PRO EXPERIMENTIS

Rapid Purification of Lactate Dehydrogenase X from Mouse Testes by Two Steps of Affinity Chromatography on Oxamate-Sepharose[1]

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Summary. At concentrations of 200 μM NADH and 0.5 M NaCl LDH-X is separated from the other LDH isozymes of mouse testes on oxamate-sepharose. In a second step LDH-X is bound to the same matrix at lower NADH and NaCl concentrations and the pure enzyme can subsequently be eluted.

Fertility inhibition by active and passive immunisation against the sperm specific lactate dehydrogenase (EC 1.1.1.27; LDH) isozyme X (LDH-X) has been reported in mice[3–6] and rabbits[7]. Investigations on the mechanism of this type of fertility inhibition[5,6,8] can only be performed after purification of LDH-X and the production of antibodies against this LDH isozyme. Usually testes tissue serves as the main source for the isolation of mouse LDH-X by conventional procedures[9–11]. We have recently reported the separation of LDH-X from other LDH isozymes of mouse testes by affinity chromatography on oxamate-sepharose as part of the isolation of pure mouse LDH-X[12]. More recently Goldberg[11] described the purification of LDH-X from spermatozoa, which contain LDH-X as the only LDH isozyme, by one step of affinity chromatography under different conditions on the same matrix. From careful studies on the binding of LDH-X and of LDH 1–5 to oxamate-sepharose at different NaCl and NADH concentrations, we determined the conditions for a rapid purification of mouse LDH-X from testes extracts by two steps of affinity chromatography.

Materials and methods. Basic chemicals were purchased from the Merck AG, Darmstadt, NADH and pyruvate from Böhringer, Mannheim, hexane diamine from EGA-

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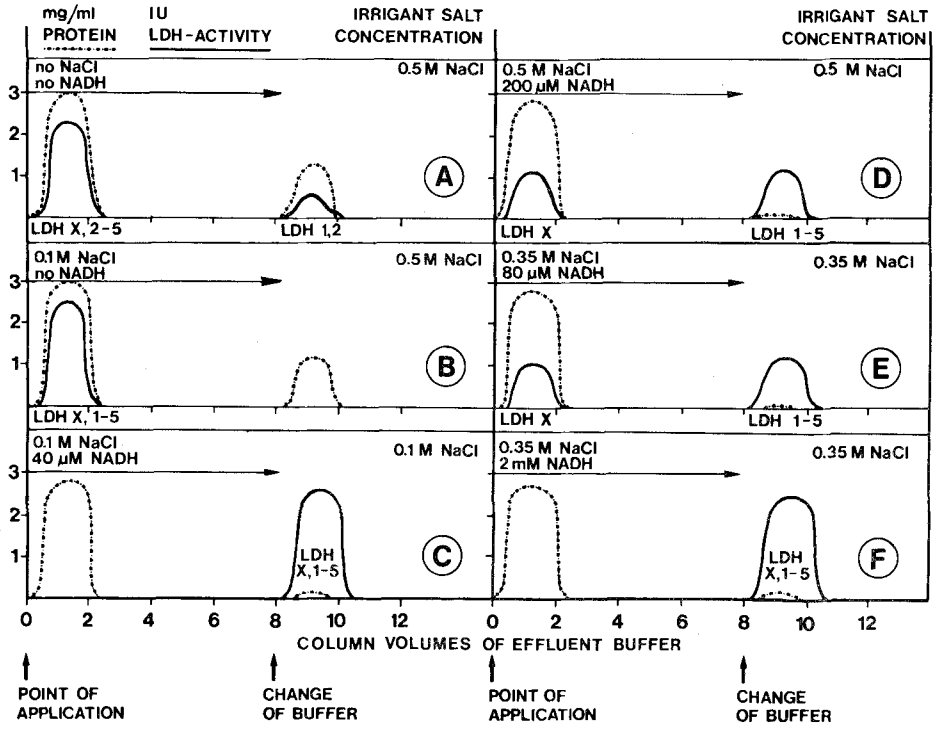

Fig. 1. Affinity chromatography studies of mouse testes homogenate using oxamate-sepharose. The irrigating buffer consisted of 0.02 M potassium phosphate buffer, pH 6.8 adjusted to different NaCl and NADH concentrations as indicated. The column contained 10 ml sepharoselinked oxamate. 5 ml samples were applied to the column and 2.5 ml fractions were collected and assayed for protein and LDH activity. The LDH isozyme patterns were identified after electrophoresis on polyacrylamide gels.

Chemie, Steinheim, cyanogen bromide from Serva, Heidelberg, and Sepharose-4B as well as 1-ethyl-3-(-3-dimethylaminopropyl)-carbodiimide from the Sigma Chemical Co., Munich. Affinity chromatography of mouse testes of the NMRI strain (breeder: Schwencke, Naunheim, Germany) was performed as previously described[12] except for the following changes: The 35,000 $\times g$ supernatant of the testes homogenate in 0.02 M phosphate buffer, pH 6.8, was adjusted to the described NADH and NaCl concentrations without any previous $(NH_4)_2$ SO_4 precipitation step and then applied to the affinity column. The preparation of the oxamate-sepharose was carried out as described in the preceding report[12]. However, the concentrations of potassium oxalate and water-soluble carbodiimide in the second coupling step were increased as recommended recently by O'CARRA et al.[13]. LDH was assayed according to EPSTEIN et al.[14]; protein concentrations were measured by the LOWRY method[15]. Conditions for polyacrylamide electrophoresis and the identification of LDH activity and protein bands have been described previously[12]. Purified LDH-X was concentrated by vacuum dialysis at 4 °C.

