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Original Research Article

Gluconeogenic Fructose 1, 6-bisphosphatase from *Ginkgo biloba* L.: Isolation and characterization

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Abstract: Fructose 1,6-bisphostase (FBPase) was partially purified from mature leaves of *Ginkgo biloba* which is a rare medicinal plant effective as antioxidant, adaptogenic and memory enhancer. The purification procedure involved homogenization, low speed centrifugation, streptomycin sulphate precipitation, ammonium sulphate fractionation and chromatography on Bio Gel-A 0.5 m. The level of purification was about 22 fold and the recovery was about 56% at the final stage of purification. D-fructose-1,6-bisphosphate (FBP) was found to be the specific substrate for *Ginkgo biloba* FBPase. Among the other hexose phosphates, D-glucose-6-phosphate, D-fructose-6-phosphate and D-galactose-6-phosphate could not act as substrates for this enzyme. However, this enzyme showed traces of activity in presence of D-fructose-1-phosphate. With the increase in substrate (FBP) concentration from 0 to 8 mM the activity of the enzyme increased linearly. The FBPase activity was directly proportional to the time of incubation upto 60 minutes and with respect to protein concentration upto 400 µg. The Bio Gel A 0.5 m purified *G. biloba* FBPase showed a K_m values of 1.86 mM for D-fructose-1,6-bisphosphate. The FBPase required Mg^{2+} as a cofactor. Mn^{2+} and Mg^{2+} appreciably stimulated the FBPase activity in a concentration guided manner. The monovalent cations, K^+ and Na^+ had little effect while Zn^{2+} was extremely inhibitory to the enzyme.

Key wods: *Ginkgo biloba*, enzyme purification, enzyme characterization, fructose 1,6-bisphosphatase, D-fructose-1,6-bisphosphate, gluconeogenesis

Introduction

Fructose -1,6- bisphosphatase enzyme (FBPase, EC: 3.1.3.11) in soluble and particulate forms, is considered to be related with two vital biochemical events namely gluconeogenesis and photosynthesis. This enzyme also plays a key regulatory role in the sucrose biosynthetic pathway (Huber *et al.,* 1985; Daie, 1993). In higher plants, at least two forms of the enzyme have been identified: chloroplastic and cytosolic (Kelly *et al.,* 1976). Of these, the cytosolic form of the enzyme is homologous to the enzyme present in the gluconeogenic pathway (Ladror *et al.,* 1990).

FBPase was originally considered as a specific hexose bisphosphatase. Later on, it was named as fructose-1,6-bisphosphatase (Gomori, 1943) which is markedly specific for fructose -1,6- bisphosphate (FBP) [Mokrasch and McGlivery, 1956] that transforms the substrate to fructos-6 phosphate and inorganic phosphate. The gluconeogenic FBPase is an allosteric enzyme which is strongly inhibited by the negative modulator AMP and stimulated by 3-phosphoglycerate and citrate.

FBPase has so far been isolated and characterized from a considerable number of microbial, plant and animal sources. The enzyme was purified and its properties investigated from a bacteria *Acinobacter iwoffi* (Mukkada and Bell, 1971). Fujita and Freese (1979) have purified and characterized the enzyme from *Bacillus subtilis* and Funayama *et al.,* (1979) purified the same from *Saccharomyces cerevisiae*. In *Mangifera indica* the activity of the enzyme was found to be at the maximum during fruit ripening. It was activated by citrate and inhibited by AMP and Zn^{2+}. Yeast cell grown in sugar medium exhibited FBPase activity (Gancedo and Gancedo, 1971). Activity of the enzyme was also found in germinating seeds of *Sida spinulosa* and appreciable titre of activity was also detected from the cotyledons of *Corylus avellana* (Li and Ross, 1988).

