Isolation of Human Lactate Dehydrogenase Isoenzyme X by Affinity Chromatography

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Human isoenzyme LDH-X (lactate dehydrogenase isoenzyme X) was isolated from seminal fluid of frozen semen samples by affinity chromatography by using oxamate-Sepharose and AMP-Sepharose. In the presence of 1.6 mM-NAD⁺, isoenzyme LDH-X does not bind to AMP-Sepharose, whereas the other lactate dehydrogenase isoenzymes do. This is the crucial point in the isolation of isoenzyme LDH-X from the other isoenzymes. The purified human isoenzyme LDH-X had a specific activity of 146 units/mg of protein.

A special form of lactate dehydrogenase, isoenzyme LDH-X, has been found in testicular tissue and spermatozoa of several mammalian and avian species (Goldberg, 1963; Blanco & Zinkham, 1963; Zinkham *et al.*, 1964). The usual form of lactate dehydrogenase occurs in five types, each being one of the possible random tetrameric combinations of two different polypeptide subunits, designated A and B (Markert, 1963). Mouse isoenzyme LDH-X, also a tetramer, is composed of only one kind of subunit, designated C, which is different from subunits A and B. It is a product of a separate gene which is active only during the primary spermatocyte stage of the spermatogenic cycle (Goldberg & Hawtrey, 1967). This isoenzyme differs from the other lactate dehydrogenase isoenzymes in chemical and enzymic properties (Goldberg, 1972). Furthermore, it has been demonstrated that isoenzyme LDH-X is immunologically distinct from isoenzymes LDH-1 and LDH-5, which are composed solely of B and A subunits respectively (Goldberg, 1971).

It has been shown that isoenzyme LDH-X is auto- and iso-antigenic in mice and rabbits, and immunization with it decreases fertility in both male and female animals (Goldberg & Lerum, 1972; Goldberg, 1973; Lerum & Goldberg, 1974), though Erickson *et al.* (1975) found that passive immunization with rabbit anti-(isoenzyme LDH-X) did not lead to a decrease in fertility in the mouse. [A substance is called auto-antigenic when it is possible to elicit an immune response in the individual possessing the

substance (e.g. sperm enzymes in male animals); iso-antigenic is used for a substance when it is possible to elicit an immune response in an individual of the same species not possessing the substance.] These unique properties make this enzyme of interest as a possible vaccine for fertility regulation.

It seems possible that some infertile men or women could possess antibodies to human isoenzyme LDH-X that are responsible for, or are a contributing factor to, their infertility. Even if not a naturally occurring cause of infertility, it is also possible that iso- or auto-immunization with isoenzyme LDH-X might be able to cause infertility in humans.

The aim of the studies was to isolate human isoenzyme LDH-X in order to develop a radio-immunoassay for the detection of antibodies against this enzyme.

The present paper describes the isolation of human isoenzyme LDH-X by affinity chromatography by using oxamate-Sepharose and AMP-Sepharose (Spielman *et al.*, 1973; O'Carra & Barry, 1972; Mosbach *et al.*, 1972). It is found that in the presence of 1.6 mM-NAD⁺ isoenzyme LDH-X does not bind to AMP-Sepharose, whereas the other LDH isoenzymes do. This is the crucial point in the isolation of isoenzyme LDH-X from the other isoenzymes.

Materials and Methods

Frozen semen samples were obtained from the infertility clinics of the Onze Lieve Vrouwe Gasthuis and the Wilhelmina Gasthuis in Amsterdam, and all samples were used, irrespective of quality. AMP-Sepharose and Sepharose 4B were from Pharmacia (Uppsala, Sweden). The ligand concentration was 2 μ mol of AMP/ml of Sepharose. Insolubilized

Abbreviations used; isoenzyme LDH-X, lactate dehydrogenase isoenzyme X.

