Properties of Fructose-1,6-bisphosphatase from Carp, Eel and Rainbow Trout Liver

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Fructose-1, 6-bisphosphatase (EC 3.1.3.11) from rainbow trout liver, eel liver, and carp hepatopancreas was purified by ammonium sulfate fractionation, followed by phosphocellulose column chromatography, and Sephadex G-200 gel filtration. Specific activity of the rainbow trout enzyme was comparable to that of the eel enzyme, while the carp enzyme was about one fifth of the above two fish species. Maximal activity of the enzyme was found around pH 7.4, irrespective of fish species. The K_m values of the rainbow trout, eel and carp enzymes for FBP were 5.6, 8.2, and 12.5 μM , respectively. Fish enzyme required Mg^{2+} for activation. In the case of carp enzyme, Mn^{2+} was also effective for activation. The activation constants for Mg^{2+} and Mn^{2+} ranged from 1 to 10 mM and from 0.2 to 0.3 mM, respectively. The activities of the rainbow trout and eel enzymes were further activated by the addition of K^+ and NH_4^+ in the presence of Mg^{2+} whereas both monovalent cations inhibited carp enzyme. Na^+ inhibited the activity of the fish enzyme in the presence of Mg^{2+} . AMP strongly inhibited the enzyme activity. In the presence of 1 mM AMP and 0.5 mM Mg^{2+} at pH 7.5, the enzymatic activity was inhibited by 57% in rainbow trout, 100% in eel, and 65% in carp.

Fructose-1, 6-bisphosphatase (D-fructose-1, 6-bisphosphate 1-phosphohydrolase, EC 3.1.3.11, FBPase) has been described as one of the key, rate-limiting enzymes in gluconeogenesis.¹⁾ FBPase has been investigated in numerous biological sources such as liver,²⁻⁷⁾ kidney,⁸⁾ and muscle.^{3,9)} In view of the regulation of its catalytic activity, it is of interest to clarify its inhibition by either FBP or AMP or both.²⁾

Extensive studies of the fish enzyme have been performed using crude extracts and there is limited information on the properties of purified fish enzyme.¹⁰⁻¹⁴⁾ Moreover, some regulating factors affecting the enzyme activity such as substrate concentration, coenzymes, and metal ions should be investigated.

As a series of studies on the mechanism of carbohydrate metabolism in fish liver, our previous report has characterized liver phosphofructokinase from rainbow trout, eel and carp, and revealed a marked species-specificity.¹⁵⁾ To clarify further the mechanism of carbohydrate metabolism in fish liver, we purified FBPase from rainbow trout, eel and carp in the present study, and examined their enzymatic properties.

Materials and Methods

Materials

Live specimens of rainbow trout *Salmo gairdnerii*, eel *Anguilla japonica*, and carp *Cyprinus carpio* were purchased from a commercial market. The acetone-dried powder of liver or hepatopancreas was prepared following the method of Pontremoli,⁴⁾ and was kept at -20°C .

FBPase Assay

FBPase activity was measured at 25°C according to the method of Pontremoli⁴⁾ by monitoring the rate of reduction of NADP^+ at 340 nm. For the routine assay, the reaction mixture contained 50 mM triethanolamine-HCl buffer (pH 7.5), 5 mM MgCl_2 , 1.4 units glucosephosphatase isomerase, 0.7 units glucose-6-phosphate dehydrogenase, 0.25 mM fructose-1, 6-bisphosphate (FBP), 0.25 mM NADP^+ . The reaction was started by the addition of the enzyme solution to the above mixture (0.6 ml in final volume). One unit of enzyme was defined as the amount which converts 1 μmol of FBP to fructose-6-phosphate (F6P) per min under the assay condition.

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Purification of FBPase

All purification steps were carried out at 0–4°C. The acetone-dried powder was extracted with 5 volumes of 20 mM Tris-HCl buffer (pH 7.5) containing 2 mM EDTA under gentle stirring for 60 min. The suspension was centrifuged for 60 min at 25,000×g. To the supernatant was added solid ammonium sulfate to bring it to 30% saturation. After 30 min, the precipitate was removed by centrifugation at 10,000×g for 20 min. The supernatant obtained was again treated with solid ammonium sulfate in the same manner described above. The precipitate at 70% saturation was collected by centrifugation, dissolved in a small volume of 10 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA (buffer A), and was dialyzed overnight against buffer A. The dialyzed enzyme solution was applied to a phosphocellulose column (3.1×24 cm) equilibrated with buffer A. The adsorbed enzyme was eluted with a linear gradient of KCl from 0 to 0.75 M, in a total volume of 400 ml of buffer A. The active fractions from rainbow trout and eel were further purified by gel filtration as in the following procedure: The pooled enzyme solution was concentrated by ultrafiltration. The concentrated enzyme solution was applied to a Sephadex G-200 column (2.5×32 cm) equilibrated with 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA (buffer B), and the proteins were eluted with buffer B.

