

FLAVIN MONONUCLEOTIDE PHOSPHATASE FROM GOAT LIVER: A POSSIBLE TARGET FOR DIVALENT HEAVY METAL CATIONS

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ABSTRACT

Objective: A comparative study of the tissues from several animals showed the presence of phosphatase(s) hydrolyzing FMN. Liver is a rich source of this enzyme. The FMN phosphatase of goat liver was not studied earlier. Present study deals with the purification and characterisation of this enzyme using cell-free homogenate as well as the effect of divalent metal cations on the activity of the partially purified enzyme.

Methods: The enzyme FMN phosphatase was purified from goat liver and it was characterised. The effects of various divalent metal cations on the activity of the partially purified enzyme were also studied.

Results: From the post mitochondrial fraction, FMN phosphatase was purified up to 785 folds by salt fractionation using ammonium sulphate. The enzyme showed a linear activity with the increasing concentrations of FMN and protein to produce riboflavin and inorganic phosphate. The optimum pH and temperature were found to be 5.0 and 50°C respectively. Among the compounds tested for its hydrolytic activity, FMN ($K_m = 2 \times 10^{-4}$ M) was the most important physiological substrate although the enzyme hydrolyzed p-nitrophenyl phosphate maximally. The enzyme was found to be inhibited by the divalent cations in increasing orders of inhibition, $Ca^{2+} > Zn^{2+} > Cd^{2+} > Cu^{2+}$ indicating the presence of a -SH group at or near the catalytic site of the enzyme. p-chloromercuribenzoate (PCMB) and DTNB are potent inhibitors while N-ethylmaleimide inhibited the enzyme to a lesser extent at even higher doses and their effect is reversed as well as protected by reduced glutathione indicating a sulfhydryl group involvement.

Conclusion: The present study indicates the presence of cytosolic, FMN phosphatase of low molecular weight with possibility of a sulfhydryl group at or near its active site in goat liver and it is a good target of the divalent cations.

Keywords: Riboflavin, FMN-phosphatase, Goat liver.

INTRODUCTION

Riboflavin-5'-phosphate or Flavin mononucleotide (FMN) is an essential coenzyme form of the vitamin riboflavin, and occurs widespread in plant and animal organisms [1, 34]. FMN and FAD are formed from riboflavin in liver, intestinal mucosa and other tissues. A great number of mammalian enzymes require FMN or Flavin adenine dinucleotide (FAD) as coenzymes for their activities and both the flavin coenzymes are associated with many of the components of the electron transport chain of mitochondria which is associated with oxidative phosphorylation. Riboflavin is related to the metabolic process of proteins and is an integral part of the prosthetic group of acyl-CoA dehydrogenase the enzyme which mediates the first oxidative step in the oxidation of fatty acids. Riboflavin is also vital for proper maintenance and health of ectodermal tissues like skin and oral mucosa. Therefore, for normal cellular function, maintenance of an optimum concentration of riboflavin, FMN and FAD is very important. However, tissue level of FAD, FMN or riboflavin at any point of time depends on the degree of activities of FAD-pyrophosphatase (which hydrolyzes FAD to FMN) and FMN-phosphatase (which hydrolyzes FMN to riboflavin). The biosynthesis of FMN from riboflavin and adenosine-5'-triphosphate is catalyzed by flavokinase whose activity is often interfered by FMN phosphatase which converts FMN into riboflavin. The existence of FMN-phosphatase has been shown in several plants as well as in animal organs.

Enzyme reactions are inhibited by metals which may form complex with the substrate, or combine with the protein-active group of the enzymes, or react with the enzyme-substrate complex [2]. Several cations inhibit the FMN phosphatase from goat liver. Sensitivity to cations generally emphasizes the sulfhydryl nature of an enzyme. The effect Zn^{2+} on the activity of FMN phosphatase is of some interest, as this cation is the most effective activator of both flavokinase from liver [3] and Cd^{2+} can elevate the level of various hepatic alkaline as well as acid phosphatases (the group to which

FMN-phosphatase belong) but their increased levels can be reduced to normal by the hepatoprotective effect of *Berberis asiatica* [4] and vitamin B₆ kinases from mammalian tissues [5,6] where contaminating phosphatases often mask kinase activity. Although Zn^{2+} rather than the usual phosphokinase activator, Mg^{2+} [7], is specifically required by B₆ kinase from mammals, a large part of the apparent activation by Zn^{2+} of liver flavokinase is undoubtedly due to suppression of interfering FMN phosphatase.

The activity of the enzyme FMN phosphatase has been reported from invertebrates like *Tetrahymena pyriformis* (protozoa), earthworm (annelida), several arthropods like sow bug, mealworm and harvestman, mollusca like *Limax sp.* to vertebrates like Rainbow trout (osteichthyes), Bullfrog (amphibian), Snapping turtle (reptilian), Chicken (aves), Rat and Guinea pig (mammalian) [8]. However, to the best of our knowledge and belief no report exists in respect of the detailed analysis on enzymatic properties and mechanism(s) of action of mammalian FMN-phosphatase. Furthermore, there is no information to date about goat liver FMN-phosphatase which, our initial studies showed, is a good source of the enzyme. Hence, we have tried to purify and characterize this enzyme and also study the effect of divalent metal cations on the activity of the purified enzyme.

MATERIALS AND METHODS

Materials

All chemicals used in the present study were of analytical grade. Benzyl alcohols, β -mercaptoethanol, Coomassie Brilliant BlueR-250 were procured from SRL, India Limited. Hydrated copper sulphate, sodium acetate, potassium dihydrogen phosphate, hydrochloric acid, sulphuric acid etc. were procured from Merck, India Limited.

