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FRUCTOSE-1,6-DIPHOSPHATASE FROM RAT LIVER

PURIFICATION AND PROPERTIES

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SUMMARY

1. Fructose-1,6-diphosphatase (D-fructose 1,6-diphosphate 1-phosphohydrolase, EC 3.1.3.11) is purified 350-fold from rat liver. The enzyme is found in an homogeneous form by disc gel electrophoresis and sucrose gradient centrifugation.

2. The molecular weight of the active enzyme is approx. 143 000 and most likely it is constituted by four peptide chains of identical size.

3. No difference in catalytic and molecular properties are found in the enzyme purified from healthy and triamcinolone-treated rats.

INTRODUCTION

Fructose-1,6-diphosphatase (D-fructose 1,6-diphosphate 1-phosphohydrolase, EC 3.1.3.11) has been isolated from a number of different sources. The mammalian enzymes exhibit many similarities, in particular rabbit [1] and rat liver fructose-1,6-diphosphatases have been shown to share several properties, namely the activity pH optimum, the K_m value, the requirement of a divalent cation, the molecular weight and the subunit composition, despite the difference in amino acid composition. Fructose-1,6-diphosphatase has been postulated to play an important role as a key enzyme in the irreversible reaction

$$\text{Fru-1,6-}P_2 \rightarrow \text{Fru-6-}P + P_i$$

of the gluconeogenic process [2, 3] and to be regulated by hormones, allosteric effectors and metabolites. The effect of glucocorticord hormones resulted in an increase of fructose-1,6-diphosphatase activity [4] which might depend on an increased enzyme biosynthesis or an activation of preexistent enzyme molecules. It was of particular interest to clarify the hormonal action on hepatic fructose-1,6-diphosphatase, and the purpose of the present study has been the investigation of the catalytic and molecular properties of the enzyme purified from rat liver, when diabetes has been induced by a triamcinolone injection.

Abbreviation: PHMB, *p*-hydroxymercuribenzoate.

EXPERIMENTAL PROCEDURE

Materials

D-Fructose 1,6-diphosphate, $NADP^+$, *p*-hydroxymercuribenzoate (PHMB), sodium dodecylsulfate, EDTA and Tris were obtained from the Sigma Chemical Co. Crystalline aldolase, hexose-phosphate isomerase and glucose-6-phosphate dehydrogenase were purchased from the Boehringer Mannheim Corp. Whatman phosphocellulose P11 was obtained from C. Erba, Milan, and charged as previously described [1]. 2-Mercaptoethanol was from Eastman Organic Chemicals. All reagents for the enzyme assay and purification were dissolved in quartz-distilled water. Rats were a gift from the Richter Ormonoterapia, Milan and Triamcinolone (Ledercort) was a gift from Lederle, Cyanamide Italia S.p.A., Catania.

Methods

For the routine assay of fructose-1,6-diphosphatase activity the rate of formation of fructose 6-phosphate was measured spectrophotometrically by following the reduction of $NADP^+$, at 340 nm, in the presence of excess of glucose-6-phosphate dehydrogenase and hexose-phosphate isomerase. The reaction mixture (1 ml) contained 0.04 M Tris–HCl buffer (pH 7.5), 0.1 mM $NADP^+$, 0.1 mM EDTA, 2 mM $MgCl_2$, 2 μg each of glucose-6-phosphate dehydrogenase and hexose-phosphate isomerase and 0.1 mM fructose 1,6-diphosphate. The assay was carried out at 23 °C and the reaction was started by the addition of fructose 1,6-diphosphate.

1 unit of enzyme is defined as the amount that will hydrolyze 1 μmole of fructose 1,6-diphosphate per min under the above conditions. The specific activity is expressed as units per mg of protein. Protein concentration was determined by the method of Bucher [5] or Lowry et al. [6], or calculated from the absorbance at 280 nm of the protein solutions.

Polyacrylamide gel electrophoresis was performed at 23 °C in the standard 7.5% gel at pH 8.6 according to Davis [7] or in sodium dodecylsulfate–10% polyacrylamide gel at pH 7.0 according to Weber and Osborn [8].

Sedimentation in a sucrose density gradient was performed at 4 °C for 15 h at 39 000 rev./min as described by Martin and Ames [9]. Amino acid analyses were carried out by the method of Spackman et al. [10] and samples, after extensive dialysis, were hydrolyzed at 110 °C in 5.7 M HCl for 24, 48, 72 h. Free thiol groups were determined according to the method of Boyer [11], tryptophan by the bromosuccinimide method [12], tryptophan and tyrosine by the method of Beaven and Holiday [13].

