GLUTAMATE DEHYDROGENASE ACTIVITY IN CRUDE PLANT EXTRACTS

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SUMMARY

The glutamate dehydrogenase deamination assay based on the increase of absorbance at 340 nm, caused by the transformation of NAD into NADH in the presence of L-glutamate applied to crude plant extracts, is unreliable without dialysis of the extract to eliminate disturbing reactions.

A low molecular weight substance, probably a phenolic compound or aromatic amine, was isolated from *Petunia* leaves which could act as a substrate or activator of another dehydrogenase. This dehydrogenase which interferes with the glutamate dehydrogenase deamination assay may use many amino acids and amines as substrate.

The use of a 1% concentration of insoluble polyvinylpyrrolidone in the extraction buffer causes a higher glutamate dehydrogenase activity for both amination and deamination.

The glutamate dehydrogenase is partially present in a latent form.

1. INTRODUCTION

Determinations of enzyme activities in crude plant extracts are unreliable since during homogenization of plant material substances are liberated which affect the enzyme activity.

The influence of acidic constituents of the vacuoles, of carbohydrates, of protease activity and of interaction of plant proteins has been summarized previously by STAHMANN (1963).

The liberation of polyphenol oxidases and their substrates causes the formation of quinones which may condense with proteins (WILLIAMS 1963; GOLDSTEIN & SWAIN 1965; JONES *et al.* 1965; LOOMIS & BATTAILE 1966; FIRENZUOLI *et al.* 1969). This inactivation may be prevented by adding low amounts of polyvinylpyrrolidone (PVP) during the extraction procedure (HULME & JONES 1963; ANDERSEN & SOWERS 1968) or by adding reducing agents like metabisulphite, dithionite and ascorbic acid (ANDERSON & ROWAN 1967).

The determinations of enzyme activities in crude plant preparations are also disturbed by the presence of factors which interfere with the assays. Particularly the spectrophotometric assay of a dehydrogenase like glutamate dehydrogenase based on measurement of the changes in absorbances at 340 nm, caused by transformation of the coenzyme, NAD(P) or NAD(P)H, is affected by the presence of greenmatter, of turbidity(SANWAL & LATA 1964), and of activity of other dehydrogenases, like malic dehydrogenase and lactic dehydrogenase (BERGMEYER 1962; HEINEN 1963).

The influences of different treatments like dialysis, addition of PVP and stora-

ge at 4 °C on the glutamate dehydrogenase (GDH) activities found in leaf extracts were investigated since they showed strong variations. Moreover, the reliability of the spectrophotometric GDH assay applied to leaf extracts was studied.

2. MATERIAL AND METHODS

2.1. Plant material

Petunia hybrida plants (clone W 166K) were grown as described previously (ROGGEN 1967). Spinach plants (*Spinacea oleracea L.*) and runner-bean plants (*Phaseolus multiflorus* Lam.) were grown in a greenhouse as described by HELMSING (1969). Young leaves were collected to prepare the crude extracts.

2.2. Preparation of the extracts

Petunia, spinach or runner-bean leaves (2 g fresh weight) were homogenized for 10 minutes in a mortar with pure quartz sand and 8 ml 0.1 M triethanolamine buffer (TRA), pH 7.8, containing 0.004 M EDTA. The supernatant obtained after centrifugation for 30 minutes at 25,000 × g was used for the GDH assay. All extraction procedures took place at 4 °C. Certain variations in the extraction procedure are described in the sections concerned.

In the experiments dealing with the influence of polyvinylpyrrolidone on the GDH activity different quantities of water insoluble Polyclar AT (General Aniline and Film Corporation, Delft, The Netherlands) were added to the extraction buffer.

2.3. Gel filtration of extracts from Petunia leaves

A column of 90 cm × 2.5 cm filled with Sephadex G 200 was used. The gel was prepared by swelling Sephadex G 200 (Pharmacia, Uppsala, Sweden) in the TRA buffer for two days at room temperature on a magnetic stirrer. The leaf extracts, prepared by homogenizing 8 g leaves with 17 ml 0.02 M TRA buffer, pH 8.7, containing 0.004 M EDTA were applied to the column and subsequently eluted at 4 °C with TRA buffer. Fractions of 7 ml were collected at a flow rate of 2.8 ml/cm²/h. The absorbances were read at 280 nm from a Zeiss PMQ II spectrophotometer in 1 cm quartz cuvettes.