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FBPase of chloroplastic origin was isolated from spinach leaves (Baier and Latzko, 1975). The enzyme was strongly activated by light and had a molecular weight of 160 kda with possibly 4 equal subunits. Cytosolic FBPase from spinach leaves have been purified by Zimmermann *et al.,* (1978) which showed a molecular weight of 130 kDa. This cytosolic enzyme regulated sucrose biosynthesis in plant leaves. Khayat *et al.,* (1993) purified cytosolic FBPase to 472 fold from *Beta vulgaris* L. leaves, the activity of which was light dependent in an indirect manner unlike the chloroplastic FBPase which is directly light regulated. The cytosolic enzyme was purified to homogeneity from castor oil seed endosperm (*Ricinus communis* L. cv. Hate) by Moorhead *et al.,* (1994). Cytosolic FBPase was purified from Spinach leaves which resolved to a single band of 39 kDa. This protein showed strong cross reactivity with plant cytosolic FBPase but no cross reactivity with chloroplastic FBPase (Hur and VasConselos, 1998). Cytosolic FBPase was also isolated from apple leaves (Zhou and Cheng, 2004). The enzyme was a homotetramer of 37 kDa units. It was stimulated by Mg^{+2} and Mn^{+2} but competitively inhibited by fructose-2,6-bisphosphate.

Despite many reports of the occurrence of FBPase in different plant systems, the information regarding the enzyme from gymnosperm is lacking. This is an attempt to bridge that gap by reporting the occurrence of the enzyme and its fundamental characterization from a famous gymnosperm *Ginkgo biloba* L. The study may pave ways toward understanding the purification, metabolic regulation cloning and its manipulation leading to new insights that may generate suitable applications.

Materials and Methods

Plant Material

Mature leaves of *Ginkgo biloba* were collected in ice-box and immediately brought to the laboratory for analysis. The plant material was collected from Lloyd's Botanical Garden, Darjeeling between 9.00 and 10.00 AM during the month of August-September. The ambient temperature was 21 ± 2°C with 80 ± 4% RH during the period. However, all experimental procedure was carried out at 0°C.

Partial Purification of gluconeogenic Fructose-1, 6-bisphosphatase from Ginkgo biloba

The isolated leaf tissues of the experimental plant were washed several times with cold distilled water followed by chilled 50 mM Tris-HCl (pH 7.5) buffer containing 0.2 mM ME and the partial purification of fructose-1, 6-bisphosphatase is done following the method outlined below:

Homogenate: 50gms of buffer washed, deribbed, leaf tissues obtained from *Ginkgo biloba was* homogenized with 3-volumes of 50 mM Tris-HCl (pH 7.5) the homogenate was centrifuged at 1000 RPM for 2 minute and the supernatant was collected which was the homogenate fraction.

Low speed supernatant: The homogenate fraction was then centrifuged at 10,000 RPM for 20 minutes in a Remi C-24 centrifuge. The pellet was discarded and the supernatant fraction was recovered from the centrifuge tubes. This fraction was named the 10K supernatant.

Streptomycin sulphate precipitation: In order to remove contaminating nucleic acids (if any) in the form of precipitate, streptomycin sulphate powder was gently added to the low-speed supernatant with constant stirring (by using a Remi cyclomixer) to a final concentration of 1% (w/v). After proper mixing, it was kept in an ice bucket for 20 minutes followed by a spin at 10,000 RPM for 15 minutes. The pellet was discarded and the supernatant was collected (SS fraction).

Ammonium sulphate fractionation: The streptomycin sulphate treated fraction obtained from the previous step was made 0-30% saturated with ammonium sulphate [$(NH_4)_2SO_4$] by adding requisite quantity (17.6 gm/100 ml) solid $(NH_4)_2SO_4$ salt slowly with constant stirring (using a Remi magnetic stirrer). It was kept at 0°C for 15 minutes and then centrifuged at 10000 RPM for 20 minutes. The pellet was discarded and the supernatant fraction was made 30-70% saturated with $(NH_4)_2SO_4$ by adding requisite quantity (27.3 gms per 100 ml) solid salt slowly with constant stirring. It was kept at 0°C for 15 minutes and then centrifuged at 10000 RPM for 20 minutes. The pellet obtained was collected and dissolved in minimal volume of 50 mM Tris-HCl (pH 7.5)

buffer having 0.2 mM ME and dialyzed over-night against the same buffer (500 volumes with one change). On completion of dialysis, the 30-70% $(NH_4)_2SO_4$ fraction was recovered from the dialysis bag (A_2S-fraction).

