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Purification of human placental prostaglandin 15-hydroxydehydrogenase

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ABSTRACT

The NAD + -linked prostaglandin 15-hydroxydehydrogenase, which is responsible for the physiological inactivation of prostaglandins by catalysing the first step in the catabolism, was isolated and purified 995-fold from human placenta. The introduction of two new chromatographic steps in the purification procedure is responsible for an achieved specific activity of 1791 mU/mg. The molecular mass of the enzyme, as estimated by fast protein liquid chromatography, was 24 500 dalton. Sodium dodecyl sulphate discontinuous gel electrophoresis of the denatured enzyme revealed a molecular mass of 24 000 dalton. These data suggest that the enzyme consists of a single polypeptide chain.

INTRODUCTION

The enzyme prostaglandin 15-hydroxydehydrogenase (E.C. 1.1.1.141) was discovered in 1964 by Ånggard and Samuelsson [1] when they demonstrated that homogenates from swine lung converted prostaglandins to 15-dehydroprostaglandins. Subsequently an enzyme named 15-hydroxyprostaglandin dehydrogenase was isolated in 1966 and purified 11-fold from the lung cytosol fraction by Ånggard and Samuelsson. Since that time, the enzyme has been identified in several tissues [2–7], followed by a number of successful purifications of the enzyme from bovine lung [8–10], swine lung [11–13], swine kidney [14] and placenta [15–20]. Considerable differences concerning the molecular mass and the enrichment of the enzyme were noted in those publications.

This paper reports the purification of prostaglandin 15-hydroxydehydrogenase from human placenta in highly purified state, using an improved rapid purification technique with fast protein liquid chromatography (FPLC). Sodium dodecyl sulphate (SDS) discontinuous gel electrophoresis of the enzyme was performed to establish the degree of purification.

Using this highly purified protein, studies of the amino acid sequence of the prostaglandin 15-hydroxydehydrogenase might now be possible, giving more information concerning the active site of the enzyme and its regulation.

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EXPERIMENTAL

Materials

DEAE-Trisacryl-M, Sephadex G-75, Blue Sepharose CL-6B and Superose 12 were purchased from Pharmacia-LKB (Uppsala, Sweden), prostaglandin E_2 (PGE_2) from Sigma (Munich, F.R.G.) and NAD^+ and all other reagents from Merck (Darmstadt, F.R.G.).

Spectrophotometric assay

The activity of prostaglandin 15-hydroxydehydrogenase was measured spectrophotometrically following the formation of NADH at 340 nm.

All assays were conducted with blank controls (denatured enzyme solutions which had been boiled for 30 min). The reaction mixture contained 100 m*M* Tris–HCl buffer (pH 7.4), 20 m*M* NAD^+, 4.7 m*M* PGE_2 and 10–400 μl of enzyme in a total volume of 1 ml. The incubation was carried out in the absence of prostaglandin at 37°C for 5 min and the reaction was initiated by the addition of prostaglandin. One unit of enzyme activity is defined as the amount of enzyme which catalyses the formation of 1 μmol of NADH per minute under standard assay conditions.

Protein determination

The protein concentration was determined by the measurement of the absorbance at 280 and 260 nm followed by calculation according to Kalckar [21]. Low concentrations of protein were determined by the method of Bradford [22] using bovine serum albumin (BSA) as a standard.

Electrophoresis

SDS discontinuous gel electrophoresis was performed in 13% gels by the method of Laemmli [23]. The protein bands were stained with silver nitrate.

Fast protein liquid chromatography

The molecular mass of prostaglandin 15-hydroxydehydrogenase was determined using FPLC (Pharmacia). The gel filtration column (30 × 1 cm I.D., Superose 12) was calibrated with dextran blue, BSA, ovalbumin, chymotrypsinogen A and ribonuclease A. Prostaglandin 15-hydroxydehydrogenase was identified by measuring the activity in each fraction.

Enzyme isolation and purification

The method used for the isolation and purification of prostaglandin 15-hydroxydehydrogenase is a modification of the method described by Schlegel and Greep [16].