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oxamate (oxamate-Sepharose) was made as described by Spielman *et al.* (1973). Ligand concentration obtained was 7 μ mol of bound oxamate/ml of Sepharose. Human serum albumin and 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide was from Sigma (St. Louis, MO, U.S.A.).

Isolation of human isoenzyme LDH-X

Frozen human semen samples were thawed, pooled and centrifuged for 20 min at 30000g and 4°C. About 500 ml of seminal fluid was fractionated by $(\text{NH}_4)_2\text{SO}_4$ precipitation (Goldberg, 1972). The precipitate obtained between 40 and 60% saturation with $(\text{NH}_4)_2\text{SO}_4$ was dialysed against 0.05 M-sodium phosphate buffer, pH 6.8, containing 0.5 M-NaCl. This buffer was used for all of the following chromatography steps. During the isolation procedure the temperature was kept at 4°C.

The affinity chromatography procedures used in the isolation of isoenzyme LDH-X from the $(\text{NH}_4)_2\text{SO}_4$ fraction were based on previously developed methods. An oxamate-Sepharose column had been used by O'Carra & Barry (1972) to isolate LDH isoenzymes. In the presence of 0.2 mM-NADH, lactate dehydrogenase isoenzymes bind to the column; they are eluted by buffer alone. Lactate dehydrogenase isoenzymes also bind to AMP-Sepharose when added in the presence of buffer alone, and buffer containing 0.5 mM-NADH effects their elution (Mosbach *et al.*, 1972).

Other methods

Protein concentrations were determined by the method of Lowry *et al.* (1951), with human serum albumin as the protein standard.

Reaction rates for lactate dehydrogenase were measured as the rate of change in A_{340} of NADH. The reaction medium contained: 0.05 M-sodium phosphate, pH 7.5, 0.6 mM-sodium pyruvate and 0.15 mM-NADH. For K_m determinations the pyruvate was between 10 mM and 10 μ M. One unit of enzyme activity oxidizes NADH at the rate of 1 μ mol/min at 25°C. Molar absorption coefficient for NADH is 6.22×10^3 litre $\cdot$ mol⁻¹ $\cdot$ cm⁻¹ (Horecker & Kornberg, 1948).

Testes were obtained from autopsy. The homogenate was prepared as described by Goldberg (1972).

Polyacrylamide-gel electrophoresis and staining of lactate dehydrogenase isoenzymes was performed as described by Dietz & Lubrano (1967).

Results

Polyacrylamide-gel electrophoresis, with staining for lactate dehydrogenase activities, showed the presence in seminal plasma from frozen pooled semen, and in testis homogenate, of isoenzymes

LDH-1, -2, -3, -X and -4. The relative amount of isoenzyme LDH-X in frozen semen samples and testis homogenate was about the same, 10–15% of the total lactate dehydrogenase activity, as estimated by staining after gel electrophoresis. Therefore frozen semen samples were used for the isolation of isoenzyme LDH-X, since the total lactate dehydrogenase activity of semen is far higher than in testis homogenate.

An oxamate-Sepharose column (15 mm $\times$ 60 mm) was used to separate lactate dehydrogenase isoenzymes in the 40–60%-satd. $(\text{NH}_4)_2\text{SO}_4$ fraction of seminal plasma. However, addition of 0.2 mM-NADH to the seminal-plasma fraction was inadequate to enable binding of lactate dehydrogenase isoenzymes to the column, since the NADH was degraded by seminal-plasma enzymes (Mann, 1964). It was found that, if the seminal fraction was mixed with an equal volume of buffer (see the Materials and Methods section) containing 0.5 mM-NADH immediately before it was loaded on to the oxamate-Sepharose column, there was still adequate NADH present during the time of passage of sample through the column to enable complete binding of lactate dehydrogenase to the column. The mixing of sample and buffer solution containing NADH was achieved with a T-piece joining the tubing coming from the containers with the seminal fraction and with the buffer plus 0.5 mM-NADH. After the lactate dehydrogenase isoenzymes had bound to the column, buffer containing 0.2 mM-NADH was used to wash out unbound seminal proteins.