Molecular sieve chromatography through BioGel A-0.5m: The bulk volume of the dialyzed $(NH_4)_2SO_4$ fraction obtained from the previous step was loaded onto a column (0.6 x 7.0 cm) of BioGel A-0.5m [before this experimentation, the column material was pre-equilibrated with 50 mM Tris-HCl buffer (pH 7.5) containing 0.2 mM ME.] Proteins were eluted with the same buffer in fractions of 2.0 ml at a flow rate of 10 minutes per tube. Twenty such fractions were collected. Fractions containing Fructose-1, 6-bisphosphatase activity were pooled together and dialyzed against 1.0 litre of 50 mM tris-HCl buffer (pH 7.5) containing 0.2 mm ME. This preparation was concentrated and used as the enzyme source (BioGel-fraction) for the experiments of characterization.

Enzyme activity assay

The FBPase activity was assayed by the procedure of Udvardy *et al.,* (1982) with slight modification. The enzyme assay mixture contained 50 mM tris-acetate (pH 7.5), 1 mM FBP, 10 mM $MgCl_2$, 1 mM EDTA, 5 mM G-6-P and an appropriate aliquot of enzyme protein in a total volume of 1.0 ml. The mixture was incubated at 37 ºC for 1h after which the reaction was terminated by adding 0.2 ml of 20 % chilled TCA. The mixture was spun at 3,000 rpm for 5 minutes and the pellet discarded. A volume of 2.8 ml Pi-reagent was added to the deproteinized supernatant followed by a second incubation at 37 ºC for 1h for the oxidation of FBPase reaction product D-fructose-6-phosphate, with concomitant release of inorganic phosphate. The activity of the enzyme was determined by estimating the product-specific release of inorganic phosphate from D-fructose-6-phosphate by FBPase reaction. The amount of inorganic phosphate released from the FBPase reaction product was estimated by the method of Chen *et al.,* (1956) and the protein was determined according to the method of Bradford (1976) with BSA as a standard. The content of protein in different fractions obtained from column chromatography was also estimated.

Results

Purification of Fructose-1, 6-bisphosphatase from Ginkgo biloba

The enzyme, FBPase was isolated and purified from the mature leaves of *Ginkgo biloba*. The summary on the purification of FBPase is given in Table 1. Chromatographic profile of proteins resolved from $(NH_4)_2SO_4$ fraction of the crude homogenate of *Ginkgo biloba* leaves is shown in Fig. 1. The BioGel A-0.5m chromatography has revealed that the FBPase from *Ginkgo biloba* was retained and eluted in a single peak with the extraction buffer. In the present study, an overall purification of the enzyme to about 22 fold with about 56% recovery based on total activity was achieved (Table 1).

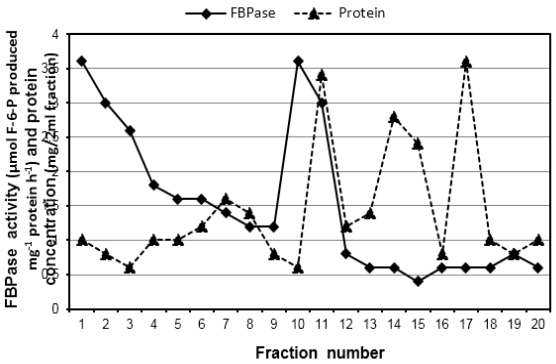

Figure 1: Elution profile of cytosolic FBPase from *Ginkgo biloba* leaves in BioGel A-0.5m column. FBPase activity defined as µ mol of F-6-P produced mg^{-1} protein h^{-1}; protein content expressed as mg/2ml fraction.

