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Comparative analysis of dipeptidyl aminopeptidase activity in haematological tissues of vertebrates

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Abstract:

Blood and bone marrow evaluation is very important means to analyze physiological conditions of mammals as well as other group of vertebrates. Dipeptidyl peptidase IV, a serine peptidase with N-terminal exopeptidase activity, is an important indicator of hematopoietic environment. DPP-IV enzyme activity was measured in blood and bone marrow tissue of rohu (*Labeo rohita),* toad (*Bufo melanostictus*); calotes (*Calotes versicolor)*; chicken (*Galus galus*); and mice (*Mus musculus*). The optimum temperature for enzyme reaction in both tissues was 37^{o}C. In both blood and haematopoietic tissues DPP-IV activity were linear upto 20 minutes of time. Both blood and bone marrow cell DPPIV displayed classical Michaelis Menten kinetics with a variable Vmax and Km values in different animal groups. The proteolytic activity of the enzyme was inhibited in a concentration dependent manner by a specific inhibitor diprotin A, which indicate the presence of DPPIV in all the samples with maximum inhibition in mice blood and bone marrow samples. We have found a significant variation in enzyme activity in different vertebrate groups the significance of which is still unknown.

Key words: DPPIV, hematology, hematopoiesis, vertebrate.

1. Introduction:

The family of dipeptidyl aminopeptidase (DPP) enzymes includes four activities, associated, at least to four different serine proteases all characterized by their ability to release N-terminal dipeptides from a variety of small proteins. One of the members of this group is DPP IV which cleaves preferentially substrate containing the sequence X-P at the amino terminus. Thus DPP-IV can be classified as a post proline cleaving enzyme, which represents an important family of regulatory proteases. As membrane anchored enzyme, DPPIV can determine the tissue microenvironment and availability of cytokines which are responsible molecules for hematopoiesis [1], [2]. It has a co-stimulatory activity for proliferation of granulocytes and macrophages [3] as well as immature thymocyte [4]. In man DPPIV activity has been associated to a 105-110 K Da cell surface protein, known to be identical to the adenosine deaminase binding protein and to the T cell activation antigen, CD 26 [5], [6], [7], [8]. The DPP IV activity has been suggested to be essential for T-cell activation, although its role in the different stimulation mechanisms has not been completely elucidated. It seems clear that this activity plays an important role in antigen-induced T cell proliferation [9]. A number of evidences have shown it as a reliable enzyme cytochemical marker of T lymphocytes that has received much more attention [10], [11], [3]. Recently evidence was provided for the regulatory role of DPPIV activity in chemokine function, however the effect of these chemokines on T cell proliferation is not well understood. The failure to unravel the role of DPPIV, is, in part due to the fact that no immunologically active substrate have been identified. In addition to CD 26, two novel proteins bearing DPPIV activity have been identified. Duke-Cohan and co-workers [12], [13] have reported a 175 KDa membrane bound and soluble form of DPPIV, which can be secreted from activated peripheral blood T cells. Blanco and co-workers [8] has reported the existence a CD 26 like protein in

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lymphocytic cell line which has DPP IV activity. Both the enzymes have same substrate affinity. . In fact, DPP IV activity may cleave some bioactive peptides, such as substance P, chorionic gonadotropin hormone, monomeric fibrin and glucose-dependent insulinotropic polypeptide [8]. Other N-terminus Xaa-Pro potential DPP IV substrates are some cytokines, such as interleukin-1β, interleukin-2, tumour necrosis factor β, granulocyte colony-stimulating factor, and chemokines that inhibit HIV infection (SDF-1, RANTES, MIP-1A and MIP-1β) [14], [15]. Its functions are related to many physiological and pathophysiological conditions [16], [17] especially in cancer [18]. Since post proline cleavage is a limiting step in peptide degradation [19] cleavage of chemokines may affect, not only selectivity, but also half-life of active peptides.

DPP-IV enzyme has many functions in mammals but the evidences for the presence of DPP IV in lower vertebrates are scanty except a few. Recent studies have shown that snake venom is a good source of DPP IV [20]. DPP IV activity showed a significant presence in plasma and whole blood in American alligator *(Alligator Mississippians)* [21].

The aim of our study was to find out the level of DPP IV activity in haematopoietic tissue as well as in peripheral blood of different vertebrate groups including lower ones. We have addressed the question of relative presence of activity of DPPIV in the hematopoietic environment. This analysis will enable us to assess the physiological condition of hematological tissues of all the vertebrate groups and may be considered as important parameter in pathological conditions.