Methods

Fresh goat liver was obtained from local slaughter house, kept in ice and brought to our laboratory in ice-bucket. The tissue was washed

in ice-cold saline, weighed and minced to very small pieces, then homogenized in 4 volumes of cold 0.05 M potassium phosphate buffer; pH 7.4. The homogenate was centrifuged in cold (4°C) at 10,000 rpm for 45 minutes. The supernatant was collected and stored at -20°C until further use.

Assay for FMN phosphatase

The mixtures for determining the FMN-phosphatase activity contained 2×10^{-4} (M) FMN, 0.75 (M) sodium acetate buffer, pH 5, and a known amount of protein in a final volume of one ml. The mixture was incubated at 37°C for 30 minutes and the reaction was terminated by addition of 400 μ l of 17.5% TCA. The solution was then centrifuged at 2000rpm for 10 minutes to obtain an aliquot that was collected from the top while the protein portion precipitated at the bottom. 0.5ml of the aliquot was made to react with 125 μ l of K_2HPO_4 to counter the acidic effect of TCA and then 1.25ml benzyl alcohol was added to dissolve the riboflavin liberated due to action of the enzyme on FMN. The mixture was then shaken vigorously to obtain a thorough mixing and then centrifuged at 2000rpm to obtain separate layers of aqueous and benzyl alcohol phase. The enzyme was assayed spectrophotometrically at 450nm according to the procedure as adopted by Bandyopadhyay *et al.* [9, 10]. Specific activities are defined as units of activity/mg protein.

Determination of inorganic phosphate

The amount of inorganic phosphate liberated due to hydrolysis of FMN by the enzyme was measured by the method of Fiske & Subbarow (1925) [11].

Determination of protein

Protein concentration was determined by the method of Lowry *et al.* [12]. For column effluents, the protein content was estimated from the absorbance at 280nm and Bradford's method using bovine serum albumin as standard [13].

Purification of FMN phosphatase

FMN phosphatase from goat liver was purified as follows:

Step 1: Homogenate and high speed supernatant

Fresh goat liver (10 g) was washed in 0.9% NaCl and minced. Then, it was homogenized in 4 volumes of cold 50mM sodium phosphate buffer (pH 7.4) by using a motor driven, Teflon glass homogenizer. Homogenate was centrifuged at 11,000 rpm for 40 min in Remi cold centrifuge. The supernatant was collected and the pellet was discarded.

Step 2: Ammonium sulfate fractionation

The supernatant was brought from 0-55% saturation by addition of solid ammonium sulfate under continuous stirring, and the suspension was allowed to stand for 15min. Then it was centrifuged at 10,000 rpm for 20min. The supernatant was collected and solid ammonium sulfate was gradually added with continuous stirring to the supernatant to give 75% saturation. The resulting mixture was allowed to stand for 30min and then centrifuged at 10,000rpm for 40min. The precipitate was collected and dissolved in 16ml of the homogenizing buffer. The enzyme solution was dialyzed overnight against 1 litre of the buffer with 2 changes and centrifuged at 10,000 rpm to obtain a clear supernatant by removing the resulting precipitates.

Step 3: DEAE-Cellulose column chromatography

The supernatant solution obtained from the previous step was applied to a DEAE-Cellulose column previously equilibrated with the dialyzing buffer (pH 7.5). After thorough washing of the column by 100ml of the equilibration buffer, the enzyme was eluted with a linear gradient of NaCl from 0 to 1 M in the same buffer, and 5ml fractions were collected. The active fraction (0.1 M NaCl) were pooled, dialyzed against 500ml of 50mM sodium phosphate buffer (pH 7.4) and concentrated by polyethylene glycol-400.

Electrophoresis

Polyacrylamide gel electrophoresis was carried out according to the method of Laemmli [14]. The separating gel contained 10% acrylamide and the stacking gel contained 4% acrylamide. Samples

containing approximately 40 μ g protein were mixed with one-fifth volume of a sample preparing solution that contained 125mM Tris buffer (pH 6.8), 10mM EDTA, 50% glycerol and 0.5 % bromophenol blue. Electrophoresis was carried out at 100V until the dye reached the bottom edge of the gel. The gel was stained for protein for 40 min with Coomassie Brilliant Blue G-250 in 7.5% acetic acid and 40% methanol. Gels were destained overnight in an aqueous solution containing 7.5% acetic acid and 5% methanol.

Determination of various kinetic parameters of FMN-phosphatase: Km values were determined using Lineweaver and Burk plot (1934) [15]. The optimum pH of the enzyme was assessed by simply providing buffers of different pH (3, 4, 5, 6, and 7) for the FMN-phosphatase assay. The optimum temperature of the enzyme was assessed by maintaining different incubation temperatures (20°, 30°, 40°, 50°, 60° and 70° C) for the FMN-phosphatase assay.

Determination of substrate specificities for the enzyme

Beside FMN, other substrates for the enzyme like pNPP, AMP and G-6-P were used to determine the substrate specificity of the enzyme. The mixtures for determining the substrate specificity contained 2×10^{-4} M. FMN or other organic phosphate ester for carrying out the enzyme assay as described earlier. The amount of phosphate liberated from the 500 μ l aliquot of the samples was determined according to the method of Fiske & Subbarow (1925) [11] to identify the order of substrate specificity of the enzyme.