2.4. Partial purification of the low molecular weight substance A

Petunia leaves (33 g) were homogenized in 100 ml 0.005 M TRA buffer, pH 8.7, containing 0.004 N EDTA. After centrifugation (see 2.2) the supernatant was dialyzed twice against 15 volumes of the TRA buffer for 24 hours at 4 °C. The buffer containing the low molecular weight substances was lyophilized and the residue subsequently dissolved in 8 ml distilled water, fractionated on a column (100 cm × 2 cm) filled with Sephadex G 25 and eluted with distilled water at 4 °C. Fractions of 8 ml were collected at a flow rate of 5.1 ml/cm²/h. The presence of substance A was determined by testing 0.3 ml of each fraction with the GDH deamination assay (see 2.5) after adding 0.3 ml fraction with XDH (see 3.2).

These fractions were applied to a column (10 cm × 2 cm) filled with DEAE cellulose (DE–32 cellulose, microgranular standard, Whatman) and eluted with successively distilled water and 0.005 M TRA buffer, pH 5.6, containing a linear salt gradient of 0–0.1 M NaCl. Fractions of 6.7 ml were collected at a flow rate of 12.5 ml/cm²/h. The fractions containing substance A were combined and concentrated by evaporation at 50 °C.

This fraction was subsequently chromatographed on cellulose layers (0.25 mm) on glass plates (20 cm × 20 cm) and developed with the solvent system 1-butanol-acetic acid-water (BAW 4:2:1 v/v). Diazotated benzidine (Randerath 1962) was used as spraying reagent.

2.5. Glutamate dehydrogenase assay

Amination and deamination by GDH were determined according to the methods described by ROGGEN (1967), except that the pH used for the deamination was 8.7 instead of 7.8, since the pH optimum was found to lie between pH 8.5 and pH 8.9. The change of absorbance at 340 nm was measured for 5 min at 25 °C on a Bausch and Lomb Spectronic 505 connected with a Hitachi QPD 33 recorder. A decrease or increase in the absorbance represents the transformation of NADH into NAD in the case of amination and of NAD into NADH in the case of deamination respectively. The activity was expressed as Δ A340/5min/mg protein or per ml extract.

2.6. Measurement of the ammonia production

To measure the ammonia production during the deamination, the reaction was stopped by adding 0.2 ml Nesslers reagent (Merck) and the absorbance read at 420 nm after 15 minutes in order to let the colour develop (LANG 1958). In the control the Nesslers reagent was added immediatly after starting the reaction.

2.7. Protein determination

The protein content was measured according to the method of LOWRY *et al.* (1951) with crystalline bovine albumine, Cohn Fraction V (Callbiochem. Los Angeles, U.S.A.) as a standard.

3. RESULTS

3.1. GDH activities in leaf extracts of *Petunia*

Dialysis of leaf extracts against 125 volumes of the TRA buffer for 24 hours at 4 °C has no influence on the GDH amination activity (*fig. 1a*), whereas the deamination activity shows a strong decrease (*fig. 1b*).

The GDH activity in crude extracts can be increased by addition of PVP to the extraction medium as shown in *figs. 1a* and *1b*. At 1% PVP in the extraction buffer a maximum of amination and deamination activity of GDH is observed. The increase of both activities is about 30% compared with 0% PVP. With increasing concentrations of PVP the level of activity (checked up to 5% PVP) decreases gradually but not below the activity at 0% PVP.

