THE SEPARATION OF LACTATE DEHYDROGENASE X FROM OTHER LACTATE DEHYDROGENASE ISOZYMES OF MOUSE TESTES BY AFFINITY CHROMATOGRAPHY

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1. Introduction

There is now substantial evidence that the sperm 'specific' lactate dehydrogenase (LDH), isozyme LDH-X has enzymatic and immunological properties different from LDH-1 and LDH-5 (EC 1.1.1.27) [1–3]. LDH-X is encoded by a separate gene locus which is only active during the primary spermatocyte stage of the spermatogenic cycle and is apparently inactive in all other cells of the organism [4, 5]. Since quantitative and qualitative changes of LDH-X may affect male fertility [6] and since an antiserum to LDH-X seems to suppress pregnancy in the mouse [7], we were interested in purifying this enzyme. Previous attempts at isolating LDH-X from different mammalian species, e.g. those recently carried out on mouse testes [1, 3], have used nonspecific purification steps. However, when performing affinity chromatography of mouse testicular LDH according to a modification of the method of O'Carra and Barry [8] we found that LDH-X, in contrast to the other isozymes, had no affinity for the sepharose linked oxamate. This binding difference facilitates a simple separation of the sperm 'specific' LDH-X from the other LDH isozymes.

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2. Materials and methods

Chemicals used in these experiments were obtained from the Sigma Chemical Co., St. Louis, Mo., USA, except for cyanogen bromide and hexane diamine which came from Eastman-Kodak, Inc., Rochester, N.Y.

The basic preparation of mouse testes of random bred Swiss mice was performed as described by Goldberg [1]. The 40%–70% $(NH_4)_2SO_4$ precipitate was dissolved in a minimum amount of 0.5 M NaCl in 0.02 M phosphate buffer, pH 6.8, and dialysed against the same buffer. After adjusting this extract to 200 μM NADH, it was applied to the affinity column according to O'Carra and Barry [8]. The extract of about 50 g of testes was carefully layered on a column of 50 ml vol.

The affinity column was prepared following the general procedure of Cuatrecasas [9]. The LDH specificity of the column was achieved as described by O'Carra and Barry [8] except for the following *essential* alteration of the method for coupling potassium oxalate to the aminohexyl-Sepharose: the potassium oxalate was dissolved in water *without* any adjustment of the pH and water-soluble 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide was added. After an activation period of 5 min this solution was mixed with the packed aminohexyl-Sepharose and gently stirred overnight at room temperature. The next morning the trinitrobenzene sulfonate test [9] indicated a complete second coupling step. The columns were run at room temperature with the described buffer system [8].

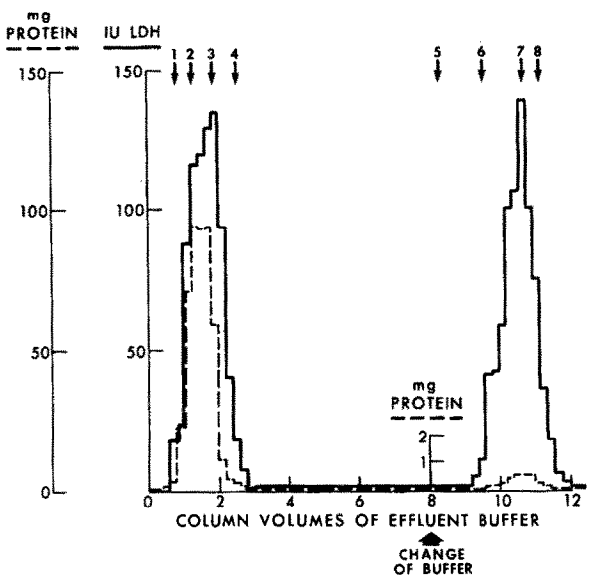

Fig. 1. Affinity chromatography of extracts of 50 g mouse testes on a 50 ml column of Sepharose-linked oxamate. The eluent was 0.5 M NaCl in 0.02 M sodium phosphate buffer, pH 6.8, adjusted to 200 μM NADH. At the arrow NADH was omitted from the eluant. The amounts of protein and LDH activity refer to 10 ml fractions. The small numbers with arrows are identical with the samples of fig. 2.

