Characterization of potato (*Solanum tuberosum*) lactate dehydrogenase isoenzymes by affinity chromatography and hybridization

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The five isoenzymes of potato (*Solanum tuberosum*) lactate dehydrogenase have been resolved by affinity chromatography. Mixtures of isoenzymes LDH-1 and LDH-5 dissociate and reassociate during freezing and thawing to produce five isoenzymes. These results indicate that potato lactate dehydrogenase isoenzymes are primary isoenzymes of the vertebrate type, which are composed of two subunit types.

The isoenzymes of vertebrate lactate dehydrogenases (L-lactate:NAD⁺ oxidoreductase, EC 1.1.1.27) have been the subject of a considerable volume of research. The principal isoenzymes, formed by random association of the H- and M-type polypeptides, have different patterns of distribution depending on the tissue from which they are obtained. The homotetramers of the two polypeptides (H₄ and M₄) have different kinetic characteristics (Pesce *et al.*, 1964), and these, together with the various tissue isoenzyme patterns, have been used to propose different metabolic functions for the different isoenzymes (Cahn *et al.*, 1962). These functions are, however, still subject to much debate (Flores, 1979a,b; Huijing, 1979; Svasti, 1979; Heffron, 1979; Knull, 1980). The characterization of vertebrate lactate dehydrogenase isoenzymes as primary isoenzymes derived from the polypeptide products of two distinct, but related, genes has been achieved by a variety of techniques. These include kinetic studies (Pesce *et al.*, 1964), hybridization (Markert, 1963) and affinity chromatography (Nadal-Ginard & Markert, 1975).

In contrast with the situation in vertebrates, little is known about the nature or function of lactate dehydrogenase in plants. Not all plants exhibit lactate dehydrogenase isoenzymes, although many do. Soya beans (*Glycine max*) and potato (*Solanum tuberosum*) tubers illustrate these two extreme situations. Germinating soya beans contain only one type of lactate dehydrogenase, and this is a homotetramer (Jervis *et al.*, 1981). Potato tubers contain up to five isoenzymes and these are claimed to be derived from subunits of the same molecular

Abbreviations used: LDH-1 and LDH-5, lactate dehydrogenase isoenzymes 1 and 5.

weight (Poerio & Davies, 1980) or two subunit types of different size (Rothe *et al.*, 1978). Few plant lactate dehydrogenases have been purified to homogeneity, and where this has been achieved (Poerio & Davies, 1980; Jervis *et al.*, 1981), the isoenzymes were not resolved. The only reported resolution of plant lactate dehydrogenase isoenzymes was achieved by Rothe *et al.* (1978) by using preparative electrophoresis. The yield of purified isoenzymes was low, as was the specific activity of the isoenzymes. This, together with their apparent existence as size isomers of mol.wts. 84 500, 106 000 and 115 000 produced from two polypeptides of mol.wts. 41 000 and 43 000, suggests that they had suffered substantial proteolytic damage during isolation, or had been partially dissociated by the low-pH treatment involved in purification.

The present paper describes the resolution of potato lactate dehydrogenase isoenzymes by affinity chromatography, their hybridization and identification as primary isoenzymes derived from two distinct polypeptides.

Experimental

Blue Sepharose CL 6B was prepared by the method of Easterday & Easterday (1974). ATP-Sepharose was prepared as described by Lamed *et al.* (1973). Chromatographic adsorbents were packed into small columns (10 cm × 0.9 cm) and equilibrated with appropriate buffer (see below). Enzyme samples, dialysed against the same buffers, were applied to the columns and the columns were then washed with starting buffer until the effluents were free from protein. Columns were eluted with concave gradients of NADH in starting buffer with

the final concentrations of NADH being approx. 1 mM (Haff & Easterday, 1978). Fractions were collected, and each fraction was concentrated by ultrafiltration to provide samples for electrophoresis.

Potato tubers (*Solanum tuberosum* var. Maris Piper) were purchased locally and lactate dehydrogenase was extracted from washed and peeled tubers as described by Poerio & Davies (1980). The extract was subjected to $(\text{NH}_4)_2\text{SO}_4$ precipitation and the protein fraction precipitating between 25 and 50% saturation was collected and used for all experiments.

Lactate dehydrogenase was assayed spectrophotometrically in the direction of NADH oxidation at 25°C as described by Davies & Davies (1972).

Hybridization of purified lactate dehydrogenase isoenzymes was performed by using the freezing-and-thawing technique of Chilson *et al.* (1965). Enzyme solutions were prepared in 0.1 M-sodium phosphate buffer, pH 7.0, containing 0.1 M-NaI and 1 mM-2-mercaptoethanol before freezing them.

Polyacrylamide-disc-gel electrophoresis was performed on 7.5%-acrylamide gels as described by Hayes & Wellner (1969). Lactate dehydrogenase activity was detected in gels by the method of Shaw & Prasad (1970).