RESULTS

Purification procedure

Extraction. Young male rats were fed a commercial diet until they reached a weight of 250–300 g. Then they were killed by blows on the head and exanguinated, the livers were quickly removed, collected and chilled. All further operations were carried out at 0–4 °C. The livers (20 g) were minced through a plastic sieve and extracted with 44 ml (w/v, 1:22) of 0.25 M sucrose containing 1 mM Tris–HCl buffer (pH 7.0) by stirring for 2 min. The homogenate was centrifuged for 60 min at

17 000 × *g*. The supernatant (40 ml) was filtered on gauze and diluted with 0.1 mM EDTA to a protein concentration of about 8 mg/ml. The pH (6.7–6.8) was adjusted with 0.1 M NaOH to 7.2–7.3.

Heat fractionation. The diluted homogenate (160 ml) was heated for 2 min at 60 °C in a water bath and immediately chilled, and the precipitate was discarded by centrifugation.

Phosphocellulose column chromatography. To the heat fraction (150 ml) wet phosphocellulose was added until the pH of the solution reached 6.8–6.7. The slurry was then filtered on a glass buchner and the enzyme solution removed by gentle suction. This solution was diluted with 2 vol. of cold 0.1 mM EDTA and a second portion of phosphocellulose was added until all the fructose-1,6-diphosphatase activity was absorbed. The pH was maintained at 6.3 by the small addition of 0.1 M NaOH. The slurry was poured in the buchner and washed with 100 ml of 20 mM Tris–acetate buffer containing 0.1 mM EDTA (pH 6.3), to remove the non-adsorbed proteins. Finally the slurry was poured into a glass column (1.5 cm × 17 cm) and washed with 500 ml of 65 mM Tris-acetate buffer containing 0.1 mM EDTA, 0.1 mM 2-mercaptoethanol, (pH 6.3). When the absorbance at 280 nm was less than 0.03, the enzyme was eluted with a solution (30 ml) of 2 mM fructose 1,6-diphosphate in the washing buffer (final pH 6.8). Fractions containing fructose-1,6-diphosphatase activity were combined and brought to pH 7.5 and subsequently 20 mM $MgCl_2$ was added. The fructose 1,6-diphosphate bound to the enzyme was removed by leaving the enzyme solution at room temperature for 30 min after addition of Mg^{2+}, as previously reported [14]. The enzymatic solution was then dialyzed under vacuum against 500 ml of 50 mM Tris–HCl buffer containing 0.1 mM EDTA (pH 7.5) (with three changes) to remove salts and concentrate the eluate. The results of the purification procedure are summarized in Table I.

TABLE I

PURIFICATION OF RAT LIVER FRUCTOSE-1,6-DIPHOSPHATASE

Results are calculated for 20 g of rat liver.

Fraction	Total units	Spec. act. (units/mg protein)	Yield (%)
Homogenate	83	0.06	–
Heat fraction	70	0.09	84
Phosphocellulose eluate	21.14	15.00	26
Dialysis	30	21.00	36

Homogeneity and molecular weight

The homogeneity of the enzyme preparation was tested on polyacrylamide gel at pH 8.6, and one single sharp band was observed. If compared with rabbit liver fructose-1,6-diphosphatase, the rat enzyme showed a faster migration to the anode. However, after dissociation in sodium dodecylsulfate at pH 7.0 the single band that was obtained migrated in the same position as the one obtained from the rabbit liver

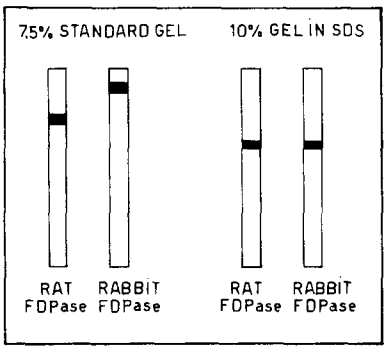

Fig. 1. Disc gel electrophoresis of fructose-1,6-diphosphatase (FDPase). Electrophoretic migration of rat and rabbit liver fructose-1,6-diphosphatase on polyacrylamide gel in Tris–glycine buffer containing 1 mM EDTA (pH 8.6) (left) and in 0.1 % sodium dodecylsulfate–gel after dissociation in phosphate buffer (pH 7.0), containing 1 % sodium dodecylsulfate (SDS), as described by Weber and Osborn [8], (right). All the gels were stained with Coomassie brillant blue.

enzyme, (molecular weight of 36 000 see Fig. 1). Sedimentation analysis in the sucrose density gradient of the peak corresponding to fructose-1,6-diphosphatase yielded a s_{20} value of 7.3 (see Fig. 2) which corresponds to a molecular weight of 143 000 ($\pm$ 2 %) assuming a spherical conformation and a partial specific volume of 0.725/cm for the enzyme.