Contrary to the results of O'Carra and Barry [8], we were unable to achieve the second coupling step after adjusting the potassium oxalate to pH 4.7. The addition of dicyclohexyl carbodiimide caused the decomposition of oxalic acid into carbon dioxide and carbon monoxide [13, 14]. This decomposition also occurred with water-soluble 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and with both free oxalic acid and potassium oxalate adjusted to pH 4.7. Potassium oxalate is stable in the presence of water-soluble carbodiimide when the pH is not further adjusted, and a successful coupling of the oxalate to the amino-Sepharose could be performed only under these conditions.

Fractions of 10 ml of the effluent were collected and assayed for LDH activity at 25°C according to Epstein et al. [12]; protein was measured by the Lowry method [13]. Polyacrylamide gel electrophoresis and the identification of LDH activity of the gels was performed as described by Epstein et al. [14]. For the identification of LDH-X activity on the gels, OH-valeric acid was used in the incubation mixture instead of lactic acid according to Goldberg and Hawtrey [5]. The gels were stained for protein, after fixation in 20% trichloroacetic acid (TCA) for 1 hr, with a solution containing 0.1% Coomassie blue in 20% TCA for 3 hr. The destaining was performed in 10% TCA. The fractions which contained high LDH activity were concentrated by vacuum dialysis at 4°C.

3. Results

The LDH activity and protein content of the fractions obtained by affinity chromatography of mouse testicular extracts are shown in fig. 1. In contrast to the patterns described by O'Carra and Barry [8] for purified LDH-1 and for placenta homogenate, we found that about 50% of the total testicular LDH activity was not bound to the column in the loading buffer which contained 200 μM NADH. This first peak of LDH activity was not perfectly coincident with the protein. The protein peak usually had its maximum a little earlier than the LDH activity. The remaining activity was bound to the aminohexyl-linked oxamate and came off the column as a second peak when buffer without any NADH was applied [8].

Identification of the isozyme pattern of the first and second LDH activity peaks was carried out by electrophoresis on polyacrylamide gels (fig. 2). Fractions from the first LDH peak contained only LDH-X while those of the second peak gave positive staining only in positions characteristic for isozymes 1–5. Closer examination reveals that after the change of the elution buffer LDH-5 is dominant in the first fractions to elute (sample 6), whereas LDH-2 comes off the column later (sample 7) and LDH-1 last (sample 8). This changing isozyme pattern indicates that LDH-1 has a higher affinity for the column than does LDH-5.

The fractions of the two LDH activity peaks were concentrated by vacuum dialysis, and small amounts of the concentrates were re-examined on polyacrylamide gels. As shown by samples A and C in fig. 3, the concentrated fractions of the second peak contained no proteins other than the LDH isozymes 1–5. The same samples also indicate that LDH-1 accounts for at least 50% of the total LDH activity of this peak. After 40 min of incubation in the OH-valeric acid-containing staining mixture there was faint staining de-