Determination of the effect of various divalent cations and other substances on the enzyme activity

The effect of different divalent cations on the enzyme activity was studied. Salts of divalent cations like cadmium chloride, hydrated copper sulphate, hydrated zinc sulphate, hydrated calcium chloride, etc (0.001, 0.01, 0.1, 1, 2, 3, 4 and 5mM concentrations) were used. The effects of some other cations like aluminium (0.001, 0.01, 0.1, 1, 2, 3, 4 and 5mM concentrations) and iron (0.001, 0.01, 0.1, 1, 2, 3, 4 and 5mM concentrations) and also some other substances like ammonium molybdate (0.001, 0.01, 0.1, 1, 2mM concentrations), SDS (0.05, 0.1, 0.15, 0.2, 0.25 and 0.3% concentrations), phosphate (2, 4, 8 and 10 mM concentrations) etc. was studied. enzyme-assay was performed after pre-incubating (15 minutes at 37°C) the enzyme with a particular concentration of the cation or other substances and the comparison of riboflavin produced with that in case of the controlled showed the effect of the respective substances on the enzyme activity.

Determination of the effect of various -SH blockers and protection from the blockage by GSH

The effect of various inhibitors like pCMB (0.001, 0.01, 0.1, 1 and 2 mM) DTNB (0.01, 0.1, 1, 2 and 3mM) and NEM (0.1, 1, 2, 5 and 10mM) on the enzyme activity was studied in the similar way as done while studying the effect of divalent cations. In order to study the effect of thiol protectors like GSH on the inhibitory effect of the various thiol inhibitors, we initially incubated the enzyme with a particular concentration of GSH (0.5, 1, 1.5 or 2mM against 2mM pCMB, 1, 2, 2.5, 3 and 4mM against 3mM DTNB and 1, 2.5 and 5mM against 10mM NEM) and observed the change in enzyme activity.

Statistical evaluation

Each experiment was repeated at least three times. Data are presented as means \pm S.E. Significance of mean values of different parameters were analyzed using one way analysis of variances (ANOVA) after ascertaining the homogeneity of variances between the groups. Pairwise comparisons were done by calculating the least significance. Statistical tests were performed using Microcal Origin version 7.0 for Windows.

RESULTS

After homogenizing the liver tissue, the FMN hydrolyzing capacity of the enzyme was observed taking different fractions of the cell which were the homogenate fraction, the nuclear-pellet portion, the nuclear-supernatant portion, the centrifuged mitochondrial fraction and the cell-free post-mitochondrial supernatant. (Table1). The maximum concentration of this enzyme was noticed in the post-mitochondrial fraction as this portion of the extract showed the

maximum hydrolyzing capacity. Nuclear and mitochondrial pellet also showed some phosphatase activity which may be due to some non-specific acid phosphatase present in those fractions.

Table 1: Intracellular localization of FMN phosphatase activity from goat liver

| Cell fraction | Protein (mg/ml) | Specific activity (μM of riboflavin/mg protein/min) |
|-----------------------------|-----------------|---|
| Homogenate | 36 | 0.029 |
| Nuclear pellet | 21 | 0.017 |
| Cell free supernatant | 56 | 0.025 |
| Mitochondrial pellet | 43 | 0.034 |
| Post mitochondrial fraction | 35 | 0.046 |

Table 2: Purification of FMN phosphatase from goat liver

| Step | Volume (ml) | Total protein (mg) | Total activity (units) | Specific activity (units/mg) | Purification (fold) |
|---|-------------|--------------------|------------------------|------------------------------|---------------------|
| Homogenate | 29 | 1044 | 30.276 | 0.029 | 1 |
| Post mitochondrial fraction | 20 | 700 | 32.2 | 0.046 | 1.586 |
| 55% (NH ₄) ₂ SO ₄ | 12 | 168 | 9.576 | 0.057 | 1.966 |
| 75% (NH ₄) ₂ SO ₄ | 10 | 86.25 | 5.693 | 0.066 | 2.276 |
| Dialysis and centrifugation | 6.75 | 81 | 8.424 | 0.104 | 3.586 |
| DEAE-Cellulose column eluate | 5 | 0.146 | 3.321 | 22.749 | 784.448 |

The major results for purification of FMN phosphatase from goat liver are summarized in Table 2 which shows that the specific activity of the crude enzyme gradually increased with partial purification of the enzyme by many folds. The dialysate showed a

specific activity of 0.104 units/mg which is 3.586 folds more than the homogenate, 2.261 folds more than the post-mitochondrial fraction and 1.576 folds more than the 75% saturated pellet. The elute fraction with the maximum specific activity showed an increase in its activity by about 219 folds than that of the dialysate and by nearly 785 folds more than the homogenate. Several rounds of purification were carried out and all gave similar results. Figure 1 shows that purified fraction of tube number 23 has maximum enzyme specific activity and minimum amount of total protein ($*p < 0.001$).

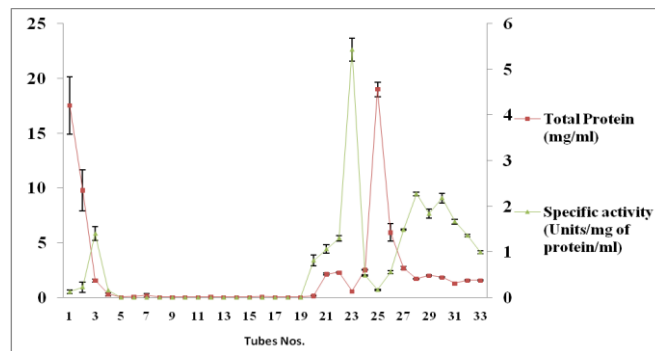


Fig. 1: Specific activity against Total protein of the different fractions of the ion-exchange eluate. T- 23 showed the peak of activity. All data are expressed as mean \pm S.E. Here every experiment was repeated three times ($*p < 0.001$).

