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Short Communication

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# Kinetic Characterization and Flavonoid Effect on Human Lymphocyte Protein Tyrosine Phosphatase

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**SUMMARY. The aim of this work was to determine the kinetic properties and evaluate the effect of some flavonoids on human lymphocyte protein tyrosine phosphatase. Tyrosine-, serine- and threonine-phosphate were hydrolyzed by this phosphatase 60%, 20% and 10%, respectively. In the kinetic studies the enzymatic activity was determined by using *p*-nitrophenylphosphate (pNPP) as substrate. The enzyme presented optimum pH around 5.0 and was inhibited by 100 $\mu$M *p*-chloromercuribenzoate (pCMB) (80%), 10 mM fluoride (35%), 10 mM vanadate (100%), and 5mM $Cu^{+2}$ (85%). The enzyme was also strongly inhibited by a tyrosine phosphatase inhibitor cocktail, but was unaffected by okadaic acid. These results confirm that the major phosphatase activity in human lymphocytes is a protein tyrosine phosphatase. Among the bioflavonoids tested only fisetin showed an inhibitory effect in order of 80% on the enzymatic activity.**

*RESUMEN*. "Caracterización Cinética y Efecto de los Flavonoides en la Proteína Linfocitaria Humana Tirosina Fosfatasa". El objetivo del trabajo fue determinar las propiedades cinéticas y evaluar el efecto de algunos bioflavonoides sobre la proteína humana linfocitaria tirosina fosfatasa. Tirosina-, serina- y treonina-fosfato fueron hidrolizadas por esta fosfatasa en un 60%, 20% y 10%, respectivamente. En los estudios cinéticos la actividad enzimática fue determinada usando *p*-nitrofenilfosfato (pNPP) como sustrato. La enzima presentó un pH optimo cercano a 5.0 y fue inhibida por *p*-cloromercuribenzoato (pCMB) 100 (M (80%), fluoruro 10 mM (35%), vanadato 10 mM (100%), y $Cu^{+2}$ 5 mM (85%). La enzima fue también fuertemente inhibida por un cóctel de inhibidores de tirosina fosfatasa, pero fue insensible al ácido okadaico. Estos resultados confirman que la mayor actividad fosfatasa en los linfocitos humanos es una proteína tirosina fosfatasa. Entre los bioflavonoides ensayados solamente la fisetina presentó un efecto inhibitorio en el orden del 80% sobre la actividad enzimática.

## INTRODUCTION

A variety of cellular functions including gene transcription, cell differentiation, contractility, neurotransmission and memory regulation involve phosphorylation of proteins which is dependent upon the relative activity of protein kinases and phosphatases [1,2]. Abnormal protein phosphatase activity could be implicated in diseases such as cancer [3], diabetes [4], and inflammation [5].

Phosphatases are hydrolases that catalyze the hydrolysis of monoester phosphates and are divided into three groups: acid phosphatases, alkaline phosphatases and protein phosphatases.

Protein phosphatases are classified according to their substrate specificity, dependence upon metal ions for activity and sensitivity to inhibitors. Protein serine/threonine phosphatases are divided into type 1 protein phosphatase (PP1) and type 2 (PP2A, PP2B and PP2C). The PP2A does not require divalent metal ions for its activity [1].

Protein tyrosine phosphatases (PTP) are characterized by about 240 amino acids and with a preserved domain having a signature sequence in the catalytic site (H/V)C(x)5R(S/T). Moreover, the PTPs share a standard active site constituted by a cystein and an arginin that are

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essential to the enzymatic catalysis so that there is no homologous sequence relating the PTPs to the serine/threonine phosphatases. Concerning the classification, the PTPs can be divided into three groups: tyrosine specific phosphatases (CD45 and PTP(), dual specificity phosphatase (cdc25-cell-division control) and low molecular weight phosphatases [1,6].

Protein serine/threonine phosphatases are divided into at least seven subtypes: PP1, PP2A, PP2B, PP2C, PP5, PP6 and PP7 [1]. They are a group of regulatory enzymes that have an important role in a variety of cellular functions, such as cellular growth, differentiation, metabolism, cellular cycle, communication cell-cell among others.