The effect of storage at 4 °C is also shown in *figs. 1a* and *1b*. It is observed

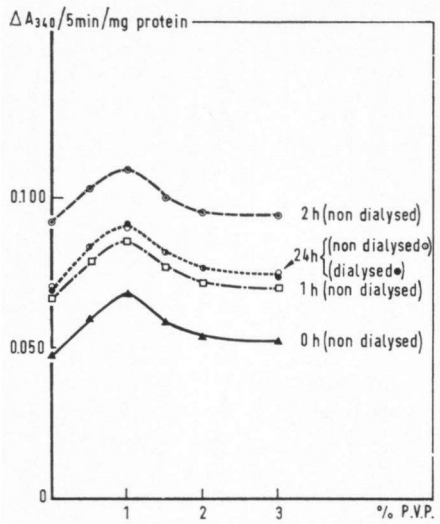

Fig. 1a

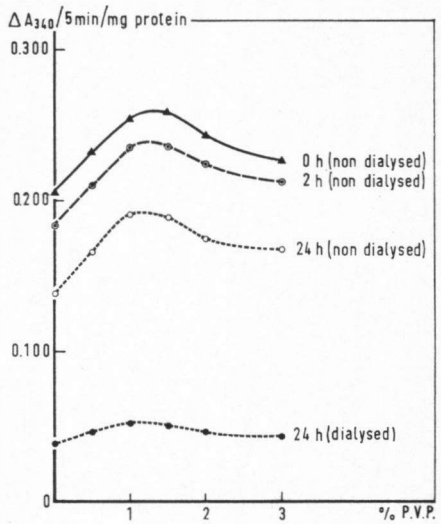

Fig. 1b

Fig. 1. GDH amination (a) and GDH deamination (b) activity after different intervals of storage at 4 °C of extracts prepared with an increasing percentage of PVP (w/v) in the TRA buffer. GDH deamination activity after dialysis of the extract was determined during 30 minutes.

that the GDH amination activity increases rapidly during the first two hours of storage whereas the deamination shows a slight decrease. In previous experiments it was established that the GDH amination reaches its maximum activity at two hours of storage, whereafter it gradually decreases. The deamination activity starts to decrease immediatly after the preparation of the extract.

3.2. Fractionation of *Petunia* leaf extracts on a Sephadex G 200 column

Leaf extracts were fractionated in order to obtain a partially purified GDH of which the ratio amination/deamination could be compared with this ratio of the GDH in crude extracts.

The results of the gel filtration of leaf extracts are shown in *fig. 2.* The GDH was eluted with approximately 150 ml buffer. It appears that the GDH amination activity is approximately 12 times higher than the deamination activity.

Since dialysis of leaf extracts causes a decrease of the deamination activity (see 3.1) it was assumed that a low molecular weight substance was necessary for the deamination by GDH. Therefore combinations were made of fractions containing GDH and of fractions containing the low molecular weight substances (fraction eluted at 310 ml buffer). No activation of the deamination was found. However, the last fractions of the GDH deamination peak could be activated by adding the fraction eluted at 310 ml. Moreover a combination of the fractions eluted immediatly after the GDH peak (eluted between 180 and

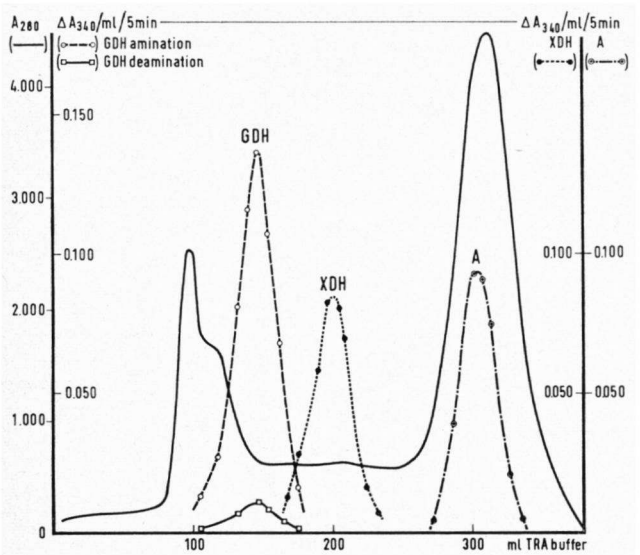

Fig. 2. Fractionation of leaf extracts on a Sephadex G 200 column. The XDH and A peaks were detected with the GDH deamination assay. XDH assay: 1.0 ml fraction + 0.5 ml fraction eluted at 310 ml. A assay: 0.5 ml fraction + 1.0 ml fraction eluted at 200 ml TRA buffer.

