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Isoenzyme pattern of glutamate dehydrogenase as a reflection of nitrogen metabolism in *Lupinus albus*.

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Abstract

Glutamate dehydrogenase (L-glutamate: NAD dehydrogenase, EC 1.4. 1.2; GDH) activity and electrophoretic separation pattern of the enzyme were studied. The enzyme was extracted from embryos of *Lupinus albus* decotyledonized and cultured for 24, 48 and 72 hours in media containing various combinations of saccharose, ammonium chloride, nitrate as well as amino acids: glutamate, aspartate, glutamine and asparagine. The absence of sugar in the medium resulted in an increase of specific activity of GDH, measured by the rate of NADH + H^+ oxidation, and induced formation of new isoenzymes of NAD^+-dependent GDH. Most significant increase in GDH specific activity and most evident appearance of new isoenzymes in the embryos were noted when sugar was substituted in the medium by any of the mentioned amino acids. Induction of new isoenzymes could also be seen when ammonium salts were present in the medium. GHD isoenzymatic patterns were obtained in various trophic conditions. It is suggested that the GDH isoenzyme patterns may serve as a nitrogen metabolism index of a tissue from which the enzyme has been extracted.

INTRODUCTION

Glutamate dehydrogenase is an essential enzyme of nitrogen metabolism in plants as well as in all living organisms and estimation of its activity is an accepted requirement in any studies of the metabolism. Various metabolic disturbances, not only in the nitrogen metabolism, result in evident changes in activity of the enzyme, as expected since the enzyme catalyzes a metabolic pathway linking as important large metabolite pools as amino acids and Krebs cycle metabolites. Although recently some authors (Miflin, Lea 1975, 1976) have challenged the suggestion that glutamate dehydrogenase is the only enzyme capable of incorporation

of ammonia into an organic form, studies on the enzyme and its numerous isoenzymes have shown that estimation of its activity and its isoenzyme pattern may be used an an index of development and differentiation of cells, tissues and organs (Hartmann et al, 1973). A hypothesis has also been put forward that individual GDH isoenzymes may differ in function, some of them exerting a catabolic effect while the other — an anabolic one (Hartmann 1973, Lee 1973). Electrophoretic studies of the enzyme should provide data indicating whether at a given time and in a given tissue synthesis of amino acids and proteins or their decomposition prevails.

Studying amino acid metabolism in *Lupinus* we have detected glutamate dehydrogenase induction caused by asparagine, aspartate or glutamate (Ratajczak, Wilanowska and Mazurowa 1976). In this work we present changes in activity and isoenzyme spectrum of GDH in *Lupinus* embryos cultured in media with various sources of carbon and nitrogen. The experiments have been performed on decotyledonized embryos so as to exclude the effect of endogenous nitrogen and carbon pools on the reaction to media.

MATERIAL AND METHODS

Seeds of *Lupinus albus* L. cv. Kali originated from Seed Centre, Breeding Station in Wiatrowo. They were allowed to swell in water for 6 hours at 24° C and then transferred to germinating beds for 18 hours incubation in dark, at 25° C. Then, germ axes of germinating seeds were isolated and separated from cotyledons. The isolated seedlings embryonal axes were placed in large culture tubes (40 in each). 40 ml of medium was added to each tube (for medium components see Table 1). Each tube was closed with gauze and the tubes with cultures were placed on a shaker the oscillations of which were regulated so as to immerse all embryos and not allow to sediment them to the bottom of the tubes. Every 4 hours media were exchanged for fresh ones.

After 24, 48 and 72 hours of culture in the media samples of the decotyledonized embryonal axes were homogenized in 0.05 M phosphate buffer, pH 7.4, using 10 ml buffer per 10 embryonal axes. Enzyme extraction, electrophoretic separation in polyacrylamide gel and the test for detecting glutamate dehydrogenase in the gel were performed as described in detail by Czosnowski (1974). For electrophoresis, the procedure of Davis (1974) was followed. Isoenzyme densitograms were obtained in the ERI-65 densitometer with an integrator (Carl Zeiss, Jena, GDR). Activities of NADH $+H^+$-dependent glutamate dehydrogenase in extracts were estimated using VSU-2P spectrophotometer, at the wavelength 340 nm, at 25° C ± 0.5° C, as described by Davies and Teixeira (1975).