Step 1. Preparation of placenta up to the first chromatography step. All purification steps were carried out at 0–5°C unless specified otherwise. Two fresh human placentas were placed on crushed ice, dissected free from membranes, washed several times with ice-cold buffer A [20% (v/v) glycerol–10 m*M* potassium phosphate–250 m*M* saccharose–1 m*M* EDTA–1 m*M* dithiothreitol (DTT) at pH 7.4] and cut into small pieces.

After homogenization with an MSE mixer and in an Ultra-Turrax (IKA-Werk,

Stauffen, F.R.G.) for 60 s, the homogenate was centrifuged at 17 000 *g* for 30 min. Cold 1 *M* acetic acid was added to the supernatant solution until the pH had decreased to 5.1 and the solution was allowed to stand overnight in a refrigerator for quantitative precipitation. The solution was centrifuged at 17 000 *g* for 30 min.

The precipitate was suspended in buffer B[20% (v/v) glycerol–10 m*M* potassium phosphate–1 m*M* EDTA–1 m*M* DTT at pH 7.4] and homogenized in a glass vessel with a PTFE pestle and the pH was adjusted to 7.4 with 1 *M* NaOH. After stirring for 1 h the solution was centrifuged at 28 000 *g* for 30 min and the supernatant solution was used for the next step.

Step 2. DEAE-Trisacryl-M chromatography. The supernatant solution was applied to a column of DEAE-Trisacryl-M (21 × 5 cm I.D.) that had previously been equilibrated against buffer B. The column had been washed with 400 ml of buffer B and the elution was carried out with a linear gradient of 1 *M* potassium chloride in buffer B at a flow-rate of 120 ml/h (chamber 1 contained 250 ml of buffer B, chamber 2 contained 250 ml of buffer B with 1 *M* KCl). Fractions of 10 ml were collected and those having enzyme activity were pooled to form the DEAE pool in a total volume of 170 ml.

Step 3. Ammonium sulphate precipitation I. Solid ammonium sulphate was added with stirring to the DEAE pool to achieve 65% saturation. After 16 h at 4°C the suspension was centrifuged at 23 000 *g* for 30 min and the precipitate was dissolved in 50 ml of buffer D [20% (v/v) glycerol–100 m*M* potassium phosphate–1 m*M* EDTA–1 m*M* DTT at pH 7.4]. This solution is termed AS-precipitation I.

Step 4. Sephadex G-75 chromatography. AS-precipitation I was applied to a column of Sephadex G-75 (100 × 5 cm I.D.) equilibrated with buffer D and chromatographed with the same medium. Fractions having enzyme activity were pooled to form the Sephadex G-75 pool in a total volume of 240 ml.

Step 5. Ammonium sulphate precipitation II. This step was done in the same way as the ammonium sulphate precipitation I. After centrifugation the precipitate was dissolved in 50 ml of buffer B. That solution is termed AS-precipitation II.

Step 6. Blue Sepharose CL-6B chromatography. AS-precipitation II was applied to a column of Blue Sepharose CL-6B (25 × 1.5 cm I.D.) that had previously been equilibrated against buffer B. The column was then washed with 50 ml of the same buffer and the enzyme was eluted with a linear gradient of 2 m*M* NADH in buffer B at a flow-rate of 8.0 ml/h (chamber 1 contained 50 ml of buffer B, chamber 2 contained 50 ml of buffer B with 2 m*M* NADH). Fractions having enzyme activity were pooled to form the Blue Sepharose pool in a total volume of 10 ml. Before using that pool in the next step, it was dialysed against buffer E [5% (v/v) glycerol–100 m*M* potassium phosphate–10 m*M* EDTA–5 m*M* DTT at pH 7.4].

Step 7. Fast protein liquid chromatography on Superose 12. The last step of the purification was carried out using the FPLC technique. A gel filtration column (30 × 1 cm I.D) of Superose 12 was used, which was equilibrated with buffer E. The enzyme was eluted at a flow-rate of 0.3 ml/min and a pressure of 0.9 MPa. The fraction size was 0.5 ml. The active fraction is termed the Superose fraction.