Since the molecular weight of a single peptide chain corresponded to 1/4 of that of the native protein, it is likely that the rat liver fructose-1,6-diphosphatase possesses a tetrameric structure, made up of four subunits of identical molecular weight.

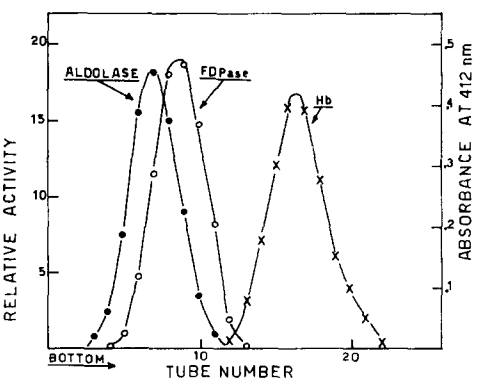

Fig. 2. Sucrose density gradient centrifugation of rat liver fructose-1,6-diphosphatase (FDPase). Sedimentation in sucrose gradient was carried out according to the method of Martin and Ames [9]. Sucrose solutions were prepared in 50 mM Tris–HCl buffer containing 0.1 mM EDTA (pH 7.5). Aldolase and human hemoglobin were used as internal standards. After centrifugation at 5 °C for 15 h at 39 000 rev./min, 25 fractions were collected and analyzed.

Amino acid analyses

Amino acid analyses were carried out as described in the section "Methods" (Table II). 24 sulfhydryl groups were detected by reacting the enzyme with PHMB;

TABLE II

AMINO ACID COMPOSITION OF FRUCTOSE-1,6-DIPHOSPHATASE FROM RAT LIVER

One sample of fructose-1,6-diphosphatase (2 mg) was dialyzed for 48 h against distilled water and evaporated to dryness. The dry material was dissolved in 5.7 M HCl, divided into 3 aliquots and hydrolyzed under vacuum for 24, 48 and 72 h at 110 °C. Number of amino acid residues per mole of enzyme is calculated for a molecular weight of 143 000, and rounded off to the nearest whole number. Tyrosine was also determined by the method of Beaven and Holiday [13]. Cysteine was determined with PHMB as described in the text. Tryptophan was detected by the method of Beaven and Holiday [13] and the bromosuccinimide method [12].

Amino acid	Number of amino acid residues per mole of enzyme
Lys	172
His	48
Arg	155
Asp	125
Thr	49
Ser	56
Glu	136
Pro	56
Gly	49
Ala	60
Val	89
Met	39
Ile	77
Leu	104
Tyr	70
Phe	60
Cyś	24
Trp	8

however, in the presence of 1 mM fructose 1,6-diphosphate, only 20 of them were available (Fig. 3).

Effect of Mg^{2+}, fructose 1,6-diphosphate and EDTA

The rat liver fructose-1,6-diphosphatase showed the maximum activity in the neutral pH region, when assayed in the presence of 2 mM Mg^{2+} (Fig. 4); the pH optimum shifted towards pH 7 at a higher Mg^{2+} concentration and towards pH 8 at a lower cation concentration, as previously reported for other mammalian fructose-1,6-diphosphatases [15, 16].

The purified enzyme, assayed at pH 7.5, showed a high affinity for fructose 1,6-diphosphate (maximum activity at 0.05 mM; with a K_m value in the order of 10^{-4} M). A higher substrate concentration resulted in an inhibitory effect as reported for fructose-1,6-diphosphatases from mammals examined so far [1, 16, 17]. Inhibition of the catalytic activity of the enzyme was also observed when fructose-1,6-diphosphatase was preincubated with its own substrate. A similar effect was previously reported by Luppis et al. [18] on partially purified rabbit liver enzyme.

