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In Vitro Dissociation-Recombination of Malate Dehydrogenase Subunits in *Corydalis solida*

A.H. Nagy, M.O. Siddiqui, Z.G. Kocsis and G. Vida
Department of Genetics, Eötvös Loránd University Budapest (Hungary)

Summary. Two allelic forms of NAD specific malate dehydrogenase were found in samples of a wild population of *Corydalis solida*. The dimeric nature and the origin of the heterodimeric form has been demonstrated by in vitro dissociation and recombination of the subunits detected by subsequent electrophoresis. The method is applicable for polyacrylamide gel electrophoresis of crude leaf extracts of individual MDH isozyme forms.

Key words: Malate dehydrogenase – Isozyme – Interallelic subunits – Heterodimer – In vitro dissociation – Subunit recombination – *Corydalis solida*

Introduction

Many enzymes occur in multiple forms, or isozymes, and many of them are multimeric, consisting of two or more polypeptide subunits. If the gene is polymorphic, the number of possible isozymes depends not only on the number of alleles but also on the number of constituent subunits required to produce the active enzyme (Scandalios 1969; Harris and Hopkinson 1976). Indirect evidence of subunit composition can be obtained by noting the correlation between the genotype and the appearance of specific isozyme bands detectable by electrophoresis. A more direct test is provided by in vitro dissociation-recombination experiments. In vitro subunit recombination of genetically determined electrophoretic variants has been reported in glutamate oxalacetate transaminase (EC 2.6.1.1) isozymes of *Triticum* species (Hart and Langston 1977). The method had previously been developed for alcohol dehydrogenases (EC 1.1.1.1) of several species (Hart 1971; Torres 1974; 1976). In this paper we describe a method for dissociation and subunit-recombination of the leaf malate dehydrogenase of *Corydalis solida*.

Malate dehydrogenase (L-malate NAD^+ oxydoreductase, EC 1.1.1.37) is known to occur in several isozyme forms in tissues of both plants and animals. Its physiological role is fairly complex (Lehninger 1972; Ting 1971; Tolbert 1971). Active enzyme forms have been found in the cytoplasm, peroxisomes, glyoxysomes, chloroplasts and mitochondria (Longo and Scandalios 1969; Yamazaki and Tolbert 1969; Ting 1968; Mukerji and Ting 1969). A multilocus system of MDH isozymes has been demonstrated in maize (Yang and Scandalios 1974; 1975). The isozyme spectra show variation with ontogenesis and are also different in cellular organelles. Mitochondrial malate dehydrogenase (m-MDH) has proven to be genetically under nuclear control in maize (Longo and Scandalios 1969). One of the m-MDH loci in maize appears to be diallelic (Yang et al. 1977).

Both the soluble cytoplasmic malate dehydrogenase (s-MDH) and the m-MDH have been found to function only in dimeric form in human tissues (Davidson and Cortner 1967). Similar observations were made on mitochondrial preparates made of mouse and calf hearts (Shows 1972; Wolfstein et al. 1969).

In our laboratory investigations were carried out on isozyme polymorphism of natural plant populations in order to measure the amount of genetic variation. During this work we found an isozyme variation of a NAD-specific malate dehydrogenase locus in the leaves of *Corydalis solida*. The aim of the present study was to expose two allelic forms of MDH to in vitro conditions in which dissociation of the isozymes into monomers and reassociation of the subunits into active enzyme would be possible. Experiments were performed with crude extracts and we found efficient methods of MDH isozyme separation in different polyacrylamide gel systems.

Material and Methods

NAD specific malate dehydrogenases of *Corydalis solida* (L.) Swartz (Fumariaceae) were studied. A large wild population was sampled which is growing in an oak-carpinus mixed forest (Quer-