Table 1: Summary of partial purification of Fructose-1, 6-bisphosphatase from *Ginkgo biloba* leaves

Purification step	Protein content (mg/ml)	Specific activity [µ mol F-6-P produced $(mg)^{-1}$ protein h^{-1}]	Total activity [µ mol F-6-P produced $(mg)^{-1}$ protein h^{-1}]	Recovery (%)	Purification (fold)
Homogenate	3.4	1.36	740.50	100.00	1.00
10K-supernatant	4.8	1.60	675.84	91.26	1.17
SS-fraction	4.0	2.80	728.00	98.30	2.05
A_2S-fraction	7.2	1.00	13.60	1.83	0.73
BioGel-fraction	3.5	29.60	414.40	55.96	21.76

Characterization of the partially purified enzyme

Requirements for FBP for G. biloba FBPase activity: The *G. biloba* FBPase have been found to utilize FBP as the exclusive substrate and other hexose phosphates could not replace it. When the specific substrate of the enzyme, FBP was not added to the reaction mixture, synthesis of F-6-P could not be detected. However, this enzyme could very slightly utilize D-fructose-1-phosphate (Table 2). Deduction of tris buffer resulted in the loss of enzyme activity by about 58%. Similarly, deduction of $MgCl_2$ and EDTA caused the reduction of enzyme activity by 68% and 50% respectively (Table 3).

Table 2: Effect of some substrate isomers on Fructose-1, 6-bisphosphatase activity from *Ginkgo biloba*

Compound	Concentration (mM)	Specific activity [µ mol F-6-P produced $(mg)^{-1}$ protein h^{-1}]	Percent activity
D-fructose 1,6-bisphosphate	10.0	3.8	100.00
D-fructose-1-phosphate	10.0	0.002	0.05
D-fructose-6-phosphate	10.0	0.00	0.00
D-glucose-1-phosphate	10.0	0.00	0.00
D-glucose-6-phosphate	10.0	0.00	0.00
D-galactose-6-phosphate	10.0	0.00	0.00

Table 3: Effect of composition of incubation medium on *Ginkgo biloba* fructose-1, 6-bisphosphatase activity.

Conditions	Specific activity [µ mol F-6-P produced $(mg)^{-1}$ protein h^{-1}]	Percent activity
Complete set	3.8	100.00
Without substrate (FBP)	0.00	0.00
Without buffer (tris-acetate)	1.60	42.10
Without $MgCl_2$	1.20	31.5
Without EDTA	1.88	49.47
Heat killed enzyme	0.00	0.00

Enzyme stability: Stability of *G. biloba* FBPase varied with the enzyme at different stages of purification. While the low speed supernatant remained active for 10-12 days with insignificant loss of activity when stored at −20 ºC, the BioGel-A 0.5m purified fractions maintained only about 70% of its activity up to 2-3 days when stored at identical temperature. Addition of enzyme stabilizer, 2-mercaptoethanol (ME) or dithiothritol (DTT) increased the shelf-life of the enzyme by 4-5 days.

Enzyme concentration and the time linearity: The *G. biloba* FBPase exhibited enzyme activity linearity up to 400 µg of protein concentration under optimal assay conditions. The rate of enzyme reaction proceeded linearly up to 60 minutes with FBP as the substrate

Effect of temperature and pH: The enzyme was remarkably active between the temperature ranges of 20-40 ºC with 30 ºC as the temperature maximum (Figure 2). Temperature maxima of 35ºC was determined for the cytosolic enzymes from both higher as well as primitive plants. The *G. biloba* FBPase exhibited optimum activity at a pH range of 7.5 – 8.0 (Figure 3). FBPase from other plant sources exhibited similar pH optima.

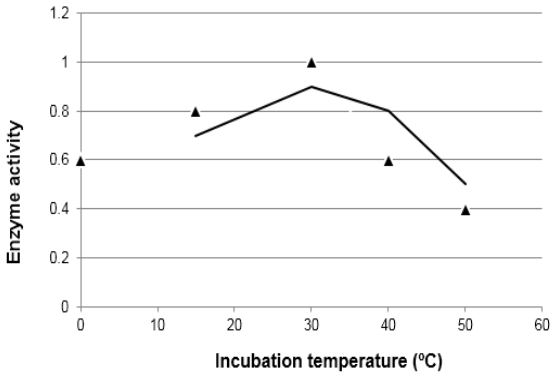

Figure 2: *Ginkgo biloba* FBPase enzyme activity at different incubation temperature (enzyme activity defined as µ mol of F-6-P produced mg^{-1} protein h^{-1}