230 ml) and the low molecular weight fraction at 310 ml caused an increase of the absorbance at 340 nm in the GDH deamination assay. This increase has been indicated as XDH (an unknown dehydrogenase activity) in *fig. 2*. By assaying the combination XDH and the fractions containing the low molecular weight substances, using the GDH deamination assay, an activity was found which was called substance A.

3.3. Purification of substance A

The first step in studying the reaction of XDH plus substance A in the GDH deamination assay, was the partial purification of substance A.

Fig. 3 shows the elution pattern of the Sephadex G 25 column. Further purification of substance A on a DEAE cellulose column resulted in two substances (A-I and A-II) which react positively when assayed for substance A (*fig. 4*).

Substance A-II was chromatographed on cellulose plates developed with BAW (4:2:1 v/v).

Spraying with diazotated benzidine revealed seven spots with hRf values of 22 (yellow), 28 (orange), 34 (orange), 41 (yellow), 47 (red), 56 (yellow) and 65 (yellow) respectively. After elution with distilled water only the red spot (hRf 47) reacts positive on the A assay. Rechromatography of this spot with other solvent systems for hydrophilic plant compounds (e.g. propanol-ammonia-water 6:2:2 v/v, 30% acetic acid and benzol-methanol-acetic acid 45:8:4 v/v) did not succeed since in that case several spots were found which did not react in the A assay. Apparently substance A becomes unstable during the purification.

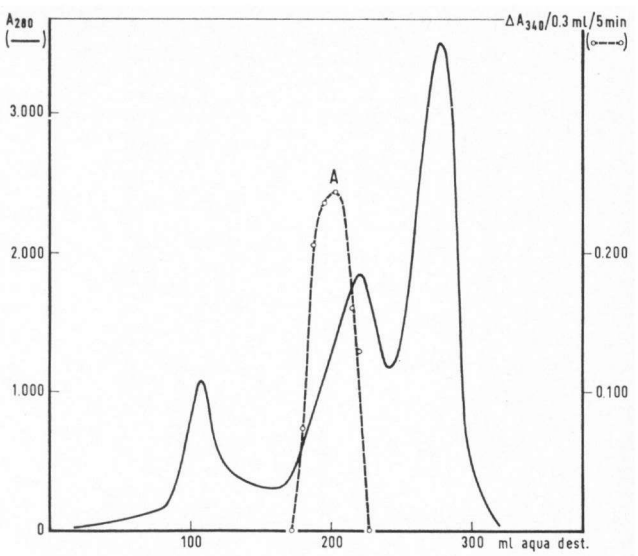

Fig. 3. Fractionation of the low molecular weight fraction (substance A) on a Sephadex G 25 column.

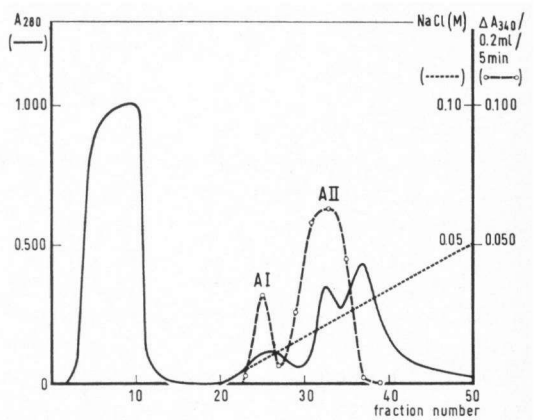

Fig. 4. Fractionation of substance A obtained from the Sephadex G 25 column on a DEAE cellulose column.

3.4. Presence of substance A and XDH in spinach and runner-bean leaf extracts

The presence of both XDH and A is necessary for the unknown reaction which interferes with the GDH deamination assay (see 3.2).

Addition of the purified substance A or XDH, obtained from *Petunia*, to spinach or runner-bean leaf extracts caused in both cases an increase of the activities in the GDH deamination assay which means that both substance A and XDH are present in spinach as well as runner-bean leaves.