Native polyacrylamide gel electrophoresis was performed with the dialysate as well as DEAE cellulose column purified active fraction. The column purified fraction was subjected to native polyacrylamide gel electrophoresis after concentrating the fraction with PEG 400 in order to increase the protein content of the fraction. Gels were stained with Coomassie Brilliant Blue G-250. Dialysate preparation showed several protein bands on staining whereas the column purified fraction showed one single protein band on polyacrylamide gel. Unstained lane was cut into pieces, eluted with buffer to get the purified enzyme and the activity was assayed. It was found that only the piece corresponding to 2cm from the origin showed FMN phosphatase activity (Fig. 2).

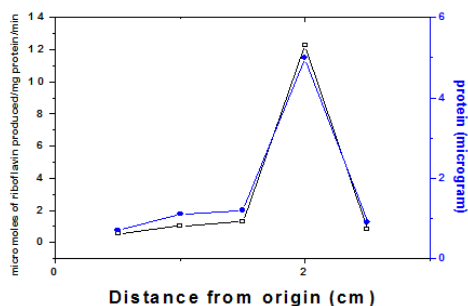


Fig. 2: Protein and activity profile of purified FMN phosphatase on

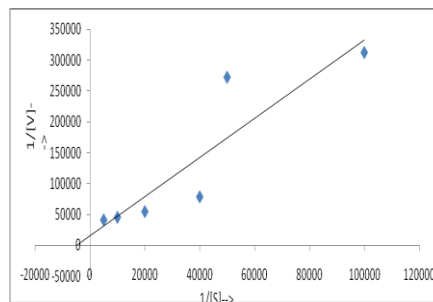
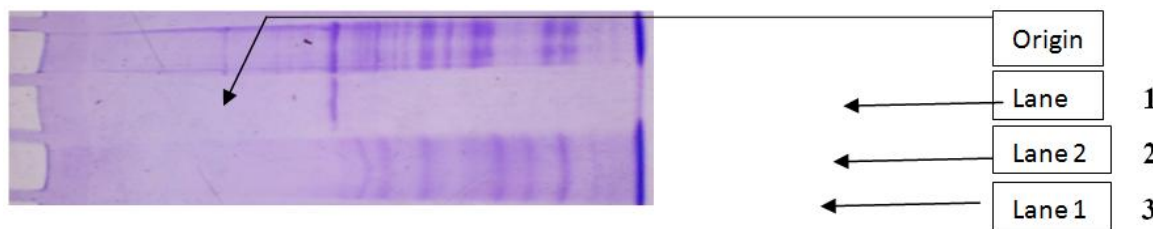


Fig. 3: Determination of the Km value using Lineweaver and Burk plot



Polyacrylamide gel electrophoresis. Protein-band with the specific enzyme activity located at 2cm from the origin. Lane 1-Marker, Lane 2-FMN-phosphatase from goat liver, Lane 3- Protein bands of the dialysate

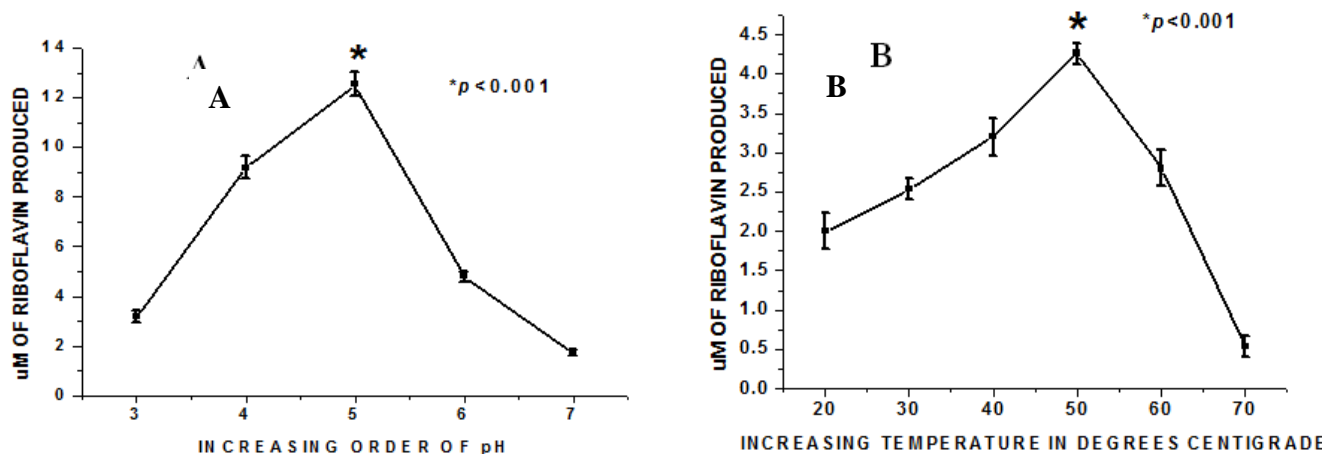


Fig. 4: Optimum pH (A) and temperature optima (B) of the partially purified FMN phosphatase enzyme of goat liver. All data are expressed as mean \pm S.E. Here every experiment was repeated three times ($*p < 0.001$).

Taking the fraction from the ion-exchange elute that showed the maximum specific activity the K_m value of the enzyme for FMN was determined using Lineweaver and Burk plot. It was found to be 0.0002M (Fig. 3).

The amount of phosphate liberated by the partially purified enzyme from the various probable substrates for the enzyme like pNPP (a synthetic substrate) and other physiological substrates like FMN, AMP and G-6-P were assessed (Table 3). FMN is established to be the best physiological substrate with a relative specific activity of 86.956 to the partially purified enzyme although pNPP, which is a synthetic substrate, was hydrolysed the most ($*p < 0.001$).