2. Materials and methods:

2.1. Animals:

The animals used in these experiments were fish (rohu; *Labeo rohita*); amphibian (toad; *Bufo melanostictus*); reptile (calotes; *Calotes versicolor)*; bird (chicken; *Galus galus*); and mammal (mice; *Mus musculus*). The number of the animal species taken was twenty five each from a commercial dealer and fed usual equilibrated diet. All the animals except fish were maintained at room temperature (28-31°C) in the animal house. Fishes were purchased from market in regular basis. Animals were apparently healthy, with no signs of any disease.

2.2. Chemicals:

DMF, FBB and gly-pro-4 methoxy-2 naphthylamide were purchased from Sigma Chemicals, St. Louis, MO. All other chemicals were of analytical grade.

2.3. Blood sampling:

Blood samples were collected by cardiac puncture in mice, calotes, toad and chicken [22], [23] and from caudal vein of fishes [24]. Sodium citrate (0.01% final concentration) was used as anticoagulant. Blood was immediately stored in an ice bath until use. All analyses were completed within two hours of withdrawal.

2.4. Preparation of tissue extracts:

20 mg of bone marrow was separated from femur of mice, calotes, toad and chicken and in case of fish same amount of head and body kidney [25] was separated. The tissue was washed thoroughly with 0.15 M NaCl, taken in 100mM phosphate buffer, pH 7.2 and homogenized. The homogenized tissue was centrifuged at 5000 rpm for 20 min. at 4°C. Then the supernatant was collected for enzyme assay.
To collect blood cells 500 µl of blood was taken in a centrifuge tube and centrifuged (at 5000 rpm) at 4°C for 8 minute; the cell pellet was dissolved in 500 µl of 0.1 M phosphate buffer pH 7.2 and then further processing was done as the case of bone marrow.

DPPIV activity was assayed using gly-pro-4-methoxy-2-naphthylamide as the substrate. The incubation mixture was prepared by dissolving 3 mg of substrate in 0.2 ml DMF and added to 4.6 ml phosphate buffer, pH 7.2 and mixing well. This solution was added to 5 mg of FBB salt in 0.2 ml of DMF. The pH of the resulting solution was adjusted to 7.2 [18]. 400 µl of this incubation mixture was added with tissue extracts in variable concentration (about 30-60 mg of protein) and was incubated for 20 min. at 37°C. The reaction was stopped by cooling the

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assay mixture in ice bath [26]. The amount of product was evaluated by comparing differential absorbance at 345 nm against a control sample having identical mixture without the enzyme source. Protein determination was performed by standard method of Bradford [27] using BSA as standard.

One enzyme unit was equivalent to 0.01ΔOD/min. The specific activity of DPPIV was defined as enzyme units/mg of protein.

2.5. **Statistics:**

Data obtained from three replicate enzyme assays from a single species showed a similar tendency and therefore a mean of all three data was considered as one experiment. All data were expressed as mean ± SD of twenty five such experiments taking blood and hematopoietic tissue from each donor species. The significance of difference of enzyme activity was determined by one way ANOVA within and between different species was made by using Tukey's multiple comparison tests using SPSS (Version. 10.1). The level of significance chosen was $p<0.05$.

3. Results:

3.1. Enzyme activity in bone marrow tissue:

The DPPIV activity was increased linearly up to 20 minute of incubation period (Fig.1).

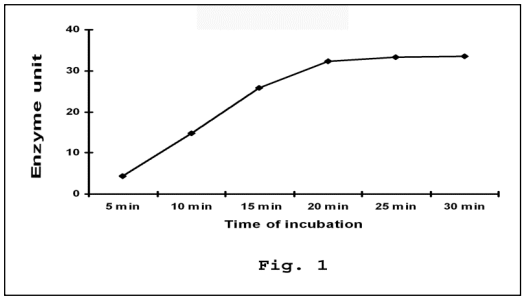

Figure1: Time kinetics for DPPIV
Identical enzyme-substrate mixture was prepared in six tubes which were incubated at 37ºC for different time intervals. The enzyme unit was calculated from differential absorbance value between substrate concentrations in experimental sample against an identical blank.

The temperature dependent study showed that the optimum temperature for the enzyme activity was at 37ºC for all the vertebrate groups (data not included). The bone marrow DPP IV activity follows the typical Michelis-Menten kinetics having the Km value of the enzyme for the substrate was 3.12 mM in mice, in chicken 0.42 mM; in calotes 2.34 mM; in toad 1.12 mM and in rohu 0.51mM (Fig.2).

