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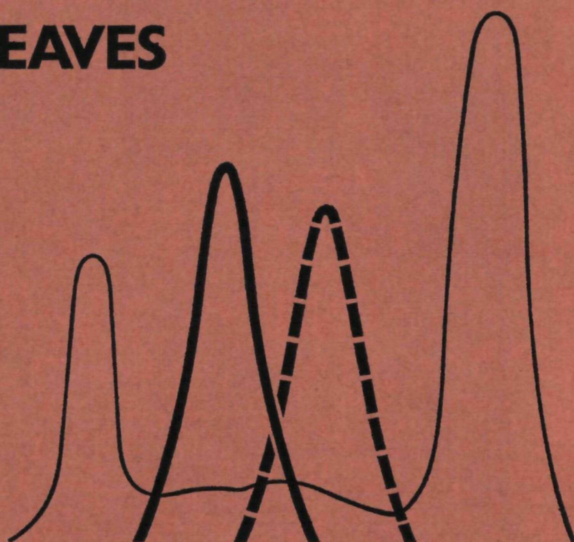
GLUTAMATE DEHYDROGENASE FROM POLLEN STYLES AND LEAVES



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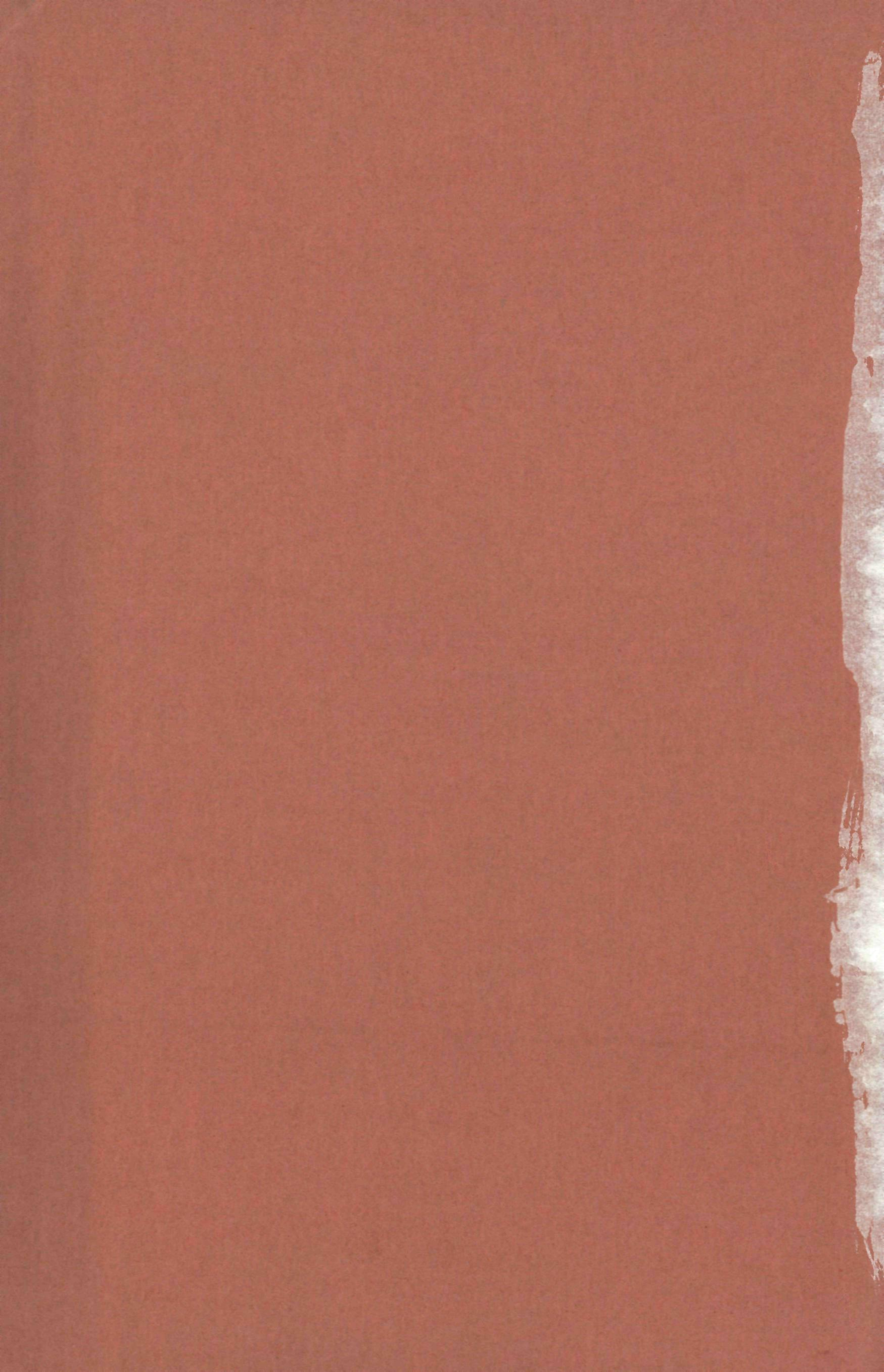