Table 3: μ M of phosphate liberated by the partially purified enzyme from different substrates: pNPP, FMN, AMP and G-6-P showing the substrate specificity of the enzyme. All data are expressed as mean \pm S.E.

| Substrate (200 μ M) | Phosphate liberated (μ M) | Relative substrate specificity |
|-------------------------|--------------------------------|--------------------------------|
| pNPP | 107.1 \pm 0.40415 | 100 |
| FMN | 93.13 \pm 1.21289 | 86.956 |
| AMP | 50.4 \pm 1.21244 | 47.059 |
| G-6-P | 26.6 \pm 1.21244 | 24.837 |

The optimum pH and temperature for FMN phosphatase activity was found to be pH 5.0 and 50°C respectively, above and below which the enzyme activity declines (Fig. 4A and 4B).

The effect of several divalent cations on the activity of FMN phosphatase was studied (Table 4). Copper cations showed an increasing graded inhibitory effect on the partially purified enzyme activity with increasing concentrations of the cations. At 5mM concentration, copper almost completely inhibited the activity of the partially purified enzyme inhibiting it by 96%. Like the Copper cations, the cadmium cations also showed an increasing graded inhibitory effect on the enzyme activity with increasing

concentrations of the cations. At 5mM concentration, cadmium appeared to inhibit the activity of the partially purified enzyme by 92.15%. Zinc cations showed a graded increase in its inhibitory effect on the partially purified enzyme with its increasing concentrations. At 5mM concentration; zinc appeared to inhibit the activity of the partially purified enzyme by 66.67%. Calcium produced positive influence on the enzyme at its lower concentrations. At 5mM concentration, calcium appeared to inhibit the activity of the partially purified enzyme by 27% although it inhibits the enzyme maximally (40.33%) at 2mM concentration. Enzyme reactions are found to be inhibited by

metals which may form complex with the substrate, or combine with the protein-active group of the enzymes, or react with the enzyme-substrate complex [2]. To understand the nature of inhibition of cations like copper, cadmium and zinc, the results of their inhibitory effects at a particular concentration (1mM) against a rising concentration of the substrate was displayed on a Lineweaver-Burk plot (Fig 5). An increase in the slope in the presence of the inhibitor indicates a reduction in the reaction speed at low substrate levels.

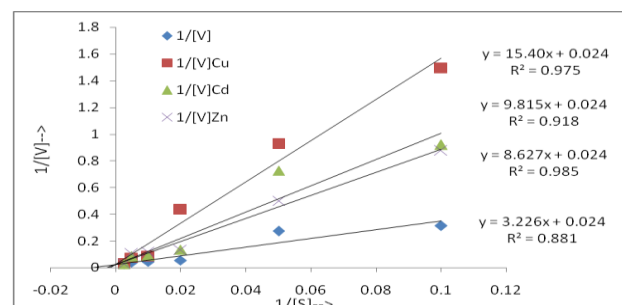


Fig. 5: Lineweaver Burk-plot showing increase in slope of the K_m -graph due to competitive inhibition by divalent heavy metal cations like copper, cadmium and zinc.

Table 5: Inhibition of Partially Purified FMN-Phosphatase from Goat-liver by other Cations and other substances. All data are expressed as mean \pm S.E. Here every experiment was repeated three times ($*p < 0.001$)

| Inhibitors | % of Inhibition |
|-----------------------------------|--------------------|
| Hydrated aluminium chloride (5mM) | 82.71% \pm 1.24% |
| Hydrated ferric chloride (5mM) | 100% \pm 0.00% |
| Hydrated ammonium molybdate (2mM) | 100% \pm 0.00% |
| SDS (0.3%) | 100% \pm 0.00% |
| Phosphate (10mM) | 54% \pm 1.15% |

The effects of some other cations like aluminium and iron and also some other substances like ammonium molybdate, phosphate on the SDS partially purified enzyme was studied (Table 5).

The action of thiol inhibitors like pCMB, DTNB and NEM on the activity of the partially purified enzyme was studied using their graded concentrations. It was also looked into the matter whether protection of the activity of the partially purified enzyme could be afforded against the inhibitory action of the thiol inhibitors by the thiol protector like GSH on pre-incubation of the partially purified enzyme with it (Table 6).

Table 4: Inhibition of Partially Purified FMN-Phosphatase from Goat-liver by divalent Cations. All data are expressed as mean \pm S.E. Here every experiment was repeated three times (* $p < 0.001$)

| Groups | μM of Riboflavin produced | % inhibition |
|---------|--------------------------------------|--------------|
| Control | 20.4 ± 0.23 | ---- |
| 0.001M | 18.8 ± 0.23 | 7.84 |
| 0.01M | 12.53 ± 0.48 | 38.53 |
| 0.1M | 10.93 ± 0.35 | 46.42 |
| 1M | 10.13 ± 0.13 | 50.34 |
| 2M | 9.73 ± 0.13 | 52.38 |
| 3M | 5.6 ± 0.46 | 72.54 |
| 4M | 4.13 ± 0.13 | 79.75 |
| 5M | 1.66 ± 0.23 | 92.15 |

EFFECT OF CADMIUM

| Groups | μM of Riboflavin produced | % inhibition |
|---------|--------------------------------------|--------------|
| Control | 20 ± 0.23 | ---- |
| 0.001M | 19.73 ± 0.13 | 1.35 |
| 0.01M | 18.4 ± 0.46 | 8 |
| 0.1M | 14.4 ± 0.23 | 28 |
| 1M | 11.2 ± 0.46 | 44 |
| 2M | 4.4 ± 0.69 | 78 |
| 3M | 3.2 ± 0.23 | 84 |
| 4M | 1.47 ± 0.13 | 92.67 |
| 5M | 0.8 ± 0.23 | 96 |