Tabela 1

Culture variants

Letter symbol of a variant	Final concentration of substances (mM) in basal medium*
A	—
B	Sodium nitrate 7 Saccharose 60
C	Ammonium chlorate 7 Saccharose 60
D	Sodium nitrate 7
E	Ammonium chlorate 7
F	Asparagine 35
G	Sodium aspartate 35
H	Glutamine 35
I	Sodium glutamate 35
J	Asparagine 35 Saccharose 60

* Heller 's medium served as a basal medium (Heller 1954) except for nitrate which was absent. Variant D of our media corresponded to the complete Heller's medium.

Estimation of enzyme activity and electrophoresis were performed directly after homogenization of the plant material and centrifugation of the extract, i.e. not later than 1 hour after collecting samples for analysis.

Protein content was estimated according to Lowry et al (1951).

RESULTS

Glutamate dehydrogenase activity in *Lupinus* embryos axes was 268 ± ± 35 mU/mg protein at the moment of their isolation from cotyledons. One miliunit (mU) corresponds to one nanomole of NADH + H^+ oxidized in one minute.

From the point of view of molecular structure, the enzyme was homogenous. While the densitogram in Fig. 1 showed only a single, clearly defined band, several new isoenzymes appeared during culture of isolated embryonal axes in media containing various sources of carbon and nitrogen. Densitograms for all culture variants are presented in Fig. 2. Densitogram fragments far from the point of applying the extract have been cut out since none of GDH isoenzymes could be detected in that region. An analysis of perpendicular columns in the Fig. 2 demonstrates time-related changes in GDH isoenzyme composition in a given medium.

Evidently, in the majority of variants new isoenzymes appeared and, with elapsing time of culture, their activity increased while activity of the original enzyme form decreased. A horizontal analysis of Fig. 2, par-

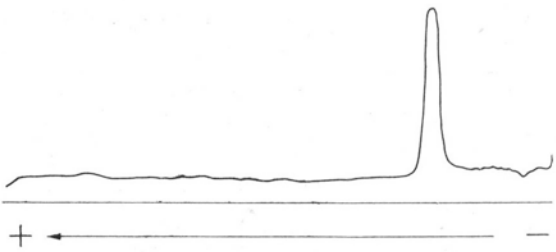

Fig. 1. Densitogram obtained for *Lupinus albus* embroys after 24 hours of seed swelling (time O in our cultures). Optical density of polyacrylamide gel was recorded after electrophoretic fractionation of embryonal extract and after developing a reaction for NAD+-dependent GDH, using 530 nm filter

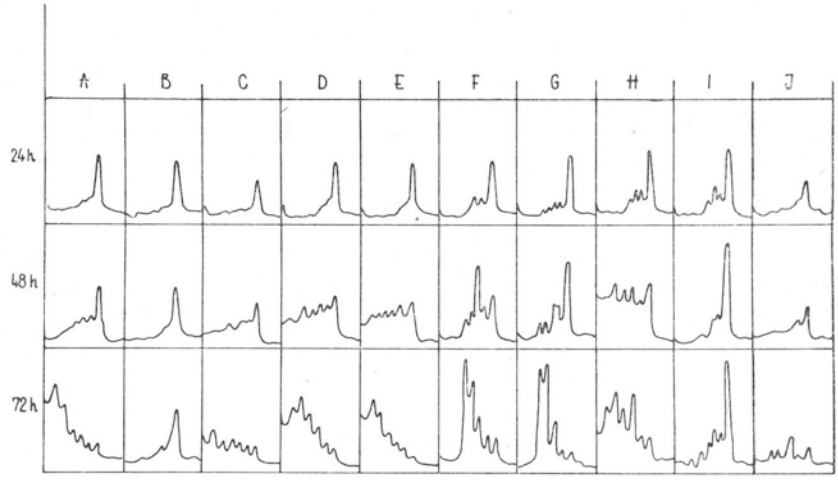

Fig. 2. Cumulative table of isoenzyme pattern changes of NAD+-dependent GDH of *Lupinus albus* embryos at different times and variants of culture

ticularly in the lowest densitograms presenting GDH isoenzyme pattern in individual cultures at 72 hours of culture permits to define the isoenzymes which appeared due to a given substance added to medium.