Results and Discussion

As shown in Table 1, FBPase activity in the crude extract of the carp hepatopancreas was lower than that of the trout and eel livers. As reported previously,¹³⁾ the distribution pattern of phosphofructokinase in fish liver showed the contrary for FBPase. Shimeno¹⁴⁾ observed that FBPase activity of omnivorous fish such as carp and crucian carp is lower than that of carnivorous fish such as yellowtail and spotted mackerel. The

present results might be attributed to the food habit of fish rather than to its location. Specific activities of the rainbow trout and eel enzymes showed the same levels as that of rat⁴⁾ and bovine⁶⁾ enzymes.

FBPase activity was eluted as single peak from the phosphocellulose column at the KCl concentration of around 0.5 M for rainbow trout and eel, and at 0.75 M for carp, respectively (Fig. 1). Olson and Marquardt³⁾ eluted FBPase of chicken breast muscle from the phosphocel-

Fig. 1. Phosphocellulose column chromatography of the rainbow trout, eel, and carp liver fructose-1,6-bisphosphatase. The precipitate by ammonium sulfate fractionations (30–70% saturation) was dissolved in and dialyzed against 10 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA, then applied to a phosphocellulose column (3.1×24 cm) equilibrated with the same buffer. Elution was carried out with a linear gradient of KCl from 0 to 0.75 M in a total volume of 400 ml of the above buffer. Fractions of 5 ml were collected.

Table 1. Activities and specific activities of fructose-1,6-bisphosphatase in fish liver extracts

Species	Activity ($\mu\text{mol}/\text{min} \cdot \text{g}$ tissue)	Specific activity ($\mu\text{mol}/\text{min} \cdot \text{mg}$ protein)
Rainbow trout	3.2×10^{-3}	0.09
Eel	3.3×10^{-3}	0.12
Carp	1.5×10^{-3}	0.02

Activity was measured in the presence of 0.25 mM FBP and 5 mM Mg^{2+} at pH 7.5 and 25°C.

lulose column at 0.4 M KCl. The carp enzyme preparation eluted from the phosphocellulose column showed some contaminations. Their further purification was discontinued due to the scarcity of the available sample. The rainbow trout and eel enzymes were further purified by Sephadex G-200 gel filtration, and FBPase activity from both species was detected as single peak (data not shown). The estimated molecular weight of fish FBPase of about 145,000 by Sephadex G-200 gel filtration is close to the value obtained for beef (130,000),⁵⁾ rabbit (130,000–140,000),⁶⁾ and chicken (140,000)⁷⁾ liver enzymes.

FBPase exhibited maximal activity at pH 7.4, irrespective of fish species (data not shown). FBPase activity was not detected at pH values below 6, whereas 60–80% of maximal activity was detected above pH 8. Shimeno¹⁴⁾ determined the optimum pH of the spotted mackerel liver enzyme to be around 7. Rainbow trout¹⁰⁾ and salmon¹²⁾ enzymes, on the other hand, showed maximal activity at pH 9.5 in the presence of Mg^{2+} as activator. It is generally accepted that "neutral FBPase" having maximal activity at a pH range between 7.0 and 7.5 is a physiological enzyme.^{8,16)} Nakashima and Horecker¹⁶⁾ reported that "alkaline FBPase" was induced by the action of internal proteinase during heat treatment or acetone fractionation in the purification process. In order to clarify the effect of acetone treatment on the enzyme activity during purification, activity ratios (activity at pH 7.4/activity at pH 8.8) were compared using the rainbow trout enzyme. The ratios calculated for the crude extract without acetone treatment, with acetone treatment, and that for the purified enzyme were 1.9, 2.0, and 1.8, respectively, inferring that no modification of enzyme occurred during the purification steps.

The effect of substrate concentration on the activity was measured in a range of 1–100 μM FBP at pH 7.5 and 25°C. Fig. 2 shows Lineweaver-Burk plots obtained from the initial velocities of the enzyme reaction. The K_m values differed among the three fish species examined: 5.6 μM for rainbow trout, 8.2 μM for eel, and 12.5 μM for carp. These values agree with those of the rat FBPase.^{2,17)} However, Behrisch and Hochackha¹⁹⁾ determined the K_m value of the rainbow trout enzyme to be ten-fold higher than the value in the present study. The reason of such discrepancy in the K_m values of the rainbow trout enzyme is not clear. Fish FBPase showed the maximal activity at a FBP concentration range from 20 to 30 μM , beyond which the activity decreased with increasing concentration (Fig. 2). At 100 μM FBP, the activities of the rainbow trout, eel, and carp enzymes were depressed by 35, 25, and 16% of their maximal activities, respectively. A number of workers have noted the same inhibitory effect of excessive FBP on FBPase activity.^{2,10–12)}