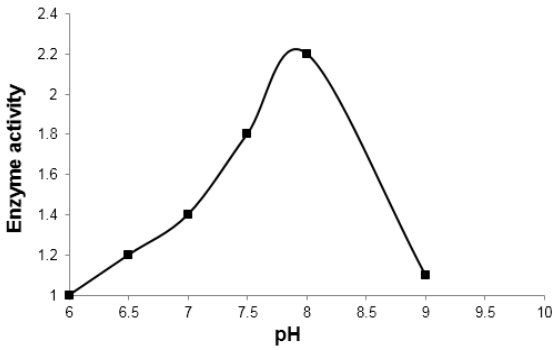

Figure 3: *Ginkgo biloba* FBPase enzyme activity at different pH (enzyme activity defined as µmol of F-6-P produced mg^{-1} protein h^{-1}

Effect of monovalent and divalent cations: Effect of different metal ions on the enzyme activity was tested. Of the monovalent cations tested, K^+ and Na^+ had no effect. Among the divalent cations, Ca^{2+} had no effect, Mg^{2+} and Mn^{2+} were stongly stimulatory while Zn^{2+} was strongly inhibitory to the enzyme activity. Mg^{2+} was stimulatory to the enzyme activity in a concentration dependent manner upto 10 mM while the same was true for Mn^{2+} upto 6 mM concentration. (Table 4).

Table 4: Effect of monovalent and divalent cations on Fructose-1, 6-bisphosphatase activity (using 5 mM FBP as substrate) from *Ginkgo biloba*

Concentration of cations (mM)	Specific activity [µ mol F-6-P produced (mg)$^{-1}$ protein h^{-1}]						
	K^+	Na^+	Ca^{2+}	Mg^{2+}	Mn^{2+}	Cu^{2+}	Zn^{2+}
0	0.60	0.60	0.60	0.60	0.60	0.60	0.60
2	1.20	0.60	1.20	1.00	0.40	0.20	0.20
4	0.60	0.20	1.00	1.00	1.40	0.14	0.12
6	0.20	0.40	1.20	1.80	2.80	0.12	0.10
8	0.40	0.40	1.00	2.60	1.00	0.10	0.10
10	0.60	0.40	0.80	3.20	1.40	0.10	0.08

Determination of K_m value: The activity of the BioGel purified *G. biloba* FBPase was found to increase with respect to the concentration of FBP upto 8 mM when tried between concentration ranges of 0 to 10 mM of substrate. Thereafter, the enzyme activity remained unchanged. The average K_m value for FBP was determined to be approximately 1.86 mM in accordance with the rate equation of Michaelis-Menten (Figure 4).

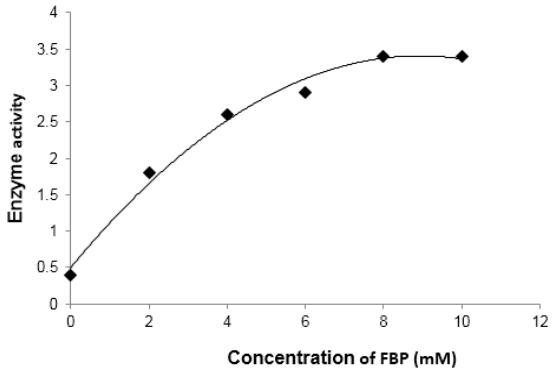

Figure 4: Effect of varied substrate (FBP) concentration on *Ginkgo biloba* FBPase activity (enzyme activity defined as µ mol of F-6-P produced mg^{-1} protein h^{-1}

Effect of varied concentrations different salts: Salts of different chemicals at 0 to 100 mM concentrations were tested. The *G. biloba* FBPase activity was stimulated by NH_4Cl in a concentration guided manner up to a concentration of 50 mM and by $MgCl_2$ upto a concentration of 25 mM. The level of stimulation was 4 times and 6 times respectively. EDTA was stimulatory upto 25 mM, beyond which it seemed to be toxic. Lithium had shown no effect on the enzyme activity (figure-not shown).