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ceto-Carpinetum) on the hill 'Feketefej', near Budapest, altitude ca. 300 m above sea level. Freshly collected green shoots and seeds were extracted using the modified extraction medium of Nagy et al. (1973) which contains 0.04 M tris-HCl (pH 7.8) and 0.01 M $MgCl_2$, 0.25 mM EDTA, 5 mM dithiothreitol, 5 mM β-mercaptoethanol and 0.4 M sucrose. Fresh leaves were extracted by adding 10 ml of the buffer to 1 g of leaf material. Extraction of a single seed was carried out using 0.2 ml buffer. Before extraction the seed coat was removed. The extracts were subsequently centrifuged for 20 min at 5000xg at 2-5°C and the supernatant was used for electrophoresis. For in vitro dissociation and recombination of MDH subunits we modified the procedure of Hart (1971) which was originally described for ADH. The leaves were homogenized (1 g/10 ml) in a buffer containing 0.1 M sodium phosphate (pH 7.0), 1.5 M NaCl, 0.1 M β-mercaptoethanol and 0.4 M sucrose. Extracts were centrifuged (5000xg, 20 min) and aliquots of the supernatants were further used either alone or mixed. Extraction was carried out at a low (0 to +5°C) temperature. Homotype extracts (controls) as well as mixed extracts were stored at −20°C for 12 hours. The extracts were then dialysed for 4 hr at 0-5°C, against a sodium phosphate buffer (0.1 M; pH 7.0) supplemented with 0.1 M β-mercaptoethanol and 0.4 M sucrose. Subsequently NaCl was added to the samples (final concentration 2.0 M) which were than stored again at −20°C for 12 hours. Finally, the dialysis was repeated and the MDH isozymes of the samples were analysed electrophoretically. The electrophoresis was carried out in tubes (length 80 mm; inner diameter 6 mm) using a 6% polyacrylamide gel according to Davis (1964). Samples (20 µl) were layered on to a spacer gel and were run at 2 mA/tube for 2.5 hr using a Shandon Southern V 500/150 power supply. Seeds of various MDH phenotypes were analyzed on polyacrylamide slab gels (80 x 80 mm) consisting of a reverse continuous polyacrylamide gradient ranging from 7% at the top to 5% at the bottom. The slab gels were prepared with UNISCIL Gradipore equipment. Electrophoresis was conducted at 1 mA/cm. This reverse continuous gradient gel gives a good separation of isozymes from such small samples as the seeds of *Corydalis solida* (about 1 mg). NAD-MDH specific stain was developed in 30 min at 37°C after both disc and slab electrophoresis. The reaction mixture consisted of a 0.1 M glycine-NaOH buffer (pH 6.5) plus 0.5 M DL-malic acid, 0.25 mg/ml NAD, 0.3 mg/ml p-nitro tetrazolium blue and 0.05 mg/ml phenazine methosulphate. The reaction was terminated by 7% acetic acid.

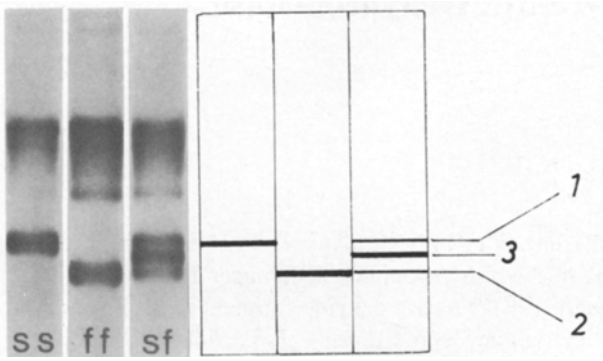

Fig. 1. NAD-MDH isozymes of leaf extracts of *Corydalis solida;* homozygotes (ss, ff) are represented by a single band at the lower region (1, 2 respectively), while the heterozygote (sf) shows a triple band in the same place (1-3)

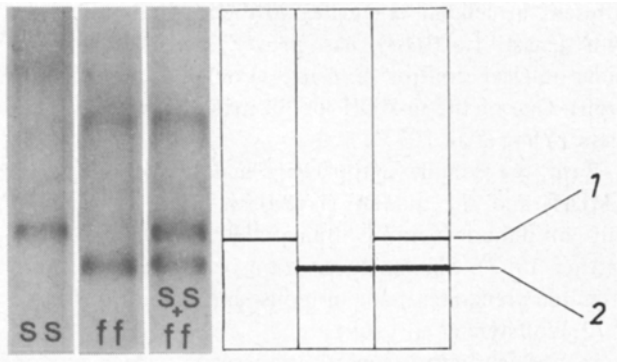

Fig. 2. The two kinds of homodimeric forms and their mixed zymogram

Results

MDH Isozymes in Leaves and Seeds

Representative MDH zymograms of leaf extracts of *Corydalis solida* are shown in Fig. 1. There are two homodimeric phenotypes: a slow and a fast moving at mobility values of 0.54 (ss) and 0.60 (ff), respectively. The third form is a characteristic triple banded phenotype, the middle band apparently representing the heterodimeric enzyme form.