To elute isoenzyme LDH-X from oxamate-Sepharose buffer alone, or buffer containing 1.6 mM-NAD⁺, was used. Elution with buffer alone released all lactate dehydrogenase isoenzymes. Elution with buffer containing NAD⁺ preferentially released LDH-3, -X and -4 isoenzymes, with isoenzymes LDH-1 and -2 being released later by buffer alone.

To separate isoenzyme LDH-X from the other LDH isoenzymes, AMP-Sepharose was used. The crude lactate dehydrogenase preparation eluted from oxamate-Sepharose was concentrated by vacuum dialysis to about 10 ml and was applied to an AMP-Sepharose column in buffer containing 1.6 mM-NAD⁺. Under these conditions isoenzyme LDH-X did not bind to AMP-Sepharose, whereas the other lactate dehydrogenase isoenzymes did, to varying degrees. This is the crucial point in the isolation of isoenzyme LDH-X free from contamination with other isoenzymes. The early eluate fractions contained isoenzyme LDH-X uncontaminated by other isoenzymes. Later fractions contained also a minor proportion of isoenzyme LDH-3 (Figs. 1 and 2). The separation of isoenzyme LDH-X from the fractions contaminated with isoenzyme LDH-3 can be effected by a second passage of the material through the AMP-Sepharose column.

Fig. 1. Elution pattern from AMP-Sepharose column.

Lactate dehydrogenase preparation obtained from an oxamate-Sepharose column was applied to the AMP-Sepharose column, (15 mm × 50 mm), and 2 ml fractions were collected. The eluent was 0.5 M-NaCl in 0.05 M-sodium phosphate, pH 6.8, containing the nicotinamide nucleotide additions indicated. Fractions 49, 50 and 51 contained too high activities to be measured in the enzyme-activity assay.

Fig. 2. Polyacrylamide-gel electrophoresis of eluate from AMP-Sepharose column

Electrophoresis was in 5.5% acrylamide. Electrophoretic migration was from top (cathode) to bottom (anode). Electrophoresis was continued for 1 h at 2.5 mA/gel. The numbers along the gel indicate the corresponding isoenzymes: (a) 20 μ l of fraction 26; (b) 20 μ l of fraction 28; (c) 10 μ l of fraction 50. All gels were stained for lactate dehydrogenase activity. See Fig. 1 for fraction numbers. Note that fraction 28 is contaminated with isoenzyme LDH-3.

from oxamate-Sepharose) on to the AMP-Sepharose column by using buffer alone (2 column volumes), whereby all lactate dehydrogenase isoenzymes bound (Fig. 1). Isoenzyme LDH-X was released preferentially by elution with buffer containing 1.6 mM-NAD⁺. With this procedure, isoenzyme LDH-3 was again the first contaminant (Fig. 2).

The enzyme activity at this stage of the isolation procedure could not be measured accurately, since NAD⁺ was present in a high concentration, which inhibits the enzymic reduction of pyruvate and also interferes with the protein determination. To remove the NAD⁺ (and if the preparation is left, there is a need to remove inactivated enzyme) the isoenzyme LDH-X preparation was applied to an oxamate-Sepharose column (10 mm × 7 mm) in the presence of an excess of NADH (0.5 mM). After washing with 0.2 mM-NADH, the isoenzyme LDH-X was eluted with buffer alone. The purity of the preparation was determined in polyacrylamide-gel electrophoresis by staining for lactate dehydrogenase activity and for protein. Only one band, corresponding to isoenzyme LDH-X, was present (Fig. 3). The yield of isoenzyme LDH-X was 70–80% in different preparations, and the purified isoenzyme LDH-X had a specific activity of 146 units/mg of protein. The Michaelis constant for isoenzyme LDH-X with pyruvate as substrate, determined by plotting v versus v/s , was found to be 46 μ M with 0.15 mM-NADH, comparable with the values obtained by Wilkinson & Withycombe (1965), 50 μ M, and by Svasti & Viriyachai (1975), 53 μ M.