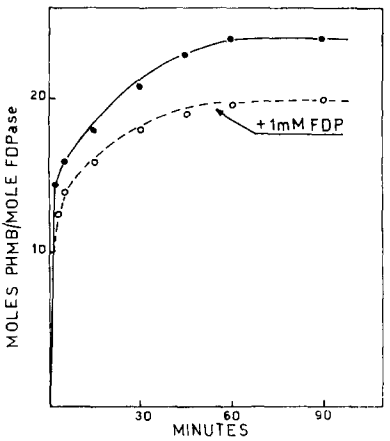

Fig. 3. Titration of sulfhydryl groups with PHMB. The reaction mixture (1 ml) contained 0.01 M Tris–HCl buffer (pH 7.5), 2.25 nmoles of fructose-1,6-diphosphatase, 100 nmoles of PHMB, 2% of *n* butanol and in the presence or in the absence of 1 mM fructose 1,6-diphosphate (FDP). Changes in absorbance were followed at 250 nm [11]. A standard curve with reduced glutathione was performed before evaluating the number of sulfhydryl bound in the assay conditions.

The inhibition produced by fructose 1,6-diphosphate is presented in detail in Fig. 5. When different concentrations of purified fructose-1,6-diphosphatase were incubated in the presence of 1 mM fructose 1,6-diphosphate at three different pH values, a decrease in the catalytic activity occurred, and particularly at pH 6.5. A slight decrease in catalytic activity was also observed after incubation of the enzyme alone especially at pH 6.5 and at the lowest enzyme concentrations. In the same

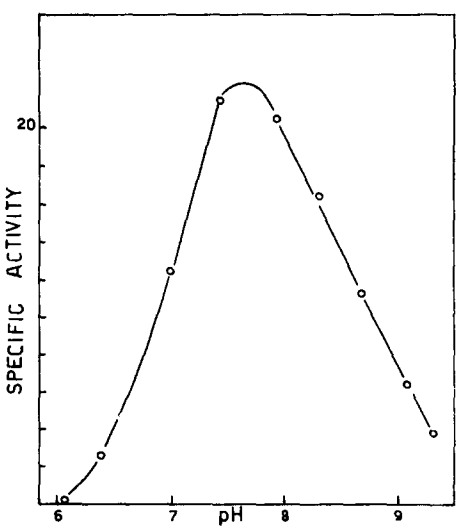

Fig. 4. The effect of pH on rat liver fructose-1,6-diphosphase activity. The enzymatic activity was measured as described in Experimental Procedure, in the presence of 0.04 M Tris–acetate buffer over the entire pH range.

experimental conditions the addition of 1 mM EDTA to the incubation mixture resulted in an activation of the higher enzyme concentrations, without loss of activity at the lower concentrations. Since fructose-1,6-diphosphatase elution from the phosphocellulose column was carried out with fructose 1,6-diphosphate, and in order to avoid the fructose 1,6-diphosphate inhibition (reported in Fig. 5), all the enzyme preparations have been allowed to stand at room temperature for 30 min in the presence of 20 mM $MgCl_2$ (pH 7.5) to completely hydrolyze the inhibiting bound fructose 1,6-diphosphate.

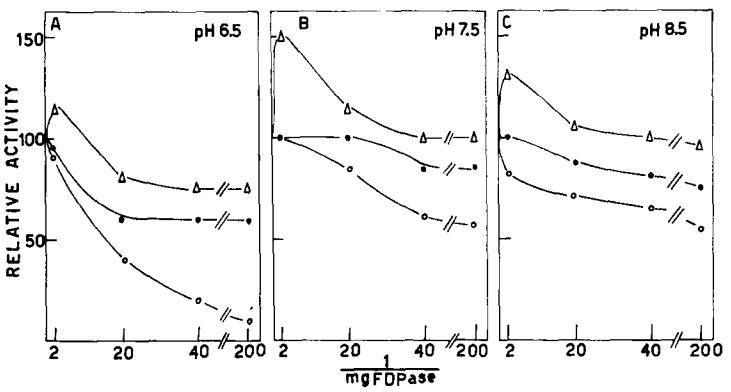

Fig. 5. Effect of fructose-1,6-diphosphatase (FDPase) concentration, pH, fructose 1,6-diphosphate and EDTA on enzymatic activity. Purified fructose-1,6-diphosphatase was incubated, at the showed concentrations, at room temperature. The incubation mixture (1 ml) contained 50 mM Tris–acetate buffer (pH 6.5) in A, or 50 mM Tris–HCl buffer (pH 7.5) in B, or 50 mM Tris–HCl buffer (pH 8.5) in C. The enzyme was then incubated without addition (●—●) and in the presence of 1 mM fructose 1,6-diphosphate (○—○), and 1 mM EDTA (△—△) at the three pHs. Aliquots of incubation mixtures were assayed as described, at timed intervals and the reported values corresponded at a 1-h incubation.