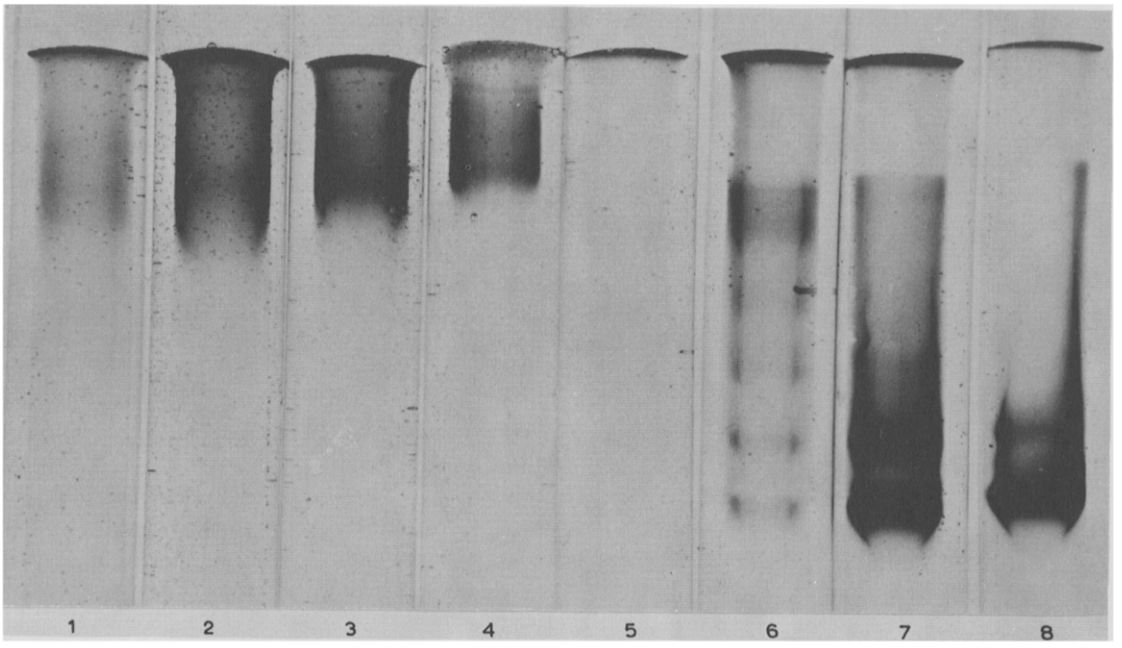

Fig. 2. LDH isozyme pattern of polyacrylamide gels of different samples of the affinity column effluent. The numbers refer to the numbers with arrows on fig. 1, indicating fractions from which samples were taken.

tectable which followed the same isozyme pattern (B). No activity was present in the LDH-X position.

With both lactate (D) and OH-valeric acid (E), the concentrated fractions of the first peak developed a positive stain only in the LDH-X position. Although the protein pattern of the first peak is very heterogeneous, there is a dark band in the position of LDH-X (F).

Further purification of LDH-X was achieved by preparative polyacrylamide gel electrophoresis of the second half of the first peak (starting at point of maximum LDH-X activity) [15].

The LDH isozymes which were isolated as described above were pure enough to be used for the preparation of specific antibodies against both LDH-1 and LDH-X of the mouse [15].

4. Discussion

The higher affinity of LDH-1 than of LDH-5 to the oxamate on the column is in good agreement with similar recent reports of the affinity of the two isozymes of man [16] and pig [17] to free oxamate. However, the fact that LDH-X of the mouse is not bound to the amino-Sepharose linked oxamate in a buffer with a high NADH concentration is not in agreement with many of the properties of this particular isozyme which have been described so far. Oxamate is a competitive inhibitor of the reaction pyruvate → lactate [18]. The K_i of oxamate for rabbit LDH-X is the same as that for LDH-1. Studies carried out on purified mouse LDH-X revealed K_m values and substrate inhibition curves with pyruvate that were similar to those obtained with LDH-1 of the mouse [3, 19]. Both LDH-X and LDH-1 have a much higher affinity for pyruvate than does LDH-5 [3, 19], and the molecular weights of all three isozymes are similar [2]. These biochemical data do not suggest an explanation of the separation of LDH-X from LDH-1 to LDH-5 by the column. It is possible, however, that structural peculiarities of LDH-X may not allow the specific binding sites of this isozyme to attach to the hexamate bound oxamate on the column in the same way as LDH-1 and LDH-5 do.

LDH-5 activity in mature testes of the mouse has so far not been identified on polyacrylamide gels since it migrates in a position close to that of LDH-X [19].