Discussion

Isoenzyme LDH-X was isolated from seminal fluid by affinity chromatography. In contrast with

It was also found practical to separate isoenzyme LDH-X from the other isoenzymes by passing the crude lactate dehydrogenase preparation (eluted

Fig. 3. Polyacrylamide-gel electrophoresis of human isoenzyme LDH-X isolated by affinity chromatography. Electrophoresis was performed as indicated in legend for Fig. 2: (a) 20 μ l of seminal fluid stained for lactate dehydrogenase activity; (b) 200 ng of isoenzyme LDH-X preparation stained for lactate dehydrogenase activity; (c) 20 μ g of isoenzyme LDH-X preparation stained for protein with Amido Black.

mouse isoenzyme LDH-X (Spielman *et al.*, 1973), human isoenzyme LDH-X is bound to oxamate-Sepharose in the presence of 0.2 mM-NADH and 0.5 M-NaCl, whereas rabbit isoenzyme LDH-X does not bind to oxamate-Sepharose in the presence of NADH of a concentration greater than 40 μ M and NaCl at a concentration greater than 0.1 M (Goldberg, 1975). NAD⁺ was added to the buffer for elution of isoenzyme LDH-X from the oxamate-Sepharose column to increase the relative amount of isoenzyme LDH-X. In this respect isoenzyme LDH-X is comparable with isoenzyme LDH-5, which is also not retarded in the presence of NAD⁺, in contrast with isoenzymes LDH-1 and -2 (O'Carra *et al.*, 1974). The retardation of isoenzyme LDH-1 on oxamate-Sepharose has been explained in terms of an abortive-complex formation (enzyme-NAD⁺-pyruvate), which is considered to be the cause of the inhibition of the enzyme activity at high pyruvate concentrations (O'Carra *et al.*, 1974). But since human isoenzyme LDH-X (Wilkinson & Withycombe, 1965; A. H. J. Kolk, unpublished work) as well as mouse and rabbit isoenzyme LDH-X are inhibited by high pyruvate concentrations, the abortive-complex formation does not explain the separation of isoenzyme LDH-X from LDH-1 on the oxamate-Sepharose column.

With the AMP-Sepharose column isoenzyme LDH-X could be separated from the other isoenzymes, because it does not bind to the column in the presence of 1.6 mM-NAD⁺. It was found that different batches of AMP-Sepharose can show different properties. Two batches bound isoenzyme LDH-X in the presence of buffer alone (batch no. 4714 and 6377), whereas another batch did not (batch no. 4859). With the latter batch, isoenzyme LDH-X passed from the column first, followed by isoenzyme LDH-3. Both kinds of AMP-Sepharose are suitable for the isolation of isoenzyme LDH-X, but the need for monitoring of eluates is pointed out.

It is possible to prepare isoenzyme LDH-X by subjecting the (NH₄)₂SO₄ fraction of seminal plasma to affinity chromatography on AMP-Sepharose. However, initial isolation of lactate dehydrogenase isoenzymes on an oxamate-Sepharose column is preferred since such a column has a far larger capacity for lactate dehydrogenase than does AMP-Sepharose, and since some batches of AMP-Sepharose do not bind isoenzyme LDH-X strongly, it then passes through the column together with other seminal plasma components. The binding capacity of the oxamate-Sepharose column (15 mm $\times$ 60 mm) was not reached by applying the lactate dehydrogenase present in 500 ml of seminal fluid with an activity of about 1.7 units/ml. The binding capacity of the AMP-Sepharose column (15 mm $\times$ 55 mm; batch no. 4714) was reached by applying the lactate dehydrogenase (and the other competing enzymes) present in 400 ml of semen, with an activity of about 0.5 unit/ml.

The successful isolation of human isoenzyme LDH-X is of value for biochemical and reproductive-immunology studies.

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