The effect of several divalent metal ions at 5 mM was examined using their chloride salts. Like all FBPase reported to date, three fish enzymes tested here required divalent cations such

Table 2. Effect of divalent metal ions on the activity of fructose-1,6-bisphosphatase from fish liver at pH 7.5

Metal ion (5 mM)	Relative activity (%) [*]		
	Rainbow trout	Eel	Carp
None	0	0	0
Mg^{2+}	100	100	100
Mn^{2+}	41	27	141
Co^{2+}	10	10	14
Ca^{2+}	0	0	0

^{*} Activity in the presence of Mg^{2+} was taken as 100%.

Fig. 2. Lineweaver-Burk plots of fructose-1,6-bisphosphatase for fructose-1,6-bisphosphate.

The activity was measured in the presence of 5 mM Mg^{2+} at 25°C in 50 mM Tris-HCl buffer (pH 7.5).

Fig. 3. Lineweaver-Burk plots of fructose-1, 6-bisphosphatase for Mg^{2+} . The activity was measured in the presence of 0.03 mM FBP at pH 7.5 and 25°C.

Fig. 4. Effect of AMP on the fructose-1, 6-bisphosphatase activity from fish livers. The activity was measured in the presence of 0.2 mM FBP at pH 7.5 and 25°C; in the presence of 0.5 mM Mg^{2+} (—○—) and 2.5 mM Mg^{2+} (—●—).

Table 3. Effect of monovalent metal ions on the activity of fructose-1, 6-bisphosphatase from fish liver at pH 7.5

Metal ion (150 mM)	Relative activity (%) [*]		
	Rainbow trout	Eel	Carp
None	100	100	100
K ⁺	161	124	82
NH ₄ ⁺	184	127	41
Na ⁺	90	45	63

* Activity was measured in the presence of 5 mM Mg^{2+} , and the value in the absence of monovalent metal ion was taken as 100%.

as Mg^{2+} for activation of the enzyme (Table 2). In the presence of 5 mM Mn^{2+} , 27–41% of the activity in the presence of Mg^{2+} was detected in rainbow trout and eel, whereas 141% in carp. Co^{2+} exhibited a slight effect on the activity (10–14%). Ca^{2+} showed no effect on the activation of fish FBPase. Likewise, the activities of rainbow trout¹⁰⁾ and salmon¹²⁾ enzymes were not activated by Ca^{2+} . From the Lineweaver-Burk plots (Fig. 3), the K_m values for Mg^{2+} were

calculated to be 1.3 mM for rainbow trout, 0.95 mM for eel, and 10 mM for carp, respectively. The calculated K_m values for Mn^{2+} ranged from 0.15 to 0.33 mM (data not shown). Shimeno¹⁴⁾ demonstrated that FBPase from the spotted mackerel was activated by Mg^{2+} , and the optimal concentration for the activation was 5 mM. He also observed that the activity in the presence of 5 mM Ca^{2+} was one eighth that in the presence of 5 mM Mg^{2+} .

Underwood and Newsholme¹⁷⁾ showed that the effect of divalent metal ions on the FBPase activity was affected in the presence of the chelating compounds or monovalent metal ions. The effect of monovalent metal ions on the FBPase activity was examined in the presence of 5 mM Mg^{2+} . As shown in Table 3, monovalent cations (150 mM) such as K⁺ and NH₄⁺ in the presence of 5 mM Mg^{2+} further activated the rainbow trout and eel enzymes ranging from 24 to 84%. In contrast, the activity of carp enzyme decreased by 18% with K⁺ and 59% with NH₄⁺ respectively. Na⁺ decreased the activities of three fish enzymes by

10–55%. Tol *et al.*⁹⁾ noted that the activity of the rabbit muscle enzyme was increased 2–3 times in the presence of 0.1 M K⁺ or NH₄⁺, while decreased the activity.

AMP is a specific, allosteric inhibitor of FBPase.²⁾ As shown in Fig. 4, fish FBPase was inhibited by AMP and the degree of inhibition varied according to the Mg²⁺ concentration. In the presence of 0.5 mM Mg²⁺ and 1 mM AMP at pH 7.5 the activities of the rainbow trout, eel, and carp enzymes were inhibited by 57, 100, 65%, respectively. Increasing the Mg²⁺ concentration to 2.5 mM, however, resulted in the weakening of inhibition, particularly in the case of the rainbow trout enzyme. This agrees with the observation of Behrisch and Hochachka¹⁰⁾ in rainbow trout. They noted that FBPase inhibition by AMP was affected by the Mg²⁺ concentration, along with reaction temperature and pH.

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