Bioflavonoids are polyphenolic compounds naturally present in vegetables, fruit and beverages such as tea and wine. More than 4,000 different flavonoids have been described and categorized into flavonol, flavone, flavan 3-ol, flavanonol, flavanone, and isoflavone [7].

It is relevant to consider the importance of studies regarding the effects of these compounds on the lymphocytes function, in relation to the pharmacological and physiological aspects, pertinent to the phosphatase activity. The aim of this work was to determine kinetic properties of the phosphatase obtained from human lymphocytes, and to evaluate the effect of flavonoids on the enzymatic activity.

## MATERIALS AND METHODS

### *Materials*

Blood samples were collected from healthy volunteers (18 to 40 years old) as approved by the Ethics Committee Nº 311/2000 (Faculdade de Ciências Médicas/UNICAMP).

The pNPP and the bioflavonoids myricetin, taxofolin, fisetin, phloretin, rutin, narigin, morin and catechin were obtained from Sigma Chemical Co. (Mo, U.S.A.). All other chemicals were of the highest purity available.

### *Blood sampling*

The blood samples were obtained by venipuncture, between 8:00 and 10:00 AM into heparinized tubes.

### *Lymphocytes isolation and enzyme obtention*

The lymphocytes were separated from venous blood, diluted 1:1 with physiological solution and centrifuged in the presence of hystopaque for 30 min at 3,000 rpm. The cells were washed twice with physiological solution and centrifuged at 3,000 rpm for 10 min and resuspended in an acetate buffer (0.01 M).

### *Protein phosphatase and flavonoids assay*

The flavonoids were used in a concentration of 1mM and incubated with the enzyme obtained by lysing the cells with 1 mM acetate buffer (pH 5.0). In a final volume of 0.5 mL the reaction mixture contained 100 mM acetate buffer (pH 5.0), 5 mM pNPP and cell extract enzyme. After 30 min at 37 °C the reaction was stopped by adding 0.5 mL of 1 M NaOH. The amount of p-nitrophenol released was determined by measuring the absorbance at 405 nm [8].

### *Statistical evaluation*

All presented data were expressed in average ± standard deviation. Significance levels among the samples were analyzed by the use of Multivariance Analysis (ANOVA), utlizing SAS software and and using significance level (alfa) = 0,05 (Dunnet's Test)

## RESULTS AND DISCUSSION

The kinetic study of acid phosphatases is very important in order to elucidate the cellular roles of these enzymes. The present report describes some properties of acid phosphatases obtained from human lymphocytes. Although several works have reported the utilization of the protein tyrosine phosphatase as a biomarker for diagnosis and monitoring of patients with carcinoma [9], little information is available concerning both the kinetics of this enzyme and the effect of flavonoids on its activity.

In relation to the human lymphocytes enzymatic specificity, some phosphorylated amino acids were tested, considering pNPP hydrolysis as 100% of enzyme activity, human lymphocytes phosphatases 60% tyrosine phosphate, 20% serine phosphate and 10% threonine phosphate, respectively (Fig. 1, $p < 0.0001$). However, it must be considered that the serine and threonine hydrolysis were underestimated due to the absence of calmodulin/$Ca^{2+}$ in the reaction medium.

The effect of some metal ions on the enzymatic activity was evaluated. A strong inhibition by $Cu^{2+}$ (in order of 85%) was observed, suggesting the presence of -SH groups in the active site of the human lymphocytes phosphatases. The other metal ions tested ($Mg^{2+}$, $Ca^{2+}$ and $K^{+}$) had no significative effect, showing that the enzyme was not dependent on these metals for

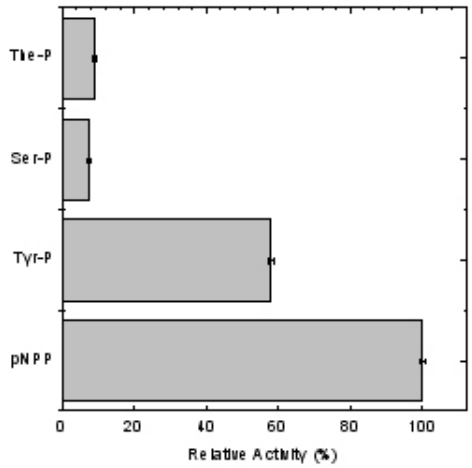


**Figure 1**. Hydrolysis of phospho amino acids by phosphatase from human lymphocytes. Enzyme activity was determined as described in Materials and Methods in the presence of 5 mM Ser-, Thr- or Tyr-phosphate. Enzyme activity in the presence of pNPP was considered as 100%. Three independent experiments were performed in trifold manner and the results in the charts represent their averages and standard deviations ($p < 0,05$)