Discussion

FBPase activity has so far been reported from almost all groups of plants but gymnosperms. The present work was undertaken with the objective of determining the occurrence of FBPase from *Ginkgo biloba* L., a gymnosperm regarded as a living fossil and to biochemically characterizes the enzyme. The enzyme activity could be detected in the leaves of *Ginkgo biloba* and the cytosolic enzyme from the same was highly specific for its substrate FBP. However, the enzyme could partially utilize fructose-1-phosphate which is in consonance with earlier studies (McGlivery, 1955).

The cytosolic enzyme from *Ginkgo biloba* was highly unstable and the partially purified enzyme could maintain its activity only for 2-3 days. In contrast, the cytosolic FBPase from spinach leaves did not lose its activity upto 2 months of storage in 50% glycerol at -20°C (Herzog *et al.,* 1984). At -20°C, the *Anacystis nidulans* FBPase could be stored for several weeks without any loss of activity (Udvardy *et al.,* 1982).

The monovalent cations used did not show any significant effect on the enzyme activity. However, among divalent cations Mg^{+2} and Mn^{+2} were distinctly stimulatory while Cu^{+2} and Zn^{+2} were decidedly inhibitory of the enzyme function. This effect was not unexpected, since in other cases like *Acinetobacter iowaffi,* the enzyme requires divalent cations, Mg^{2+} and Mn^{2+} for its activity (Mukkada and Bell, 1971). The strong inhibition due to heavy metals suggests that one or more free sulphhydryl groups are present within the active site of the enzyme

(Chhetri *et al.,* 2006). Of the other salts, LiCl and EDTA showed inhibitory action while NH_4Cl was strongly stimulatory to the enzyme. Inhibition by EDTA was also a characteristic feature of FBPase from *Acer pseudoplatanus* (Scala and Semersky, 1971) and inhibition by Zn^{+2} was observed in *Mangifera indica* FBPase activity. Li^{+1} inhibited FBPase activity in *Purococcus furiosus* (Verhees *et al.,* 2002).

In the present case, *Ginkgo biloba* FBPase displayed pH optima of 8.0 which was quite similar to the pH optima towards the alkaline range shown by the same enzyme in bacteria and lower plants viz., pH 8.0-8.5 in *Bacillus licheniformis* (Opheim and Bernlohr, 1975); pH 8.0 in *Bacillus subtilis* (Fujita and Freese, 1979); and it was 8.0 for *Saccharomyces cerevisiae* (Funayama *et al.,* 1979). The pH optimum was found to be towards neutral in FBPase extracted from the storage tissue of different fatty seeds (Youle and Huang, 1976). Cytosolic FBPase of higher plants exhibits neutral pH optima (Botha and Turpin, 1990) in contrast to the alkaline FBPase of *Ginkgo biloba*.

The K_m value for of *Ginkgo biloba* cytosolic FBPase for its substrate FBP was 1.86 which was quite different from the same from other sources as in 3.1µM from apple leaf (Zhou and Cheng, 2004); 20µM from *Bacillus licheniformis* (Opheim and Bernlohr, 1975) and 13µM from *Synechococcus leopoliensis* (Gerbling *et al.,* 1986). However, the value was nearer to the chloroplastic FBPase from spinach leaves having a value of 1.40 mM (Lazaro *et al.,* 1975).

Conclusion

The cytosolic FBPase from *Ginkgo biloba* leaves utilized D-fructose-1,6-bisphosphate (FBP) as its specific substrate. However, this enzyme showed traces of activity in presence of D-fructose-1-phosphate. The *Ginkgo biloba* FBPase was alkaline like that of prokaryotes in contrast to the same from higher plants which were neutral. The stability of enzyme was very short unlike the cytosolic enzymes form higher plants. Thus this enzyme is unique in showing some eukaryotic and some prokaryotic charaters. This may indeed be a considered an evolutionary link between gymnosperms and angiosperms.

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