EFFECT OF COPPER

| Groups | μM of Riboflavin produced | % inhibition |
|---------|--------------------------------------|--------------|
| Control | 18 ± 0.46 | ---- |
| 0.001M | 11.6 ± 0.46 | 35.55 |
| 0.01M | 10.53 ± 0.13 | 47.5 |
| 0.1M | 10.4 ± 0.23 | 42.22 |
| 1M | 6.93 ± 0.13 | 61.5 |
| 2M | 6.67 ± 0.13 | 62.94 |
| 3M | 6.53 ± 0.27 | 63.72 |
| 4M | 6.27 ± 0.27 | 65.17 |
| 5M | 6.0 ± 0.00 | 66.67 |

EFFECT OF ZINC

| Groups | μM of Riboflavin produced | % inhibition |
|---------|--------------------------------------|--------------|
| Control | 15.2 ± 0.46 | ---- |
| 0.001M | 16.8 ± 0.23 | -10.53 |
| 0.01M | 17.8 ± 0.13 | -17.57 |
| 0.1M | 18.0 ± 0.23 | -18.42 |
| 1M | 17.07 ± 0.13 | -12.3 |
| 2M | 9.07 ± 0.13 | 40.33 |
| 3M | 9.6 ± 0.23 | 36.84 |
| 4M | 10.27 ± 0.13 | 32.4 |
| 5M | 11.7 ± 0.13 | 27 |

EFFECT OF CALCIUM

K_i values of Cu^{2+} , Cd^{2+} and Zn^{2+} for the enzyme FMN phosphatase are 3.35, 0.11 and 0.78 respectively.

Table 6: Effects of thiol inhibitors on partially purified FMN phosphatase activity and protection from the blockage by GSH

| Inhibitors | Percent inhibition | Percent Protection by reduced glutathione* |
|--|--------------------|--|
| p-chloromercuribenzoate (2mM) | 100 | 98.383 |
| 5,5'-dithiobis-2-nitrobenzoic acid (3mM) | 100 | 99.03 |
| N-ethylmaleimide (10mM) | 57.333 | 95.797 |

* GSH was used at 2mM concentration for PCMB, at 4mM for DTNB and at 5mM for NEM.

It was observed that at 2mM concentration, pCMB could inhibit the partially purified enzyme activity totally. However, when the partially purified enzyme was pre-incubated with GSH it was found that the inhibitory effect of pCMB gradually declined with the increasing concentrations of GSH, decreasing the inhibitory effect of 2mM pCMB to 1.697% and protecting the activity of the partially purified enzyme by 98.383%.

Similar results were observed in case of the inhibitory action of 3mM DTNB and similar protection was provided by GSH. NEM also was observed to inhibit the activity of the partially purified enzyme to a certain extent with graded concentrations, inhibiting the activity up to 57.333% at 10mM concentration.

However, when the partially purified enzyme was pre-incubated with 5mM GSH, its inhibitory action at 10mM concentration was reduced to 4.203% only with 95.797% protection of the enzyme activity by GSH.

DISCUSSION

Riboflavin-5'-phosphate or Flavin mononucleotide (FMN) is an essential coenzyme form of vitamin, Riboflavin, and occurs widespread in plant and animal organisms [1]. A great number of mammalian enzymes require FMN or FAD as coenzymes for their activities and both the flavin coenzymes are associated with many of the components of the electron transport chain of mitochondria which is associated with oxidative phosphorylation. Again riboflavin is related to the metabolic process of proteins, oxidation of fatty acids and is also vital for proper maintenance and health of

ectodermal tissues like skin and oral mucosa. However, tissue level of FAD, FMN or riboflavin at any point of time depends on the degree of activities of FAD-pyrophosphatase (which hydrolyzes FAD to FMN) and FMN-phosphatase (which hydrolyzes FMN to riboflavin). The existence of FMN-phosphatase has been shown in several plants as well as in animal organs. However, there is no information to date about goat liver FMN-phosphatase which, our initial studies showed, is a good source of the enzyme. Hence, we have tried to observe the activity of the enzyme from the goat liver and also to understand the effect of heavy metal cations on the enzyme activity [16-19]. FMN phosphatase is an acid phosphatase which causes hydrolysis of FMN and converts it back to the riboflavin. The phosphatase activity was found to be localized in the post mitochondrial fraction of the tissue, so further studies were carried out with this fraction. From this post mitochondrial fraction, FMN phosphatase was purified up to 785 folds by salt fractionation using ammonium sulphate.

K_m value is a measure of the affinity of the enzyme for the substrate. The K_m value of the enzyme for FMN was calculated from Lineweaver-Burk plot. The K_m of the enzyme in partially purified form of goat liver is 0.0002 M for FMN whereas the K_m of the enzyme obtained from rat liver for FMN, as observed by McCormick et al (1962), is 0.001 M [8].

The optimum pH of the enzyme is found to be at 5. It is known that acid phosphatases are most active in an acidic environment, quickly losing activity at $\text{pH} > 5.5-6.0$ [20-23]. Moreover, it has also been said that the optimum for FMN hydrolysis by phosphatases in the extracts of most animal tissues is near pH 5 [8] where it is also said

that the characteristic optimum for the liver and kidney enzymes is similar to that of the acid prostratic phosphatase [24] but FMN is less readily hydrolyzed by the latter enzyme.

The FMN phosphatase from *Tetrahymena* and perhaps also from *Limax*, is said to have greater hydrolytic activity in the more acid range of pH 3 and 4 [8], which indicates that our FMN phosphatase of mammalian liver requires less acidic medium for its best activity than the above mentioned non-mammalian ones. For an enzyme the optimum pH may vary from substrate to substrate because the conformational and the ionization changes in the enzymes at different pH may make it more suitable for acting on different substrates.