Embryos cultured in amino acid-containing media (variants F, G, H, I) contained most active new isoenzymes (the sharpest peaks in densitograms). However, addition of saccharose to the media abolished the induction of new isoenzymes by amino acids, as evident from variant I in which medium contained asparagine and saccharose. The same was found for all 4 studied amino acids (variants not shown here). Independently of nitrogen source and even if nitrogen compounds were not present in

a medium (variant A), the absence of sugar in a medium always resulted in an increased activity of new isoenzymes. New GDH isoenzymes could however be induced by ammonium chloride (variant C) even in the presence of saccharose although they were less active than isoenzymes induced by ammonium chloride in the absence of saccharose. The described changes can easily be noticed upon analysis of Fig. 3 in which schematic electrophoretic fractions have been presented of GDH isoenzymes from embryos cultured for 72 hours. In the figure, specific activity of GDH has also been presented for 72 hours embryos and various medium variants. Specific activity of GDH reached peak values in embroys cultured in amino acid-containing, sugar-free media. Specific activity of GDH was unaltered if medium was supplemented with an amino acid and saccharose. Analyzing specific activities of GDH in embroys of all variants of cultures a conclusion may be drawn that the absence of sugars in the medium has always caused an increase in the enzyme activity but the increase has been particularly evident when sugar has been substituted in the medium by an amino acid.

DISCUSSION

The literature discussing properties of glutamate dehydrogenase in plants is extensive but conclusions and generalizations of individual authors frequently contradict each other. The controversis may result from the fact that divergent results have been obtained studying different plants. This argues for performing basic studies on economically important plants, species and varieties cultivated in defined climatic zones.

In this work we have described changes in activity and in isoenzyme pattern of glutamate dehydrogenase of *Lupinus albus* cv. Kali in relation to various trophic conditions. In the absence of sugar an increase in GDH activity has been noted in the embryos and new isoenzymes have appeared. GDH induction resulting from sugar absence has been described by Sahulka et al. (1974) who have performed experiments on isolated pea roots. Similarly, Duke et al. (1975) have found a decrease in GDH activity in *Lemna perpusilla* cultured in saccharose or fructose solutions. Sahulka have ascribed to sugars a role of a direct repressor of GDH synthesis and have attempted to explain by the same mechanism also other cases of increased GDH activity, i.e. GDH activation by ammonium ions and amino acids (Sahulka et al. 1974, 1975). Our studies indicate that also in *Lupinus albus* an activity of GDH and its isoenzyme pattern are, in fact, dependent on the availability of sugars. GDH isoenzyme distributions are divergent, however, for embryos cultured with nitrates and for those cultured with ammonium salts if the medium contains also sugar. Ammonium chloride has been inducing additional isoenzymes both

in the absence and in the presence of saccharose (Fig. 3). Thus, in our experiments on *Lupinus albus* we have confirmed the activating influence of ammonium ions on GDH demonstrated previously by numerous authors

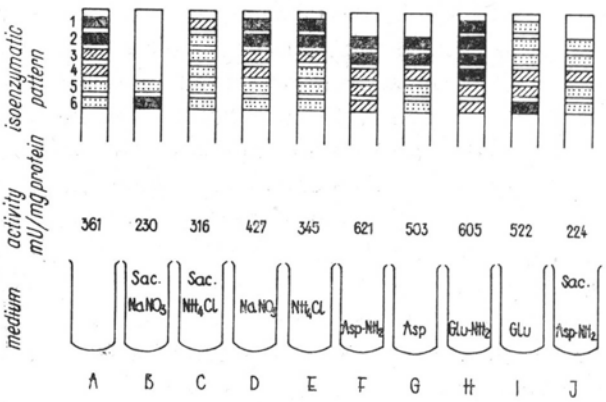

Fig. 3. Schematic presentation of NAD+-dependent GDH isoenzymes and specific activity of NADH + H+ dependent GDH in *Lupinus albus* embroys in individual culture variants after 72 hours of culture. Over the letter symbols of variants, at the bottom of the figure, the presence of factors added to medium is noted. Abbreviations: Asp = asparatate, Asp-NH_2 = asparagine, Glu = glutamate, Glu-NH_2 = = glutamine, Sac. = saccharose