RESULTS

Enzyme purification

The purification steps of the placental prostaglandin 15-hydroxydehydrogenase,

TABLE I

PURIFICATION OF HUMAN PLACENTAL PROSTAGLANDIN 15-HYDROXYDEHYDROGENASE

Fraction	Volume (ml)	Protein (mg)	Activity (mU)	Specific activity (mU/mg)	Enrichment	Yield (%)
(1) 28000 *g* supernatant	200	6060	10880	1.8	-	100
(2) DEAE pool	170	399.6	8704	21.8	12.1	80
(3) $(NH_4)_2SO_4$ precipitation I	50	248	8000	32.3	17.9	73.5
(4) Sephadex G-75 pool	240	101.4	5352	52.8	29.3	49.2
(5) $(NH_4)_2SO_4$ precipitation II	46	51.3	3901	76	42.2	35.9
(6) Blue sepharose pool	10	1.5	1312	874.7	486	12.1
(7) Superose fraction	0.5	0.068	121.8	1791	995	1.1

resulting in a 995-fold enrichment and a yield of 1.1%, are listed in Table I. The specific activity of the purified enzyme was 1791 mU/mg.

The chromatography on Blue Sephrose CL-6B and the FPLC were the most effective steps, leading to a high purity of the protein.

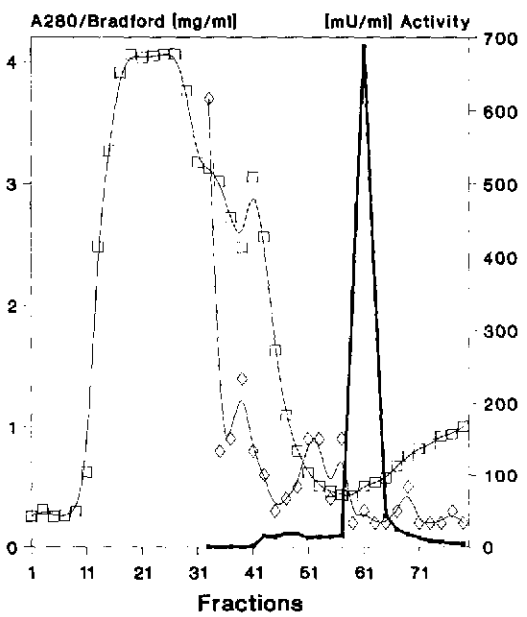


Fig. 1. Elution profile of Blue Sepharose CL-6B. ⊔ = Protein determination by measurement of the absorbance at 280 nm; ◇ = protein determination by the method of Bradford using BSA as standard; ■ = prostaglandin 15-hydroxydehydrogenase activity.

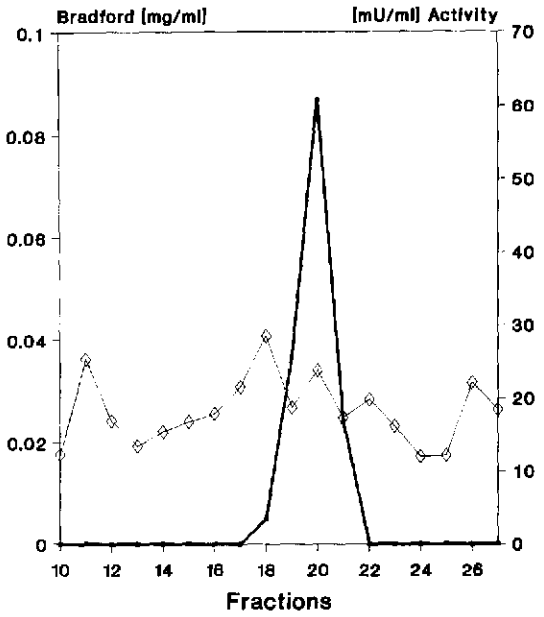


Fig. 2. Elution profile of Superose 12 using the FPLC technique. ◇ = Protein determination by the method of Bradford using BSA as standard; ■ = prostaglandin 15-hydroxydehydrogenase activity.

