result in any inhibitory effect on PK from curimbata. On several species of fishes, L-alanine has one inhibitory effect on muscle PK (SOMERO and HOCHACHKA, 1968; JOHNSTON, 1975), while in other species, L-alanine is one activator (OCAMPOS *et al.*, 1987).

L-phenylalanine (10 mM) inhibited 30% of curimbata PK activity. Fru-1,6-P_2 reverted this effect until concentrations of 20 mM.

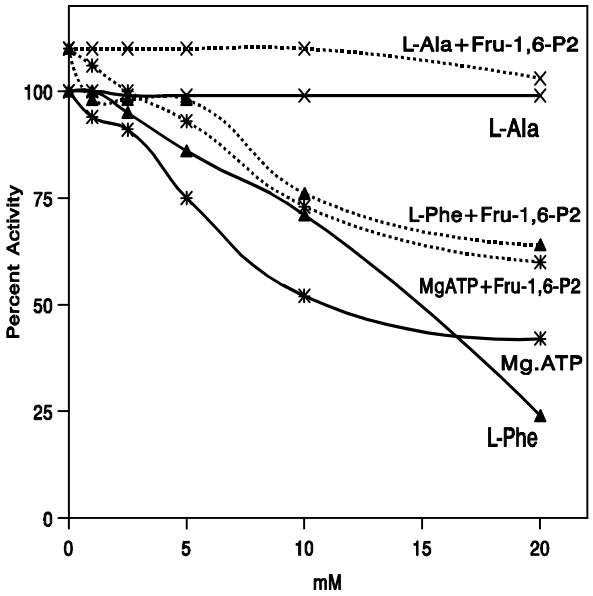

FIGURE 4 – EFFECT OF L-ALANINE, L-PHENYLALANINE AND MgATP ON PK ACTIVITY OF CURIMBATA EPAXIAL MUSCLE (PH 7.0) AT 37ºC. THE ASSAY MIXTURE CONTAINED (1.0 ml) 1.0 mM ADP, 1.0 mM PEP, 0.15 mM NADH, 3 IU/ml LDH, 8.0 mM $MgCl_2$, 70 mM KCl AND L-ALANINE OR L-PHENYLALANINE OR MGATP (0-20 mM) IN 25 mM IMIDAZOL-HCL (pH 7.0). THE EXPERIMENT WAS PERFORMED WITH AND WITHOUT ADDITION OF 0.5 mM FRU-1.6-P_2. THE REACTION STARTED BY ADDITION OF 10 μL OF PK SAMPLE, CONTAINING 0.79 mg/ml PROTEIN.

The presence of 2 mM oxalate, resulted in inhibition of curimbata PK of approximately 85% (FIGURE 5). Inhibition was partially reverted by Fru-1,6-P_2 (35%). ZAMORA (1989) detected the same degree of inhibition in ice-fish PK. The oxalate effect on PK activity is dependent of the concentration of oxalate and PEP (BLAIR and WALKER, 1984; CALLENS *et al.*, 1991).

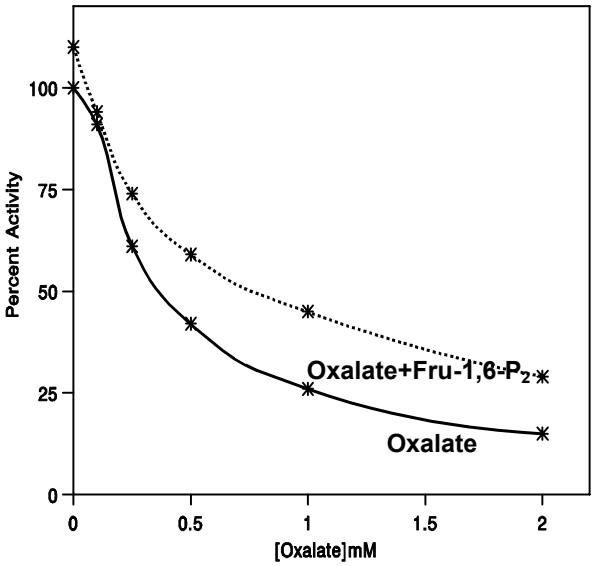

FIGURE 5 – OXALATE EFFECT ON PK FROM CURIMBATA EPAXIAL MUSCLE (pH 7.0) AT 37^{O}C. THE ASSAY MIXTURE CONTAINED (1.0 ml) 1.0 mM ADP, 1.0 mM PEP, 0.15 mM NADH, 3 IU/ml LDH, 8.0 mM $MgCl_2$, 70 mM KCl AND OXALATE (0-2 mM) IN 25 mM IMIDAZOL-HCL, pH 7.0. THE EXPERIMENT WAS PERFORMED WITH AND WITHOUT ADDITION OF 0.5 mM FRU-1.6-P_2. THE REACTION STARTED BY ADDITION OF 10 μL OF PK SAMPLE, CONTAINING 0.79 mg/ml PROTEIN.

Conclusions

Pyruvate kinase purified from the epaxial muscle of the tropical fish Curimbatá (*Prochilodus lineatus)* displays several characteristics of the M2 isoenzymes but is not inhibited by L-alanine. The enzyme is highly sensitive to oxalate and to Ni^{2+} and Zn^{2+}.

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