GLUTAMATE DEHYDROGENASE FROM POLLEN, STYLES AND LEAVES

Properties of purified and non-purified glutamate dehydrogenases from Petunia hybrida

Promotor: Prof. Dr. H. F. Linskens

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from Petunia hybrida

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE WISKUNDE
EN NATUURWETENSCHAPPEN AAN DE KATHOLIEKE UNIVERSITEIT
TE NIJMEGEN, OP GEZAG VAN DE RECTOR MAGNIFICUS
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EN NATUURWETENSCHAPPEN, VOLGENS BESLUIT VAN DE SENAAT
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GERARDUS MARTINUS MARIA BREDEMEIJER

geboren te Voorburg

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Bij Petunia hybrida en andere Angiospermen vindt tijdens de progame fase een metabolische interactie plaats tussen groeiende pollenbuizen en stijlweefsel. De pollenbuizen scheiden enzymen uit, die de middenlamellen van het geleidingsweefsel oplossen[1,2], waarna de afbraakproducten van pectine, hemicellulose en cellulose door de pollenbuizen worden opgenomen[3]. Deze afbraakproducten worden gebruikt als energiebron en als bouwstenen voor de celwandsynthese[4,5]. Behalve suikers worden zouten, water en aminozuren door de pollenbuizen opgenomen[6].

De uitwisseling van de verschillende stoffen gaat gepaard met veranderingen in enzymactiviteiten. Een aantal enzymen, waaronder glutamaat dehydrogenase, citraat synthase[7], β galactosidase en α mannosidase[8] wordt tijdens een compatibele pollenbuisgroei geactiveerd, terwijl zure fosfatase wordt geremd[7]; tijdens een incompatibele pollenbuisgroei stijgen de activiteiten van zure fosfatase en van cytochroom oxidase[9] en blijft de activiteit van een aantal andere enzymen gelijk[8].

Omtrent de oorzaken van deze activiteitsveranderingen is weinig bekend. De toename van de specifieke activiteit van het glutamaat dehydrogenase (GDH) gedurende de compatibele pollenbuisgroei door de stijl van Petunia hybrida is nader onderzocht, omdat dit enzym een sleutelpositie inneemt tussen de aminozuur- en de koolhydraatstofwisseling. Het is gebleken dat deze activiteitstoename veroorzaakt wordt door de novo synthese van pollen GDH en activering van stijl GDH[7]. Mengen van pollen- en stijlextracten is een simulatie van de uitwisseling van stoffen tijdens de progame fase. Het veroorzaakt een activering van het stijl GDH door een niet dialyseerbare stof uit de pollen[7]. Of de activering in vitro dezelfde is als de activering tijdens de progame fase, is niet bekend.

Bij de bepaling van de activering in vivo is men uitgegaan van extracten van bestoven stijlen[7]. Een dergelijk extract is in feite een mengsel van pollen- en stijlextract. Omdat het mengen van pollen- en stijlextracten een activering van stijl GDH veroorzaakt, is het mogelijk dat de homogenisering van bestoven stijlen tot eenzelfde activering leidt. Dit betekent dat de gevonden activering op een van de volgende wijzen tot stand is gekomen:

a. de activering vindt plaats tijdens het homogeniseren van de bestoven stijlen. In dit geval is de activering een artefact.
b. de activering, die door het mengen van pollen- en stijlextract ontstaat,

vindt ook plaats tijdens de pollenbuisgroei door de stijl

c behalve de activering die door het homogeniseren veroorzaakt wordt, vindt er een ander soort activering tijdens de pollenbuisgroei plaats