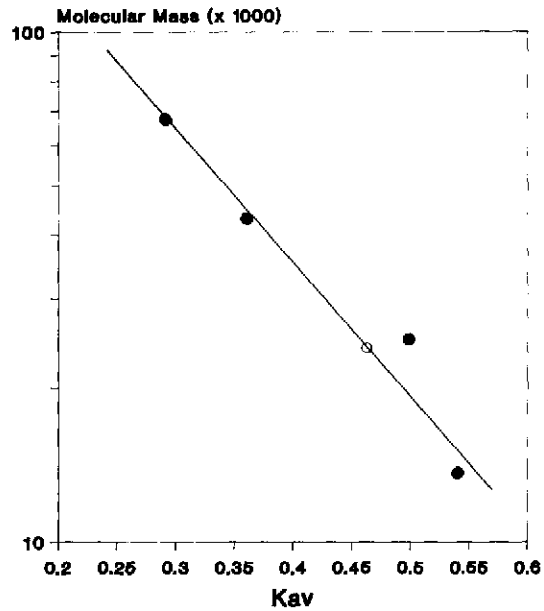
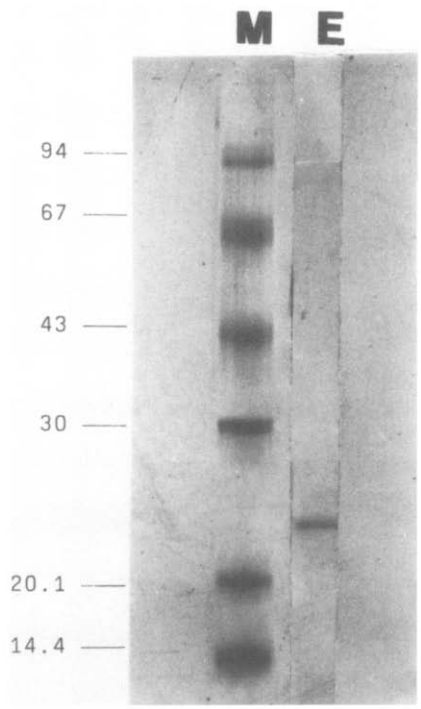


Fig. 3. Molecular mass determination of prostaglandin 15-hydroxydehydrogenase by FPLC. 500 µl of purified enzyme were applied to a Superose 12 gel filtration column (30 × 1 cm I.D.). (●) Protein standards: 1 = BSA (67000 dalton); 2 = ovalbumin (43000 dalton); 3 = chymotrypsinogen A (25000 dalton); 4 = ribonuclease A (13700 dalton). (○) Prostaglandin 15-hydroxydehydrogenase (24500 dalton). The partition coefficient K_{av} is defined by $(V_e - V_0)/(V_t - V_0)$, where V_e is the elution volume, V_0 the void volume and V_t the total volume.



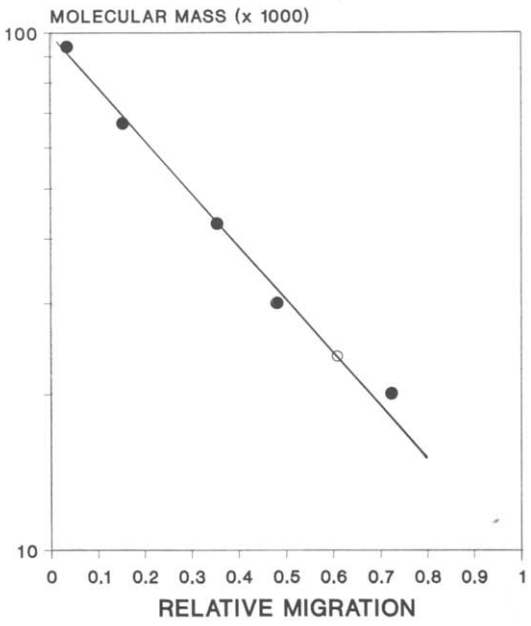


Fig. 4. SDS-gel electrophoresis of prostaglandin 15-hydroxydehydrogenase. The lines denote the positions of the marker proteins (molecular masses, indicated in kilodalton). (M) Phosphorylase *b* (94 000 dalton), BSA (67 000 dalton), ovalbumin (43 000 dalton), carboanhydrase (30 000 dalton), trypsin inhibitor (20 100 dalton), α-lactalbumin (14 400 dalton). (E) Purified enzyme.