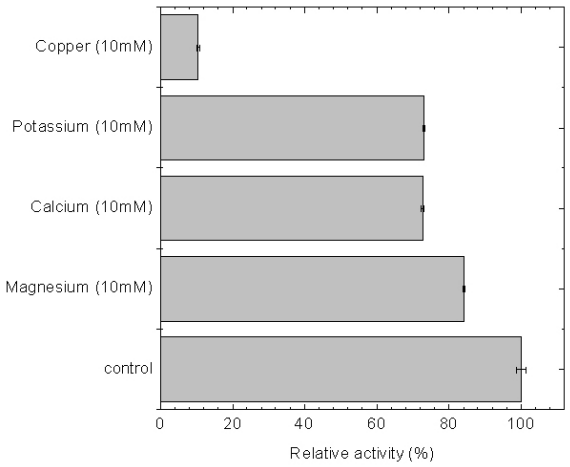


**Figure 2**. Effect of cations on the human lymphocytes acid phosphatase activity. The assay conditions were the same as described in Materials and Methods, with pNPP as substrate in the absence (100% activity) or in the presence of 10 mM cations. The experiments were performed in triplicate and bars represent the standard deviations. ($p < 0.05$).

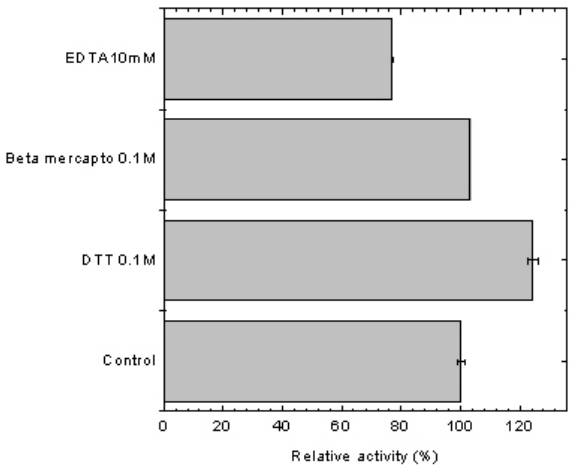


**Figure 3**. Effect of antioxidants on human lymphocytes phosphatase activity. Enzyme activity was determined with pNPP as substrate as described in Materials and Methods in the absence (100% activity) or in the presence of EDTA or antioxidant compounds. Three independent experiments were performed in triplicate and the results in the charts represent their averages and and standard deviations ($p < 0.05$).

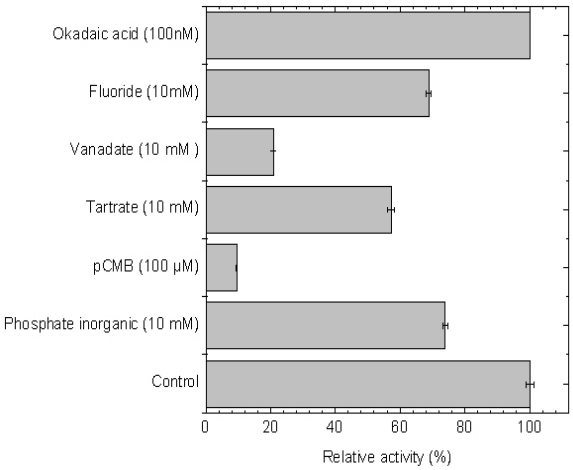


**Figure 4**. Effect of potential inhibitors on the human lymphocytes phosphatase activity. The assay conditions were the same as described in Materials and Methods, with pNPP as substrate in the absence (100% activity) or in the presence of compounds at the concentrations indicated. The experiments were performed in triplicate and bars represent the standard deviations($p < 0.05$).

the catalytic activity (Fig. 2). Some protein phosphatases (Ser/Thr) are metal dependent. PP2B and PP2C depend on calcium and magnesium for its activity [1].