The specific activity of dipeptidyl peptidase IV

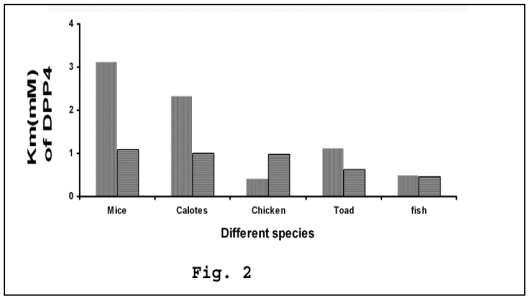

Figure 2: Variation of Km of DPPIV in blood and bone marrow
Km for the enzyme was determined for blood and bone marrow tissue of each animal using Michaelis-Menten equation. Each bar represents Km in mM and each zone represents Km in blood and bone marrow where vertically lined bar stands for bone marrow and horizontally lined bar stands for blood.

was maximum in mice, 80 ± 7.9 U/mg protein of bone marrow tissue. Whereas the amount of protein was 6.31 ± 0.59 µg/mg bone marrow tissue. In chicken the enzyme specific activity was 9.67 ± 0.85 U/mg proteins when the protein content was 7.75 ± 0.83 µg/mg bone marrow. In toad the specific activity was 25.97± 1.8 U/mg protein and the protein content was 9.62± 0.42 µg/mg bone marrow. In calotes the activity was 54.59 ± 4.3 U/mg proteins and protein content was 8.25 ± 0.73 µg/mg bone marrow and in rohu the activity was 12.5 ± 1.3 U/mg proteins and the protein content was 14 ± 0.11 µg/mg bone marrow (Fig.3).

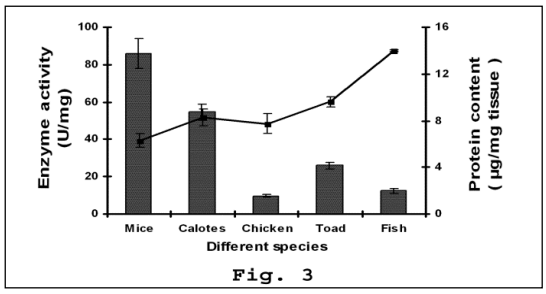

Figure 3: Comparison of protein content and enzyme activity in hematopoietic tissue
Protein content in each tissue extract was determined using Bradford's method as mentioned in materials and methods. Each point on the line represents protein content (µg/ mg tissue). Enzyme activity was expressed in unit/ mg protein (U/mg) which was represented by each bar. Values in each case were taken as mean of five replica ± S.D. and was significant ($p<0.05$).

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There was a gradual decrease in specific activity from rohu to mice except in chicken where both amount of recovered protein as well as the enzyme activity were significantly lower than both toad and calotes.

3.2. Enzyme activity in blood cells:

The DPPIV showed linear increase in activity upto 20minutes of incubation of blood proteins and maximum activity was observed at 35 minutes of incubation.

The temperature dependence curve showed the same pattern as the bone marrow tissues. The enzyme kinetic studies showed that blood DPPIV followed the same Michaelis-Menten equation having Km value of the enzyme was 1.09 in mice, 1.01 in calotes, 0.62 in toad, 0.97 chicken and 0.45 in fish (fig 2). The specific activity of dipeptidyl peptidase IV was maximal in mice, 27.1 ± 2.1 U/mg protein of blood cells. Whereas the amount of protein was 0.46 ± 0.032 µg/µl of blood. In calotes the specific activity of enzyme was 21.5 ± 1.9 U/mg protein whereas protein content was 0.4 ± 0.031 µg/µl blood. In toad the enzyme activity was 16.66 ± 1.4 U/mg protein of blood cells when the protein content was 0.33 ± 0.06 µg/µl blood; in chicken out of 0.41 ± 0.03 µg/µl protein content of blood, the specific activity was 23.03 ± 1.9 U/mg and in fish the enzyme activity was 13.29 ± 1.2 U/mg protein when protein content was 0.37 ± 0.032 µg/µl blood (Fig.4).

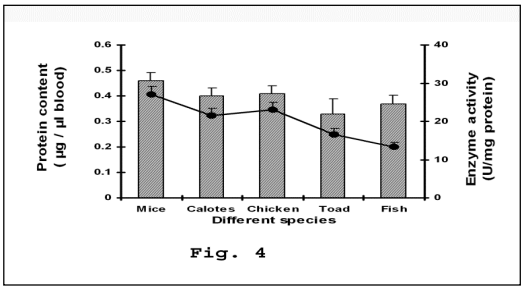

Figure 4: Comparison of protein content and enzyme activity in blood
Protein content in blood cells of each animal was determined using Bradford reagent as mentioned in materials and methods. Each bar represents protein content (µg/ mg tissue). Enzyme activity was expressed in (U/mg) which was represented by point on the line.

There was a significant decrease in enzyme activity from mice to rohu except in chicken where there was a drastic increase was found. Whereas the protein content per micro liter of blood was more or less equal in all these groups.

Table1: Inhibition of DPPIV activity in different groups of vertebrates with variable concentration of inhibitor diprotin A.