It has been found that as the kinetic energy of the reactant molecules increases with the increase of temperature, there is a gradual increase in the rate of enzyme action with the increase of temperature. The optimum temperature of the enzyme is found to be 50°C. This indicates that this enzyme is quite heat-resistant and hence a very suitable enzyme required at different steps in body metabolism wherever FMN needs to be hydrolyzed. However beyond 50°C the activity of the enzyme gradually decreases perhaps due to denaturation of the enzyme.

Although phosphomonoesterases with a wide range of specificities have been reported from extracts of animal tissues [25, 26], only in a few instances [27, 28] has FMN been included in those organic phosphate esters tested as potential substrates for well characterized phosphatases. FMN appears to be the best physiological substrate tested with this liver phosphatase although a synthetic substrate, pNPP is readily and maximally hydrolyzed. At pH 5, other organic phosphate esters like AMP and G-6-P are less effectively hydrolyzed. Since this enzyme is substrate specific, it can be said that this enzyme is not involved in trans-phosphorylation according to Morton (1953) [29].

Amongst the divalent cations, copper is the most potent inhibitor of the enzyme inhibiting the partially purified enzyme by 96%. It is similar to the finding that the FMN hydrolysis is strongly inhibited by Cu^{2+} in case of Bovine kidney low molecular weight acid phosphatase [30]. The inhibitory effect of Cd^{2+} is found to be similar to the inhibitory effect of Cu^{2+} . The effect of zinc as a divalent cation inhibitor to the enzyme was also investigated on the partially purified enzyme. It is found that similar results of inhibition of enzyme by zinc and other cations have been shown [31]. The inhibition of FMN phosphatase by Zn^{2+} is of some interest, as this cation is the most effective activator of both flavokinase from liver [8, 32] and vitamin B6 kinases from mammalian tissues [5, 6, 8, 32] where contaminating phosphatases often mask kinase activity. Although Zn^{2+} rather than the usual phosphokinase activator Mg^{2+} is specifically required by B6 kinase from mammals, a large part of the apparent activation by Zn^{2+} of liver flavokinase is undoubtedly due to suppression of interfering FMN phosphatase [7].

In the case of calcium, we find that at lower doses Ca^{2+} shows activation effect on the partially purified enzyme but this gradually switches over to inhibitory effect at higher doses (5mM) which is really of some interest as regard the method and nature of inhibition on the enzyme by this metal cation.

Since from the above discussions we come to know that the enzyme of our interest is inhibited by several divalent cations and in the order $\text{Cu}^{2+} > \text{Cd}^{2+} > \text{Zn}^{2+} > \text{Ca}^{2+}$, which is similar to the findings in the case of FMN phosphatase from rat liver [3], it can be said that sensitivity of the enzyme to such cations may emphasize the sulfhydryl nature of this goat hepatic FMN phosphatase.

Zn^{2+} , Cd^{2+} and Cu^{2+} competitively inhibit the enzyme activity which was clear from the Lineweaver Burk plot. Competitive inhibitors may work by direct competition with the substrate by binding to the active site, or by binding to a remote site and causing a conformational change in the enzyme. Both mechanisms give identical kinetic results. Similar results with cadmium and zinc have been observed with FMN phosphatase from goat heart [33].

Beside the above mentioned cations, it is found that aluminium and ferric cations also shows inhibitory effect on the enzyme

activity. At lower doses both aluminium ions and ferric ions activates the enzyme but their activating effect gradually decreases with increasing concentrations of these cations and they show potent inhibitory effect. Our deductions about the effect of aluminium ions on the enzyme activity are comparable to the findings on the FMN phosphatase from rat liver [8]. Our results from the study of the inhibitory effect of other substances like ammonium molybdate, SDS and phosphate both on the crude and partially purified enzyme indicate that ammonium molybdate and SDS are potent inhibitors to the enzyme showing 100% inhibition on the enzyme. Phosphate also inhibits the enzyme to a large extent which is similar to the findings in rat liver [8]. As is seen from data in our results for the partially purified enzyme, pCMB (2mM) and DTNB (3mM) are potent inhibitors of the goat liver phosphatase at lower doses while NEM could shows its inhibitory effect only at large concentrations (10mM) which is similar to the findings in rat liver [8]. GSH has been found to protect the enzyme and negate the inhibition caused by pCMB and also that of DTNB and help the enzyme to retain almost its original activity. These findings suggest that a sulfhydryl group may be involved with FMN phosphatase activity. A higher dose of GSH (5mM) is required to protect the enzyme against NEM which indicates that NEM being a co-valent modifier of the SH-group requires GSH at a higher dose to produce some protective effect on the enzyme activity. NEM reacts with -SH groups to form stable complex through covalent binding [22].

As we know that several metabolic pathways in living cells are dependent on the level of FAD, FMN and riboflavin in the cell, the enzymes concerned with their conversions and production like FAD pyrophosphatase, FMN phosphatase, flavokinase and FAD synthetase are of immense importance as in availability of any of these can make the metabolic pathways to come to an abrupt stagnancy and there will be a crisis in the production of energy. FMN Phosphatase, which converts FMN to riboflavin, has been found to be severely inhibited by several cations like copper, cadmium, zinc, calcium, aluminum and iron and hence exposure to such heavy cations can cause FMN Phosphatase enzyme inhibition and this may lead to metabolic failure and several disorders.

CONCLUSION

It can be concluded that a specific enzyme for hydrolyzing FMN, namely FMN phosphatase, from goat liver has been identified, characterized and purified. It is a cytosolic, acidic, heat-resistant phosphatase ($K_m = 0.0002\text{M}$) and of low molecular weight with a sulfhydryl group at or near its active site and this FMN phosphatase is a good target of the divalent cations.

