Demonstration of fructose 1,6-bisphosphatase in human term placenta

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Summary. A proteolysed form of fructose 1,6-bisphosphatase (Fru-P_2ase) has been detected and characterized in human term placenta. The extract was found to contain very low levels of activity with an alkaline pH optimum. Western blotting demonstrated a polypeptide of Mr 26,000, instead of the subunit of Mr 36,000 observed in native mammalian Fru-P_2ases.

Key words. Fructose 1,6-bisphosphatase; human placenta.

Mammalian fructose 1,6-bisphosphatases (Fru-P_2ase, EC 3.1.3.11) are tetrameric enzymes with a subunit Mr of 36,000. They have a neutral pH optimum, high affinity for the substrate fructose 1,6-bisphosphate (Fru-1,6-P_2) ($K_m \sim 1$ μM) and require Mn^{++} or Mg^{++} for activity. They are strongly inhibited by AMP and by fructose 2,6-bisphosphate (Fru-2,6-P_2) and they are susceptible to limited proteolysis with a shift of the pH optimum to the alkaline region [1, 2].

Gluconeogenesis has been demonstrated in human term placenta by the incorporation of isotopically labeled pyruvate into glucose [3]. However, Fru-P_2ase, a key enzyme of gluconeogenesis, has been reported to be absent from the placenta of rat, guinea pig and Rhesus monkey [4], or was found to be barely detectable in rat placenta during pregnancy [5, 6].

We report here the presence of an immunoreactive proteolysed enzyme in human term placenta.

Materials and methods

Human term placentas were obtained after normal delivery, cleaned, cut into small pieces and frozen in liquid nitrogen. Frozen tissue, 8 g, was homogenized in a Waring blender in 32 ml 10 mM Tris-HCl, 1 mM EDTA, pH 7.4, for 1 min. The homogenate was centrifuged at 20,000 × g for 40 min and the supernatant solution was passed through four layers of cheesecloth (extract).

Fru-P_2ase was assayed spectrophotometrically at pH 7.4 or as indicated [7, 8]. One unit of enzyme activity was defined as the amount catalyzing the conversion of 1 μmol of Fru-1,6-P_2 per min at 25 °C.

Protein was determined by the method of Bradford [9] using bovine serum albumin as standard.

SDS-polyacrylamide gel electrophoresis (15% acrylamide) was performed according to Laemmli [10] and Western blots according to Towbin et al. [11].

Antibodies were raised in guinea pigs against rabbit muscle Fru-P_2ase purified according to Black et al. [12]. The titer of the antibodies was followed by enzyme-linked immunosorbent assay (ELISA) [13]. Alkaline phosphatase-conjugated protein A was employed as a second antibody. The immunospecificity was checked by reactivity against bovine serum albumin (BSA).

After Western blotting, protein binding sites on the nitrocellulose sheet (Millipore, Corp. Bedford, MA, USA) were blocked by incubating with Western buffer (PBS-0.1% Tween 20-5% BS) containing 0.5% BSA for 1 h. The blot was further incubated with antiserum diluted 1/10 in Western buffer for 2 h, washed with the same buffer three times, and incubated with alkaline phosphatase-conjugated protein A, diluted 1/1000 in the above solution, for 2 h. It was then stained with the substrate naphthol AS-MX phosphate.

Results and discussion

Immediate freezing of the placenta in liquid nitrogen was found to be essential in the detection of the activity of Fru-P_2ase. The specific activity of the extract was 0.3×10^{-3} U/mg. The activity detected, 0.03 U/g fresh weight, was very low compared with the activity found in gluconeogenic tissues such as human liver, 1.7 U/g [14], or in non-gluconeogenic tissues such as rabbit muscle, 2.1 U/g [12].

The pH optimum of the activity in extracts (fig. 1) was found to be about 9.1, indicating the presence of a Fru-P_2ase modified by proteolysis [1].

No improvement in the total activity was observed under various other conditions of extraction tested. These included: a) pulverization in liquid nitrogen and homogenization with Polytron; b) as in (a) but at pH 8.5,

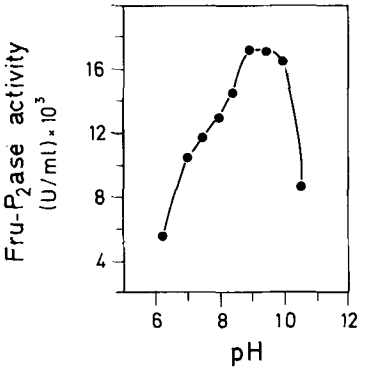

Figure 1. pH activity curve for Fru-P_2ase in extracts of human term placenta.