in several plants eg. Kanamori et al. (1972) in rice roots, Kretowicz et al. (1972) in roots of pea seedlings, Shepard and Thurman (1973) in *Lemna gibba*, Postius and Jacobi (1975) in *Cucurbita maxima*. The papers have demonstrated that ammonium ions increase GDH activity and induce the appearance of new isoenzymes. GDH stimulation bya mmonium ions results probably from *de novo* synthesis of the enzyme as shown by Barash et al. (1975) in oat leaves. An increased GDH activity and appearance of a new isoenzyme has been described by Kretowicz et al. (1974) in *Lupinus luteus*. In this work we demonstrate the presence of 6 GDH isoenzymes in *Lupinus albus*. New isoenzymes most inducible with ammonium ions and by the absence of sugars were the slowest to migrate anodally (1 and 2 in Fig. 3). Lee (1973) and Hartmann (1973) have postulated that GDH as an enzyme of both anabolic and catabolic potential contains isoenzymes catalyzing binding of nitrogen to a carbon chain eg. for glutamate synthesis and different from them isoenzymes capable of desaminative oxidation of glutamate. Since glutamate synthesis with subsequent transaminations is the main pathway of formation of all amino acids, the Hartmann-Lee hypothesis is most interesting as it suggests the occurence of a mechanism of metabolic regulation in this point. This mechanism would direct the amino acid and protein metabolism in the direction of synthesis or a catabolism. Studying *Ricinus communis* seedlings, Lee ascribes anabolic activity to the rapidly migra-

ting GDH isoenzymes and catabolic activity to isoenzymes slowly migrating toward anode. Hartmann has determined that the reverse is true for lucerne and pea: anabolic isoenzymes migrate slowly (Hartmann 1973, Hartmann et al. 1973). Thus, the migratory properties may vary between plants. However, Hartmann and Lee are in accord ascribing anabolic function to the ammonium-induced isoenzymes. Hartmann (1973) has quoted experimental results indicating that induction of new isoenzymes by ammonium ions is associated with a change in amination/ /desamination ratio in favour of the anabolic reaction. Our results seem to confirm those obtained by Hartmann for two other *Leguminosae* plants and indicate that, also in *Lupinus albus* the slowly migrating GDH isoenzymes exert an anabolic activity.

The facts that new isoenzymes are best induced by asparagine and glutamine (Figs. 2 and 3) and that their production is inhibited by saccharose are seemingly inconsistent with their anabolic activity. In recent years it has been found, however, that in plants apart from amination of 2-oxoglutarate by GDH, incorporation of ammonium to the organic forms may be stimulated by other enzymes in the two following stages: 1. incorporation of NH_3 to the amid group of glutamine, catalyzed by glutamine synthetase (GS; EC 6. 3. 1. 2), 2. reductive transfer of the glutamine amid group to 2-oxoglutarate with formation of 2 glutamate molecules. The latter reaction is catalysed by glutamate synthase (GOGAT; EC. 2. 6. 1. 53) (Miflin and Lea 1976). Interaction of the two separate pathways of ammonium assimilation to an organic form creates several possibilities of regulating the process. Miflin and Lee (1976) are of the opinion that ammonium assimilation by GS and GOGAT represents the main pathway while incorporation mediated by GDH takes place at high ammonium levels in tissues. Lee and Miflin (1975) as well as Miflin and Lee (1975) suggest that in pea roots ammonium assimilation is mediated by glutamine (catalysed by GS). At high supply in 2-oxoglutarate and a reducing agent (NAD/P/H + H^+ or ferredoxine) glutamate synthesis takes place, catalysed by GOGAT. When supply of either factor is limited glutamine amid group is transferred to an aspartate with formation of asparagine. Asparagine biosynthesis in germinating lupin most probably follows the above pathways. Lever and Butler (1971a) demonstrated that the carbon core of asparagine in germinating lupins originates mainly from aspartate. No enzymes could be detected which would be capable of linking directly ammonium to aspartates (analogous to asparagine synthetase in bacteria and animals; EC. 6. 3. 1. 1.). Interaction of ammonium incorporation pathways was studied in *Lemna minor* by Rhodes et al. (1976). They demonstrated that in conditions of low ammonium availability GS and GOGAT activities were increasing which indicated that in these conditions the enzymes were responsible for ammonium assimilation. Increase in the ammonium supply was followed by a decrease