Results and Discussion

When a mixture of all five potato lactate dehydrogenase isoenzymes was applied to a column of Blue Sepharose CL 6B in 40 mM-Tris/acetate buffer, pH 8.0, containing 2 mM-EDTA and 2 mM-2-mercaptoethanol, all five isoenzymes were bound to the adsorbent. Elution of the column with a concave gradient of NADH (250 ml) in the same buffer with a final NADH concentration of 1 mM resulted in lactate dehydrogenase being eluted in a broad peak. Fractions (5 ml) of the column effluent were collected, concentrated by ultrafiltration and subjected to electrophoresis. The resulting gels were stained for lactate dehydrogenase activity and the results are shown in Fig. 1. Partial resolution of the isoenzymes has been achieved, and the order of elution was identical with the order of electrophoretic mobility of the isoenzymes, with LDH-1 being eluted first and LDH-5 being eluted last. This order of elution is identical with that observed both by Nadal-Ginard & Markert (1975) and by Haff & Easterday, (1978) for isoenzymes of vertebrate lactate dehydrogenase.

When potato lactate dehydrogenase isoenzymes were applied to ATP-Sepharose in 50 mM-potassium phosphate buffer, pH 6.0, containing 2 mM-EDTA and 2 mM-2-mercaptoethanol and the column was subsequently eluted with a concave gradient of NADH in the same buffer, the isoenzymes were again resolved. The pattern of elution was identical with that obtained from Blue Sepharose.

These results strongly suggest that potato lactate dehydrogenase isoenzymes are derived from two distinct polypeptides having different structural and kinetic characteristics. In order to confirm this, samples of LDH-1 and LDH-5 obtained by gradient elution of Blue Sepharose columns were mixed and dialysed against 0.1 M-sodium phosphate buffer containing 0.1 M-NaI. The mixtures were then frozen rapidly and thawed slowly three times, as described by Chilson *et al.* (1965), and then subjected to electrophoresis. Activity stains of the gels showed that, during freezing and thawing, dissociation and random reassociation of the LDH-1 and LDH-5 subunits had occurred, giving rise to all five isoenzymes normally found in potato tubers (Fig. 1).

Potato lactate dehydrogenase has recently been purified to homogeneity by affinity chromatography on both Blue Sepharose and ATP-Sepharose (Jervis *et al.*, 1981). Binding and elution of the enzyme is very sensitive to pH with both adsorbents. Although the enzyme binds readily to Blue Sepharose over a wide pH range (5.5–8.5), efficient elution with NADH is only possible at pH values of 8.0 and above. In the case of ATP-Sepharose, enzyme binding occurs readily at pH values of 5.5–6.5, but rapidly decreases above 6.5. Elution of bound enzyme from ATP-Sepharose is, however, easily achieved with 1 M-NADH at all pH values at which binding occurs. Jervis *et al.* (1981) did not investigate the chromatographic resolution of potato lactate dehydrogenase isoenzymes. However, the isoenzymes of bovine lactate dehydrogenase have been resolved by chromatography on Blue Sepharose by using a concave concentration gradient of NADH (Haff & Easterday, 1978). The isoenzymes of lactate dehydrogenase from several species were also resolved by affinity chromatography on Blue Dextran-Sepharose (Nadal-Ginard & Markert, 1975). The latter workers demonstrated that, regardless of species, the lactate dehydrogenase isoenzymes were eluted in the same order i.e. B4(H_4), B3A, B2A2, BA₃, A4(M_4), [nomenclature defined by Markert (1963) and Nadal-Ginard & Markert (1975)], clearly indicating evolutionary homologies among the different subunits. The same order of elution was observed by Haff & Easterday (1978).

On the basis of the results presented here, it is proposed that potato lactate dehydrogenase isoenzymes are primary isoenzymes derived from random association of the polypeptide products of two different, but related, genes. This proposal is contradictory to the finding of Rothe & Corper (1978) that potato lactate dehydrogenase isoenzymes are secondary isoenzymes formed as a result of the reduction of disulphide bonds within the lactate dehydrogenase molecule.

It is further proposed that, in view of the similarity in the elution patterns of potato and vertebrate

Fig. 1. Polyacrylamide gels stained for lactate dehydrogenase activity

(a) a, 25–50%-satd.-(NH₄)₂SO₄ protein fraction; b, lactate dehydrogenase adsorbed on to ATP-Sepharose at pH 8.0; c, lactate dehydrogenase isoenzymes not adsorbed on to ATP-Sepharose at pH 8.0; d–h, fractions from Blue Sepharose column eluted with NADH gradient (d, fraction 23; e, fraction 33; f, fraction 38; g, fraction 45; h, fraction 50); i, LDH-1 and LDH-5 mixture before freezing and thawing; j, LDH-1 and LDH-5 mixture after freezing and thawing. The lactate dehydrogenase isoenzymes are numbered 1–5 on the basis of electrophoretic mobility (Markert, 1963). Copies of the original photographs on which the drawings are based will be sent to readers requesting reprints. (b) shows densitometric tracings corresponding to (i) LDH-5 alone, (ii) LDH-1 alone, (iii) LDH-1 and LDH-5 before freezing and thawing [i.e. (a) i above] and (iv) LDH-1 and LDH-5 after freezing and thawing [i.e. (a) j above].

lactate dehydrogenase isoenzymes from affinity adsorbents, the two polypeptides of potato lactate dehydrogenase resemble the products of the vertebrate A and B lactate dehydrogenase genes. If this is correct, then the metabolic functions of the potato lactate dehydrogenase isoenzymes will be of considerable interest, especially in view of the traditional concept of the functions of vertebrate lactate dehydrogenase isoenzymes (Pesce *et al.*, 1964; Everse & Kaplan, 1973).

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