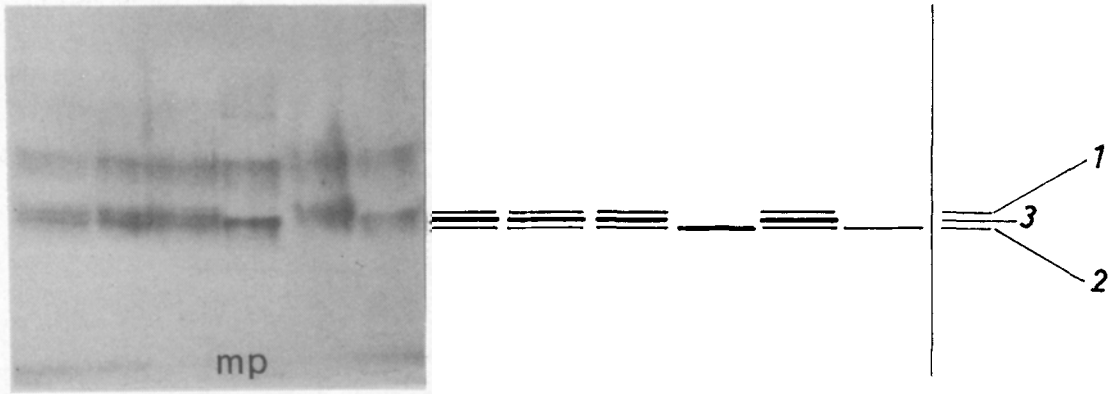

Fig. 3. NAD MDH zymograms of seeds and their mother plant of *Corydalis solida* (mp = mother plant; 1 = ss, 2 = ff; 3 = sf enzyme forms)

The difference between the mobilities of the slow and fast moving homodimeric forms are also shown in Fig. 2 where extracts of the two homozygotic plants were mixed and electrophoresed. The same MDH isozymes were found in the seed extracts (Fig. 3). The open pollinated homozygotic plant (ff) produced some triple banded phenotypic seeds.

The presence of some additional, invariable slow moving MDH isozymes are also visible in the upper region of zymograms of both leaves and seeds (Fig. 1, 3). These isozymes, however, were active only in the young tissues, and were usually destroyed during storage.

Frequency Distribution in a Natural Population

Isozymes were analysed in extracts of field-collected plants. NAD specific MDH genotypes and allele frequencies, calculated from data of 300 *Corydalis solida* plants from a single population, are presented in Table 1. Assuming a single locus with two alleles (s, f) the genotype frequencies of our sample correspond to the Hardy-Weinberg equilibrium values, expected from the calculated allelic frequencies ($\chi^2 = 0.364$ P ~ 0.95).

Table 1. Genotype and allele frequencies of leaf MDH isozymes in a population of *Corydalis solida* (calculated from 300 plants)

Genotype	Frequency	Allele	Frequency
ss	0.661	s	0.807
sf	0.292		
ff	0.047	f	0.193

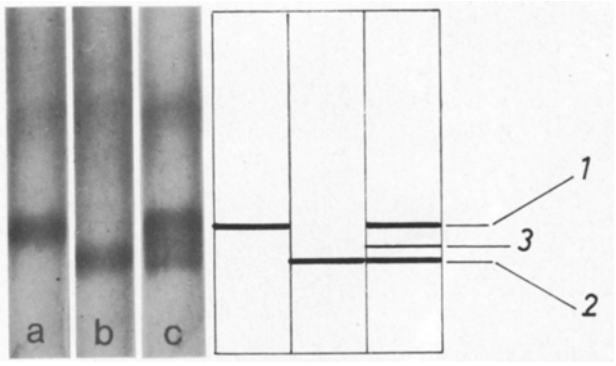

Fig. 4. NAD specific MDH isozymes of *Corydalis solida* after in vitro dissociation-recombination.
a, b: extracts of the pure homodimeric forms;
c: zymogram of the mixture of the two homodimeric forms after treatment. The middle band (3) appeared as a result of in vitro recombination of the subunits (synthetic heterodimer)

In Vitro Dissociation-Recombination of Subunits in Crude Extracts

Results of in vitro dissociation and recombination are illustrated in Fig. 4. It may be seen that the procedure did not result in a change in the electrophoretic mobilities of the homodimeric forms (ss or ff extracts) but lead to the appearance of a band with an intermediate electrophoretic mobility. The zymogram is similar to that of the sf triple banded phenotype except for the relatively pale appearance of the heterodimeric middle band. This is probably due to the incomplete dissociation of the homodimers.

Discussion

The NAD specific MDH isozymes, extracted from leaves of *Corydalis solida*, have different (0.54 and 0.60) electrophoretic mobilities and appear to be allelic. The subunits of the two homodimeric forms can freely combine in extracts forming a heterodimer with an intermediate mobility value. The same MDH isozymes were found in seeds (Fig. 3) and bulbs (data not presented). Our results of in vitro dissociation and recombination prove the functionally dimeric nature of the variable MDH isozymes of *Corydalis solida* and support our conclusion on the allelic relationship of the genetic determinants of the subunits. Heterozygotic plants with s and f subunits in vivo form dimers in all three combinations as expected from the binomial form $(s + f)^2 = s^2 + 2sf + f^2$ (Table 1). This observation is in accordance with our assumption of a nuclear, diallelic MDH locus responsible for isozymes at the 0.54 (ss), 0.60 (ff) and intermediate 0.57 (sf) mobility values.

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Dr. A.H. Nagy
Department of Genetics
Eötvös Loránd University
Muzeum krt. 4/a
1088-Budapest (Hungary)

Dr. M.O. Siddiqui
Department of Botany
Patna University
Patna 800005 (India)