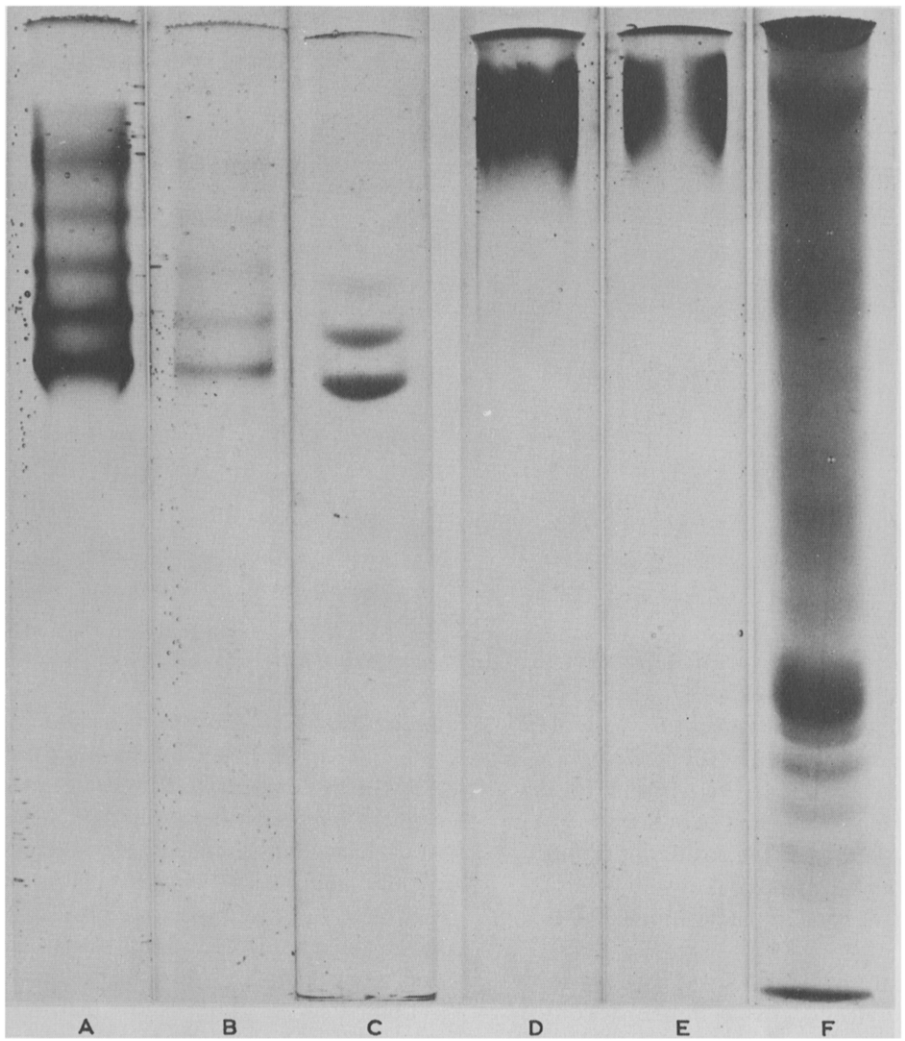

Fig. 3. Polyacrylamide gel patterns of concentrated LDH activity-containing fractions. A, B, C = pooled second (eluant without NADP); LDH activity peak; D, E, F = pooled first peak. A and D-lactate staining; B and E = OH-valeric acid staining; C and F = protein stain (Coomassie blue).

Our separation of LDH-X from the other isozymes allowed for a demonstration on polyacrylamide gels of a measurable LDH-5 activity in mature testes of the mouse, similar to what was previously shown by starch gel electrophoresis [3]. If the LDH-X of other species can be separated from the other LDH isozymes by affinity chromatography, this method might permit the detection of the sperm 'specific' isozyme in species or perhaps even in organs in which electrophoretic similarities to one of the other 5 isozymes has previously not allowed an identification of LDH-X to be made.

Acknowledgements

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References

[1] Goldberg, E. (1972) J. Biol. Chem. 247, 2044.
[2] Markert, C.L. (1971) in: Advances in the Biosciences, (Raspé, G., ed.), Vol. 6, p. 511, Pergamon Press–Vieweg, New York, Braunschweig.
[3] Battellino, L.J. and Blanco, A. (1970) J. Exptl. Zool. 174, 173.
[4] Zinkham, W.H., Blanco, A. and Clowry, L.J. (1964) Ann. N.Y. Acad. Sci. 121, 571.
[5] Goldberg, E. and Hawtrey, C.O. (1967) J. Exptl. Zool. 164, 309.
[6] Zinkham, W.H. (1972) Johns Hopkins Med. J. 130, 1.
[7] Goldberg, E. (1972) Science 176, 686.
[8] O'Carra, P. and Barry, S. (1972) FEBS Letters 21, 281.
[9] Cuatrecasas, P. (1970) J. Biol. Chem. 245, 3059.
[10] Zetsche, F. and Friedrich, A. (1939) Chem. Ber. 72, 363.
[11] Tanimora, T., Kasai, Y. and Tamura, Z. (1972) Chem. Pharm. Bull. 20, 1845.
[12] Epstein, C.J., Wegienka, E.A. and Smith, C.W. (1969) Biochem. Genet. 3, 271.
[13] Lowry, O.H., Rosebrough, N.J., Latt, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265.
[14] Epstein, C.J., Kwok, L. and Smith, S. (1971) FEBS Letters 13, 45.
[15] Spielmann, H., Erickson, R.P. and Epstein, C.J., in preparation.
[16] Wilkinson, J.H. and Walter, S.J. (1972) Enzyme 13, 170.
[17] Holbrook, J.J. and Stinson, R.A. (1973) Biochem. J. 131, 739.
[18] Nora, W.B., Wiener, A.D., Glaid, A.J. and Schwert, G.W. (1959) J. Biol. Chem. 234, 1143.
[19] Hawtrey, C.O. and Goldberg, E. (1970) J. Exptl. Zool. 174, 451.