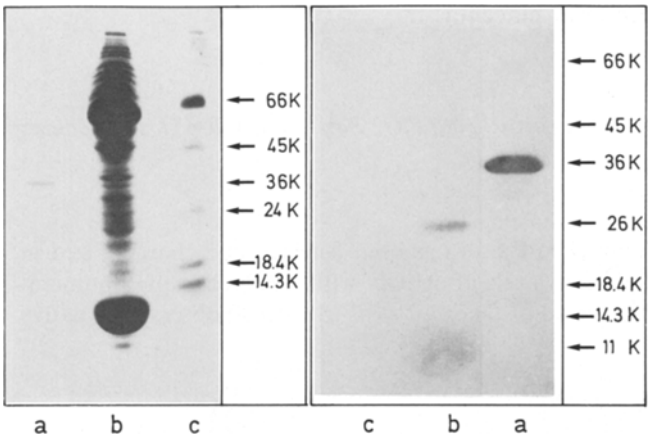

Figure 2. Left: SDS polyacrylamide gel electrophoresis of rabbit muscle Fru-P_2ase, 20 µg (lane a), extract of human term placenta, 1.2 mg (lane b) and molecular weight standards: bovine serum albumin (Mr 66,000), ovalbumin (Mr 45,000), trypsinogen (Mr 24,000), β-lactalbumin (Mr 18,400) and egg white lysozyme (Mr 14,300) (lane c).
Right: Western blot of the gel shown on left: placental extract (lane b), rabbit muscle Fru-P_2ase (lane a), molecular weight standards (lane c).

a pH value found to inactivate a specific lysosomal protease[15,16]; c) addition of protease or cathepsin B_1 inhibitors (1 mM phenylmethanesulphonyl fluoride, [PMSF] or 0.2 ng/ml leupeptin[17], in the extraction buffer) d) as in (a) followed by heating in a water bath at 67 °C for 3 min and rapid chilling[18,19].

When a Western blot of the extract was made, immunodetection gave a band of Mr 26,000 which reacted with the antibody against rabbit muscle Fru-P_2ase. A fainter, very diffuse band at Mr 11,000 was also detected (fig. 2). The control, purified rabbit muscle enzyme, gave a single band of Mr 36,000, as reported previously for the subunit of the tetrameric native enzyme[12].

Both Western blotting and the pH profile show that the placental extract contains a smaller subunit recognizing an antibody raised against native Fru-P_2ase. A proteolysed subunit of Mr 26,000 was reported earlier on incubation of rabbit liver Fru-P_2ase with rat or rabbit liver lysosomal fractions[15,16]. Various inhibitors tried did not inhibit the proteolysis[20].

The presence of Fru-P_2ase was further investigated by studying the kinetic properties in the extract. The values for the Michaelis-Menten constants (K_m) for the substrate Fru-1,6-P_2 (pH 7.4, 26 µM; pH 9.3, 130 µM), and the inhibition by the strong inhibitor of Fru-P_2ases, AMP[21] (pH 7.4, 0.33 mM; pH 9.3, 1.7 mM) were found to be an order of magnitude higher at both neutral and alkaline pH, compared with the published values for the neutral enzyme from human liver[14]. The activity at neutral pH was inhibited by high substrate concentrations as reported by others[22]. Fru-2,6-P_2, an inhibitor of neutral Fru-P_2ase[23,24] was found not to affect the alkaline enzyme at saturating substrate concentrations, either at pH 7.4 or at pH 9.3. Alkaline phosphatase was excluded because L-phenylalanine, an inhibitor of this enzyme[25] did not affect the activity at the substrate concentrations employed either at pH 7.4 or at pH 9.3.

The kinetic evidence, the pH profile, and Western blotting indicate that the activity found in human term placental extracts has the properties of purified alkaline Fru-P_2ases, thus demonstrating the existence of this enzyme in human term placenta. Since the activity is affected by proteolysis, the amount of active Fru-P_2ase detected may not represent the total Fru-P_2ase present in placenta.

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