Concentration of diprotin A	Inhibition of enzyme activity in different vertebrate groups				
	Mice	Calotes	Toad	Chicken	Fish
2.5mM	+ +	+	+	+	+
5mM	+ + +	+ +	+ +	+ + +	+
10mM	+ + +	+ +	+ +	+ + +	+

The proteolytic activity of DPPIV was inhibited in a concentration dependent manner by a classical DPPIV inhibitor, diprotin A [28] which indicate that the activity is due to the presence of DPPIV in both bone marrow and blood samples. The maximal inhibition of the DPPIV activity was obtained with 5mM inhibitor and was appox. 23% for rohu, 30% for toad, 33% for calotes, 40% for chicken and almost 50% for mice bone marrow and more or less in same order in peripheral blood (Table 1) whereas EDTA (100mM) was found to have no significant effect on enzyme activity in all these groups (data not included) which is very similar characteristics of DPP IV. The partial inhibition in lower vertebrates indicated the presence of was probably due to the presence of other proteases, which may hydrolyze the inhibitor or other molecules that may bind to the inhibitor.

4. Discussions:

Hematopoiesis is dependent upon the tissue microenvironment composed of stroma cells, extracellular matrix and cytokines. The presence of catalytic activity of various peptidases can determine the availability of cytokines at a particular site in the hematopoietic tissue regulating the microenvironment. The DPPIV potentially generates gradient or local microenvironment of hematopoietic system as well as regulates the normal functioning of blood lymphocytes. In this study various modifications of previously published methods for showing the presence of DPPIV in peripheral blood and bone marrow cells were investigated. The

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optimal conditions for showing the enzyme activity at the cellular level were determined. Before investigating DPP IV-like activity, it was necessary to confirm that the enzyme responsible was actually DPP IV, since a combination of aminopeptidases could potentially have been responsible for cleavage of the substrate Gly-Pro-4-methoxy-β-naphthylamide The results showed significant level of activity of DPPIV in both higher and lower order of vertebrates (Fig.3 & 4). The catalytic activity of DPPIV in bone marrow tissue showed significant variation in different groups of vertebrates. The enzyme activity showed linear increase up to 20 min. of incubation period (Fig.1). In order to test the specificity of the proteolytic activity of DPPIV, we monitored its inhibition by diprotin-A, a classical competitive inhibitor of this enzyme (Table 1). Moreover EDTA (100mM) was ineffective on enzyme activity. The specific inhibition was observed in the entire vertebrate group but the maximum inhibition was found in mice samples (approx 50%). It is also point to note that the rate of competitive inhibition is quite low in lower vertebrates. So it indicates the presence of other groups of aminopeptidases in lower vertebrates as well as in mice tissues.

The highest activity was found in the bone marrow tissue of mice in comparison to other vertebrate groups. Lowest activity per unit amount of protein was found in chicken. The recovery of total protein also varied in different vertebrates. The recovery was highest in fish in comparison to others (Fig.4). The comparative analysis of Km value of this enzyme showed significant variation in different groups (Fig.2). The Km was highest (about 3.2 mM) in mice and lowest in chicken (0.42 mM). As the apparent Km reflects the relative affinity of the enzyme for a given substrate and as the assayed substrate was an artificial one, the observed differences in the apparent Km indicated different molecular association of the enzyme in different microenvironment of the tissue, it was found that total affinity of enzyme towards the substrate was lowered significantly in peripheral blood than bone marrow in mice and calotes than rohu and toad, when the Km values were more or less equal in peripheral blood of mice, calotes and chicken but lowest in fish. In peripheral blood the comparative status of this enzyme activity showed same trend but in case of chicken the activity was significantly higher than bone marrow preparation regarding comparative status of this enzyme (Fig.3&4).

5. Conclusions:

As a number of studies showed that DPPIV was normally restricted to the lymphocytes in bone marrow or peripheral blood, our study indicated that DPPIV activity varied in different tissue environment in different vertebrate groups may be due to the divergences in the spatial structure of the catalytic site of these enzymes. The apparent Km was significantly different and dependent upon the tissue origin of the cells studied. The apparent Km does not depend upon the total quantity of assayed enzyme and indirectly reflects the relative affinity to the enzyme for a given substrate. Although the assayed substrate is an artificial one and does not necessarily reflect the affinity of the enzyme for potential natural substrates, the observed differences in the apparent Km for diverse molecular back grounds indicated that the enzyme affinity was modulated by the associated molecules. Moreover as the organisms selected for this study are the representatives of particular vertebrate animal groups, so, this result reflects the status of different groups of vertebrates in different biological niche and their changing physiological conditions. The subsequent functional classification and characterization is in progress to know the status of amino peptidases population in different vertebrate groups and their relative significance in hematopoietic physiology.

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