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REFERENCES

1. Beinert H. Flavin coenzymes. The Enzymes. 2nd ed. New York: Academic Press; 1960.p. 339-416.
2. Karrer P, Schöpp K, Benz F. Synthesen von Flavinen IV. Helv Chim Acta 1935; 18:426-429.
3. McCormick DB. Flavokinase activity of rat tissues and masking effect of phosphatases. Proc Soc Exp Biol Med 1961;107:784-786.
4. Tiwari BK, Khosa RL. Evaluation of the hepatoprotective and antioxidant effect of *Berberis asiatica* against experimentally induced liver injury in rats. Int J Pharm Pharmaceutical Sci 2010; 2(1):92-97.

5. McCormick DB, Guirard BM, Snell EE. Comparative inhibition of pyridoxal kinase and glutamic acid decarboxylase by carbonyl reagents. *Proc Soc Exp Biol Med* 1960;104:554-557.
6. McCormick DB, Snell EE. Pyridoxal kinase of human brain and its inhibition by hydrazine derivatives. *Proc Natl Acad Sci* 1959; 45:1371-1379.
7. Lardy HA. The influence of inorganic ions on phosphorylation reaction. Baltimore:John Hopkins press;1951.p.477-499.
8. McCormick DB, Russell M. Hydrolysis of flavin mononucleotide by acid phosphatases from animal tissues. *Comp Biochem Physiol* 1962; 5: 113-121.
9. Bandyopadhyay D, Chatterjee A K, Datta A G. Effect of cadmium treatment on hepatic flavin metabolism. *J Nutr Biochem* 1993;4:510-514.
10. Bandyopadhyay D, Chatterjee A K, Datta A G. Effect of cadmium on purified hepatic flavokinase: Involvement of reactive-SH group(s) in the inactivation of flavokinase by cadmium. *Life Sciences* 1997; 60(2):1891-1903.
11. Fiske CH, Subbarow Y. The colorimetric determination of phosphorus. *J Biol Chem* 1925; 66(2):375-400.
12. Lowry O H, Rosebrough N J, Parr A R, Randall R J. Protein measurement with folin-phenol reagent. *J Biol Chem* 1951; 193:265-275.
13. Bradford M M. A rapid and sensitive method for the quantization of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; 72:248-254.
14. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; 227 (5259):680-685.
15. Line weaver H, Burk D. The Determination of Enzyme Dissociation Constants. *J Chem Soc* 1934; 56: 658-666.
16. Khan AR, Rubina N, Mukhtiar H, Saeed A. The Low and High Molecular Weight Acid Phosphatases in Sheep Liver. *J Chem Soc Pak* 1999;19(1):60-69.
17. Shibko S, Tappel AL. Acid phosphatase of the lysosomal and soluble fraction of rat liver. *Biochim Biophys Acta* 1963;73:76-86.
18. Heinrichson RL. Purification and characterization of a low molecular weight acid phosphatase from bovine liver. *J Biol Chem* 1969; 244:299-307.
19. Panara F, Mileti A. Subcellular localization of high-and low-molecular weight acid phosphatases from chicken liver. *Int J Biochem* 1986;18(11):1057-1059.
20. Sandoval FJ, Yi Z, Sanja R. Flavin Nucleotide Metabolism in Plants, Monofunctional Enzymes Synthesize FAD in Plastids. *J Biol Chem* 2008; 283(45):30890-30900.
21. Mitsuda H, Tsuge H, Tomozawa Y, Kawai F. Multiplicity of acid phosphatase catalyzing FMN hydrolysis in spinach leaves. *J Vitaminol* 1970;16:52-57.
22. Mitsuda H, Tomozawa Y, Tsuboi T, Kawai F. Levels of enzymes for biosynthesis and degradation of Flavins in spinachs. *J Vitaminol* 1965;11:20-29.
23. Granjeiro JM, Ferreira CV, Juca MB, Taga EM, Aoyama H. Bovine kidney low molecular weight acid phosphatase: FMN-dependent kinetics. *Biochem Mol Biol Int* 1997; 41:1201-1208.
24. Schmidt G. Acid prostratic phosphatase. *Meth Enzymol* 1955; 2,: 37-47.
25. Roche J. Phosphatases. *The Enzymes*. New York:Academic Press; 1953.p. 473-510.
26. Schmidt G. Nonspecific acid phosphomonoesterases. *The Enzymes*. 1961.p.37-47.
27. Tsuboi KK, Hudson PB. Acid phosphatase. VI. Kinetic properties of purified and erythrocyte phosphomonoesterase. *Arch Biochem Biophys* 1956;61:197-210.
28. Garen A, Levinthal C. A fine-structure genetic and chemical study of the enzyme alkaline phosphatase of *E. coli*. *Biochem Biophys Acta* 1960; 38:470-483.
29. Morton RK. Transferase activity of hydrolytic enzymes. *Nature* 1953;172:65-68.
30. Zhang ZY, Van Etten RL. Purification and characterization of a low-molecular-weight acid phosphatase--a phosphotyrosyl-protein phosphatase from bovine heart. *Arch Biochem Biophys* 1990;282:39-49.
31. Goodlad GAJ, Mills GT. The Acid Phosphatases of Rat liver. *Biochem J* 1956; 66:346-354.
32. McCormick DB. Purification and properties of flavokinase from rat liver. *Fed Proc* 1961;20:447-451.
33. Dey M, Mukherjee D, Dutta M, Mallik S, Ghosh D, Ghosh A K, Chattopadhyay A, Bandyopadhyay D. Flavin Mono Nucleotide Phosphatase from Goat Heart: A forgotten enzyme of an important metabolic pathway. *J Cell Tissue Res* 2013;13(3):3851-3858.
34. Shrinivas B, Rajesh P, Saxena M. Colostrum: All in one medicine. *Int J Pharm Pharmaceutical Sci* 2010; 2(1):31-36.