Fig. 5. Molecular mass determination of prostaglandin 15-hydroxydehydrogenase by SDS gel electrophoresis. (●) Protein standards: 1 = phosphorylase *b* (94 000 dalton); 2 = BSA (67 000 dalton); 3 = ovalbumin (43 000 dalton); 4 = carboanhydrase (30 000 dalton); 5 = trypsin inhibitor (20 000 dalton). (○) Prostaglandin 15-hydroxydehydrogenase (24 000 dalton).

An NADH gradient was introduced into the Blue Sepharose CL-6B procedure as it released the enzyme in a sharp peak from the column, leading to a 486-fold enrichment. A typical elution profile of Blue Sepharose CL-6B is shown in Fig. 1.

The elution profile of the progressive purification step on Superose 12 using the FPLC technique is shown in Fig. 2.

Determination of molecular mass

Using the FPLC technique, a molecular mass of 24 500 dalton was established for prostaglandin 15-hydroxydehydrogenase (Fig. 3).

SDS discontinuous gel electrophoresis of the denatured enzyme revealed a single band with a molecular mass of 24 000 dalton (Fig. 4). The calculation is based on the retention factor of the enzyme in relation to protein standards (Fig. 5).

DISCUSSION

Prostaglandins play an important physiological role in the female reproduction system. However, still not much is known about the regulation of the prostaglandin metabolism. As placentae tissues are the richest sources of the prostaglandin 15-hydroxydehydrogenase, it was feasible to use placentae for purification of the enzyme. The modified purification procedure resulted in an enzyme fraction with a specific activity of about 1800 mU/mg protein, which is about three times higher than reported previously [16]. Recently, Jarabak and Watkins [20] described a purified enzyme fraction with a specific activity of 8300 mU/mg. Their results are not comparable with ours, however, as they used PGE_1 as the substrate in their test system. There is also a difference in calculating the enrichment of enzyme, as we used the 28 000 *g* supernatant as the basic for calculation, because it is difficult and not reliable to measure NADH production in a mass of tissue samples.

The purification steps 6 and 7 resulted in an extremely high enzyme enrichment, which should be promising for advances in studies on amino acid sequence analysis.

The linear gradient of NADH on Blue Sepharose CL-6B led to an 11.5-fold enrichment in one step, which could not be achieved without NADH.

The concentration of glycerol could be reduced from 20 to 5% (v/v) in the elution buffer of the Superose 12 without any loss of enzyme activity. In order to conserve enzyme activity we had to increase the concentration of EDTA from 1 to 10 m*M* and that of DTT from 1 to 5 m*M* in the buffer. Frozen enzyme solutions in that buffer at −20°C were stable for more than 8 months. The fact that glycerol was not important for the protection of the highly purified enzyme may also indicate that prostaglandin 15-hydroxydehydrogenase is not organized in subunits.

Jarabak and Watkins [20] suggested that the enzyme is split into two inactive identical or nearly identical subunits with a molecular mass of 25 500 -26 000 dalton. These findings could not be confirmed in our studies. A molecular mass of 24 500 dalton for the purified enzyme could be established determined by FPLC. The molecular mass of the denatured enzyme, obtained by SDS gel electrophoresis, was almost identical with that obtained by FPLC. These results provide strong evidence that the enzyme consists of a single polypeptide chain with a size of *ca.* 24 000 dalton.

Preliminary studies on the amino acid content of the enzyme demonstrated Amadori products, which indicate that there are carbohydrate molecules associated with the protein structure (unpublished results).

Further studies on the organization of the prostaglandin 15-hydroxydehydrogenase, especially the analysis of the amino acid sequence, are necessary in order to understand the physiological control of the prostaglandin metabolism.

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