The presence of EDTA and reducing compounds such as β-mercaptoethanol and dithiothreitol did not significantly affect the enzymatic activity (Fig. 3). These results indicate that the reactions catalyzed by human lymphocytes phosphatases occurred in adequate conditions.

The study of potential acid phosphatase inhibitors is very important in order to determine which phosphatase is predominant in the fraction extracted from the human lymphocytes.

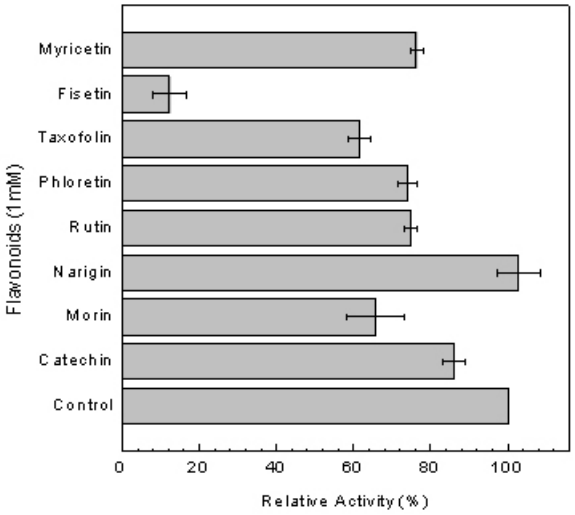


**Figure 5**. Effect of specific protein tyrosine phosphatase inhibitor on the human lymphocytes phosphatase activity. human lymphocytes phosphatase activity. Enzyme activity was determined with pNPP as substrate as described in Materials and Methods in the absence (100% activity) or in the presence of coquetel of the inhibitor PTP. The experiments were performed in triplicate and bars represent the standard deviations ($p < 0.05$).

From the inhibitors tested, phosphatases were inhibited by 100 µM pCMB (90%), and 10 mM each vanadate (80%), tartrate (40%), phosphate (30%) and fluoride (30%)($p < 0.0001$). Since vanadate is a specific protein tyrosine phosphatase inhibitor, these results suggest that the major phosphatase present in human lymphocytes could be this enzyme. This finding can be strongly supported by the high specificity of human lymphocytes phosphatase towards tyrosine-phosphate (Fig. 1) and the lack of inhibition by okadaic acid (Fig. 4), a specific inhibitor of PP1 and PP2A Ser/Thr protein phosphatases.

The presence of protein tyrosine phosphatase as a major phosphatase in human lymphocytes was highly evidenced by the inhibition observed in the presence of a cocktail containing specific inhibitors of this class of enzymes (Fig. 5).

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**REFERENCES**

1. Ferreira,C.V., Justu, G.Z., Souza,A.C.S., Queiroz, K.C.S., Zambuzzi,W.F., Aoyama, H. & Peppelenbosch, M.P (2006) *Biochem.* **88**: 1859-73.
2. Jia, Z. (1997) *Biochem. Cell Biol.* **75**: 17-26.
3. Cantley, L.C., K.R. Auger, C. Carpenter, B. Duckworth, A. Graziani, R. Kapeller & S. Soltoff (1991) *Cell.* **64**: 281-302.
4. Taylor, S.I., A. Cama, D. Accili, R. Barbetti, M.J. Quon, M. Sierra, Y. Suzuli, E. Koller, R. Levy Toledano & E. Wertheimer (1992) *Endocrinol. Review.* **13**: 566-95.
5. Parnetti, L., U. Senin & P. Meccoci (1997) *Drugs* **53**: 752-68.
6. Zhang, Z.-Y. (2001) *Curr. Opin. Chem. Biol.* **5**: 416-23.
7. Casagrande F. & J.M. Darbon (2001) *Biochem. Pharmacol.* **61**: 1205-15.
8. Aoyama, H., P.S. Melo, P.A. Granjeiro, M Haun & C.V Ferreira (2000) *Pharm. Pharmacol. Comm.* **6**: 331-4.
9. Ben-Arie, A., Z. Hagay, H. Bem-Hurt, M. Open & R. Dgani (1999) *Eur. J. Obs. Gyn. Rep. Biol.* **86**: 69-71.