in activities of the enzymes and by an increase in GDH activity. The authors drew a conclusion that glutamine synthetase (GS) and glutamate dehydrogenase (GDH) were controlled by a simultaneously acting regulatory mechanisms, glutamine rather than ammonium providing the negative control over glutamine synthetase and the positive control over glutamate dehydrogenase. A mechanism similar to that described by Rhodes et al. (1976) for *Lemna minor* may also be operative in lupin. It might explain our results, both the increase in GDH activity induced by amino acids and the intense induction of new anabolic isoenzymes by amino acids (glutamine and asparagine in particular). Positive feedback is rarely suggested in the literature as a mechanism of metabolic regulation but in the case of germinating *Lupinus* such a mechanism would well explain the enormous accumulation of asparagine which, as well known, in etiolated lupin seedlings may amount to 30% of the dry mass (Lever and Butler, 1971b).

Results presented in this paper are in agreement with estimations of sugar content and of nitrogen fractions in decotyledonized lupin embryos cultured in media containing various concentrations of amino acids and saccharose (Ratajczak and Mazurowa 1971, Ratajczak, Wilanowska and Mazurowa 1976). The estimations have shown that a turn in metabolism toward protein synthesis is regulated by sugar level but, at the same time, an important role of amino acids, particularly of asparagine, has been demonstrated for nitrogen assimilation in lupins. The main nitrogen transport path in lupins also involves asparagine (Atkins et al. 1975).

Detailed analysis of nitrogen metabolism in germinating lupins requires further studies, particularly on the GS and GOGAT system, but already the presented results show that estimation of activities of GDH and its isoenzymes may provide valuable informations on nitrogen metabolism in lupins, its disturbances or on differences in the metabolism in various species. In further studies on the effect of various factors on lupins development and metabolism we will analyze the GDH isoenzyme pattern and compare it with the patterns shown in Fig. 2 to obtain information on the metabolism status of a tissue from which the enzyme has been isolated. We believe that activity determinations and electrophoretic fractionation of enzymes will provide a tool for agricultural diagnostics, evaluation of varieties similarly as estimation of enzymes in humans has been introduced into clinical diagnostics in medicine. Glutamate dehydrogenase would serve well such a purpose, providing indices of developmental and differentiation stages as well as of metabolic status. This, however, has to be preceded by accurate estimation of enzyme properties for each of studied species and varieties.

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Wzór izoenzymów dehydrogenazy glutaminianowej jako odzwierciedlenie stanu metabolizmu azotowego w łubinie białym

Streszczenie

Przeprowadzono oznaczanie aktywności dehydrogenazy glutaminianowej (L-glutaminian: NAD dehydrogenaza, EC. 1. 4. 1. 2; GDH), oraz jej elektroforetyczne rozdziały. Enzym ekstrahowano z zarodków łubinu białego odmiany Kali, pozbawionych liścieni i hodowanych przez 24, 48 i 72 godziny w pożywkach, zawierających różne kombinacje sacharozy, soli amonowej, azotanu oraz aminokwasów: glutaminianu, asparaginianu, glutaminy i asparaginy. Brak cukru w pożywce wywoływał wzrost aktywności własnościwej GDH jak i najwyraźniejsze ujawnienie się nowych izoenzymów w zarodkach następowało wówczas, gdy cukier w pożywce zastępowano którymkolwiek z wymienionych wyżej aminokwasów. Indukcja nowych izoenzymów następowała również przy obecności jonów amonowych w pożywce. Przedstawiono zbiorczą tabelę wzorów izoenzymów GDH w zależności od warunków troficznych. Postuluje się stosowanie wzoru izoenzymów GDH jako kryterium stanu metabolizmu azotowego tkanki, z której enzym był ekstrahowany.