Results and discussion. When studying the binding of mouse testes extracts to oxamate-sepharose in the absence of NADH and NaCl, we found no adsorbtion of LDH-X and LDH 3–5 but a binding of LDH 1–2 and also of other proteins which could be eluted by 0.5 M NaCl (Figure 1A). When the same extract was chromatographed at 0.1 M NaCl, no LDH was bound in the absence of NADH (Figure 1B). However, low NADH concentrations were able to bind LDH 1–5 and LDH-X at 0.1 M NaCl (Figure 1C). The latter conditions have been used by WHEAT and GOLDBERG[16] to adsorb mouse LDH-X to oxamate-sepharose and by GOLDBERG[11] to isolate rabbit LDH-X. We had previously not found any adsorbtion of mouse LDH-X at 0.5 M NaCl and 200 μM NADH[12], but at the same time we did find a binding of LDH 1–5 (Figure 1D). WHEAT and GOLDBERG[16] suggest from their data (Figure 1C) that the binding of mouse LDH-X is exquisitely sensitive to high NADH concentrations and was not to be absorbed under our conditions (200 μM NADH; Figure 1D). Since NADH facilitates the binding of LDH 1–5 and also of LDH-X in the presence of 0.1 M NaCl (Figure 1C), and LDH-X could not be eluted by increasing NADH concentrations up to 2 mM NADH in additional experiments, we felt that the binding of LDH-X in our separation study was inhibited by the high salt concentration (0.5 M NaCl) rather than by the NADH concentration (200 μM NADH; Figure 1D). This problem was further studied at a constant concentration of 0.35 M NaCl. An adjustment of the NADH concentration to 80 μM NADH did not allow a binding of LDH-X (Figure 1E). When increasing the NADH concentration up to 2 mM NADH, however, LDH-X was adsorbed and could be eluted with LDH 1–5 as soon as NADH was omitted from the irrigation buffer (Figure 1F). Figure 1, E and F demonstrate that NADH facilitates the binding of LDH-X to oxamate-sepharose in the presence of NaCl; higher NADH concentrations, of course, had to be used than at the lower NaCl concentration in Figure 1, B and C. An inhibition of the binding of LDH-X by the high NADH concentration in our separation study[12], as suggested by WHEAT and GOLDBERG[16], therefore seems very unlikely.

The differences in the binding characteristics of LDH-X and LDH 1–5 at different NADH and salt concentrations allow a rapid purification of LDH-X from testes by two steps of affinity chromatography. The first step is performed on the 35,000 $\times g$ supernatant of mouse testes homogenate adjusted to 200 μM NADH and 0.5 M NaCl according to Figure 1D. The binding of LDH-X to the column, which is the second step of the affinity chromatography, is carried out on the unbound fractions containing LDH-X and contaminating proteins after a 1:5 dilution. The resulting NaCl and NADH concentrations (= 0.1 M NaCl and 40 μM NADH) allow a binding of LDH-X, according to Figure 1C. Pure LDH-X can finally be eluted from the column in the absence of NADH. After concentration of the purified LDH-X, no contaminating proteins could be detected on polyacrylamide gels which only revealed a single band identical with the single band that appeared after staining for LDH activity (Figure 2). The specific activity of the purified mouse LDH-X from testes was 120 IU/mg, which is similar to GOLDBERG's results[10,11] and higher than that reported by WONG et al.[9]. Both groups were using conventional isolation procedures.

The main advantage of the isolation of LDH-X by two steps of affinity chromatography is the rapidity and ease with which the pure enzyme is obtained. The binding properties of LDH-X are slightly different in every species and have to be determined before the two steps of affinity chromatography are carried out. In the rat, the purification of the testes specific LDH isozymes by our method revealed 2 bands on polyacrylamide gels (Figure 2), the latter having been described by previous investigators[17].

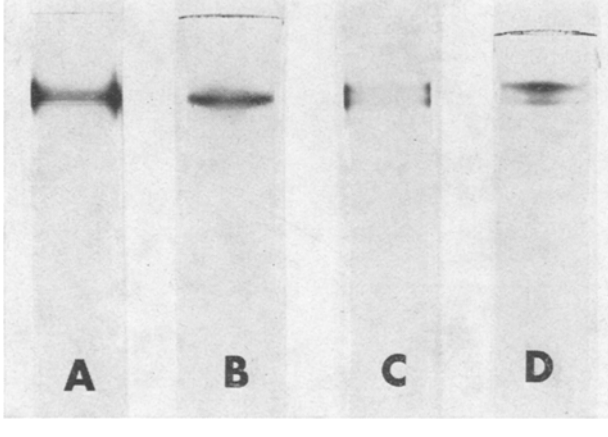

Fig. 2. Polyacrylamide gel patterns of pure testes specific LDH as obtained after the two step isolation procedure using oxamate-sepharose from mouse testes (A and B) and rat testes (C and D). A and C, LDH staining; B and D, protein staining (Coomassie blue).

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