3.5. Substrate specificity of GDH and XDH

Different amino acids and amines were used as substrate for both GDH and XDH (obtained from the Sephadex G 200 column) in the GDH deamination

assay. GDH is specific for L-glutamate, whereas XDH can use several amino acids and amines. A number of these substrates are given in *table 1.*

Table 1. Substrate specificity of XDH. The GHD deamination assay was carried out with 0.2 ml XDH + 0.1 ml A (section 3.2). TRA buffer was replaced by distilled water adjusted to the proper pH with 0.2 N NaOH.

Substrate	pH optimum	A340/5 min
L-glutamic acid	9.5 ± 0.1	0.073
L-glutamine	9.6 ± 0.1	0.088
L-valine	9.8 ± 0.1	0.108
L-proline	9.9 ± 0.1	0.125
L-phenylalanine	9.7 ± 0.1	0.102
p-amino benzoic acid	9.9 ± 0.1	0.087
ammonium sulphate	9.7 ± 0.1	0.082

3.6. Ammonia production

The reactions catalyzed by GDH and XDH, obtained from the Sephadex G 200 column, were tested for ammonia production. During the deamination of L-glutamate by GDH ammonia was formed. In the reaction with XDH, using L-glutamate as substrate, no ammonia production was observed.

4. DISCUSSION

The GDH deamination assay based on the determination of the increase of absorbance at 340 nm, caused by reduction of NAD in the presence of L-glutamate (*e.g.* STRECKER 1953; ROGGEN 1967; LÉJOHN & JACKSON 1968; EISENKRAFT 1969) applied to crude plant extracts, is disturbed by an unknown reaction which is measured with the same assay. The lower activities found after dialysis in the leaf extracts with the GDH deamination assay is not necessarily caused by an inactivation of the GDH, but may also be due to the removal of a low molecular component of the unknown reaction. Substance A is apparently transformed into several other compounds during the purification procedure. It is a phenolic compound or aromatic amine because it can be visualized by spraying with diazotated benzidine (RANDERATH 1962).

Since the use of PVP in the extraction buffer has the same effect on GDH and XDH and since in both reactions NAD is transformed into NADH it is likely that XDH is a dehydrogenase. However, one cannot exclude the possibility that the XDH activity may represent more than one enzyme. The XDH may use several amino acids and amines as reaction component. The reaction is no deamination, since no ammonia production was found. Moreover, it may use ammonia as substrate.

Whereas dialysis of leaf extracts causes a strong decrease of the "deamination", it has no influence on the amination activity of the GDH. The ratio amination/deamination changes from about 1:2 to 12:1 after dialysis. Since the latter value is the same as found for the partially purified GDH, obtained

from the Sephadex G 200 column, it is reasonable to assume that the activities measured in crude extract after dialysis represent indeed GDH activities. ROGGEN (1967) also found an increase of the ratio amination/deamination after dialysis and chromatography of crude plant extracts (styles of *Petunia*). He suggests that the decrease of the activity found with the GDH deamination assay was due to the removal of small molecules which would be necessary for the action of GDH.

The maximum activity of GDH and XDH found at approximately 1% PVP and the decrease of activity at higher concentrations of PVP are in agreement with the findings for other dehydrogenases like succinic dehydrogenase, malic dehydrogenase (JONES *et al.* 1965), lactic dehydrogenase and alcohol dehydrogenase (GOLDSTEIN & SWAIN 1965). This suggests that, like other dehydrogenases, the GDH is partially inhibited by polymeric phenols which are formed during homogenization.

The GDH in the leaf extract is partially present in an inactive form. This latency (BENDALL 1963) is partially caused by phenolic compounds (see 3.1) and partially by another factor since storage of the extract at 4 °C causes an increase of the GDH amination activity independently of the use of PVP. The GDH deamination activity could not be studied during dialysis. Storage at low temperatures of mitochondria preparations may activate latent enzymes (BENDALL 1963). It is dubious, however, if this could have happened in our preparations since it is likely that our homogenization procedure disrupts the mitochondria and if still present they would have been removed by the centrifugation at 25,000 × g.

Furthermore, storage at 4 °C may cause hydrolysis of phytate and polysaccharides in seed extracts which are known to form complexes with proteins (MIKOLA *et al.* 1962).

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