In order to explain the inhibitory effect of fructose 1,6-diphosphate, two gel filtration experiments were performed. A Sephadex G-100 column (1.2 cm × 70 cm) was equilibrated with 50 mM Tris–HCl buffer (pH 7.5), containing 1 mM EDTA or 1 mM fructose 1,6-diphosphate and 2 mg of fructose-1,6-diphosphatase was applied in each experiment. From the first filtration a peak of fructose-1,6-diphosphatase emerged with a specific activity of 22, and from the second one a peak of enzyme with a specific activity of 5. A partial recovery of the activity of the second enzyme peak was obtained after incubation with $MgCl_2$ and dialysis. No difference in molecular weight between the two enzyme fractions was apparent after gel filtration; therefore, the inhibitory effect of fructose 1,6-diphosphate does not depend on an hypothetical dissociation of the tetrameric fully active enzyme, but it is likely that it depends on an induced conformational change of the enzymatic molecule. EDTA was the most effective compound in preventing the loss of fructose-1,6-diphosphatase activity, also at a low enzyme concentration. Various buffers at different pH and ionic strengths were tested in the incubation mixture, but no prevention of the inhibition by fructose 1,6-diphosphate was obtained without EDTA [14]. 2-Mercaptoethanol also failed in preventing the substrate inactivation.

Studies on fructose-1,6-diphosphatase from rats treated with triamcinolone

Rats (250–300 g) were daily injected intraperitoneally with triamcinolone, one group with 1 mg/100 g body wt, and another with 5 mg/100 g body wt, for 5–8 days to induce diabetes [4]. The animals were killed and the blood glucose concentration was determined to check the extent of glucosemia produced by the hormone. Livers were collected, fructose-1,6-diphosphatase was prepared in the pure form as described above, and subsequently compared with the normal enzyme. Both the fructose-1,6-diphosphatases showed the same pH curve and the same affinity for fructose 1,6-diphosphate and no differences were found in the electrophoresis migration or in the molecular weight. Therefore, the catalytic and molecular properties between the enzyme derived from healthy and triamcinolone-treated animals did not show any relevant difference.

DISCUSSION

Fructose-1,6-diphosphatase from rat liver has been obtained in an homogeneous form; the pH optimum of the pure enzyme is in the neutral pH range (as for the rabbit liver and kidney enzyme [1, 17]). Under the assay standard conditions both the crude and the purified enzyme exhibited neither a double pH optimum, in the neutral and in the alkaline region as observed by Byrne [19], nor a maximum in the range of pH 9 as described by Bonsignore et al. [20]. The only relevant phenomenon observed during these studies was represented by a considerable variation in the activity of the crude homogenate in the dependence on the seasonal changes, namely a twice increase in the activity was reached in the summer time. The physiological significance of this variability, which has been also described for rabbit liver fructose-1,6-diphosphatase, is still unclear.

Purified fructose-1,6-diphosphatase from rat liver have been found to have a molecular weight of 143 000, and most likely the enzyme is composed of four subunits having the same size. Although the hepatic enzymes from rabbit and liver possess several common properties, a few differences in amino acid composition were observed. The ultraviolet spectrum for both enzymes showed a maximum at 279 nm, and for rabbit liver fructose-1,6-diphosphatase the $A_{280\ nm}/A_{260\ nm}$ ratio was 1.7, whereas for rat liver fructose-1,6-diphosphatase it was 2, which was likely considering the higher content of tyrosine and tryptophan demonstrated in the latter.

EDTA plays an essential role in preserving the catalytic activity, particularly at low enzyme concentrations; on the contrary fructose 1,6-diphosphate, which binds to the enzyme molecule causes a decrease in catalytic activity. Since molecular weight variations were not observed after fructose 1,6-diphosphate treatment, it is possible that the fructose 1,6-diphosphate inactivation is secondary to conformational changes of the enzyme molecule.

Finally no difference between liver fructose-1,6-diphosphatase produced in the physiological state and that induced by triamcinolone administration was observed.

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