Om uit te maken welke van deze mogelijkheden de juiste is, was het noodzakelijk de activering van GDH in vitro nader te onderzoeken Op de eerste plaats moest het GDH gezuiverd worden, omdat de gemeten activiteit van een enzym in een ruw extract slechts een indicatie is dat het enzym aanwezig is Deze activiteit is geen voldoende maat voor de enzymconcentratie[10] Dit feit wordt veroorzaakt door de aanwezigheid van stoffen, die met bepaalde enzymtesten interfereren De bepaling van de GDH activiteit in een ruw extract wordt bijvoorbeeld gestoord door de aanwezigheid van andere dehydrogenasen[11 12] en door troebeling in het extract[13] Bovendien bevat een ruw extract stoffen, die de enzymactiviteit zelf beinvloeden[14 19]

Om zowel de activering van het stijl GDH in een mengsel van pollen- en stijlextract, als de activering van stijl GDH tijdens de progame fase te kunnen verklaren was het, i v m de onbetrouwbaarheid van enzymbepalingen in ruwe extracten, wenselijk om

1 de spectrofotometrische GDH test, toegepast op ruwe extracten, te controleren
2 pollen- en stijl GDH te zuiveren
3 de stof te zuiveren, die de toename van de GDH desamineringsactiviteit tijdens het mengen van pollen- en stijlextract veroorzaakt
4 de invloed van een aantal in de pollen aanwezige stoffen op de GDH activiteit te onderzoeken

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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/cm^2/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/cm^2/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/cm^2/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

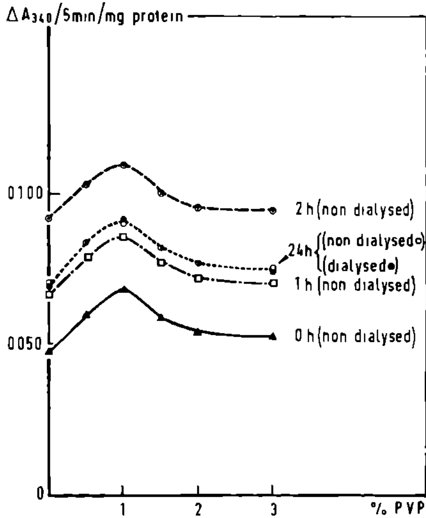

Fig. 1a

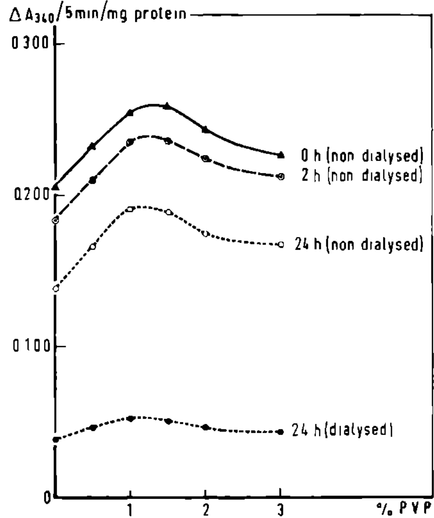

Fig. 1b

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

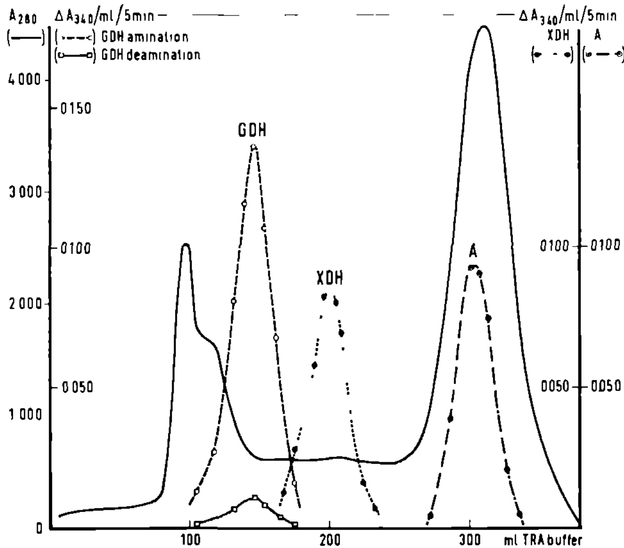

Fig 2. Fractionation of leaf extracts on a Sephadex G 200 column The XDH and A peaks were detected with the GDH deamination assay. XDH assay: 1.0 ml fraction + 0 5 ml fraction eluted at 310 ml. A assay· 0.5 ml fraction + 1 0 ml fraction eluted at 200 ml TRA buffer.

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.

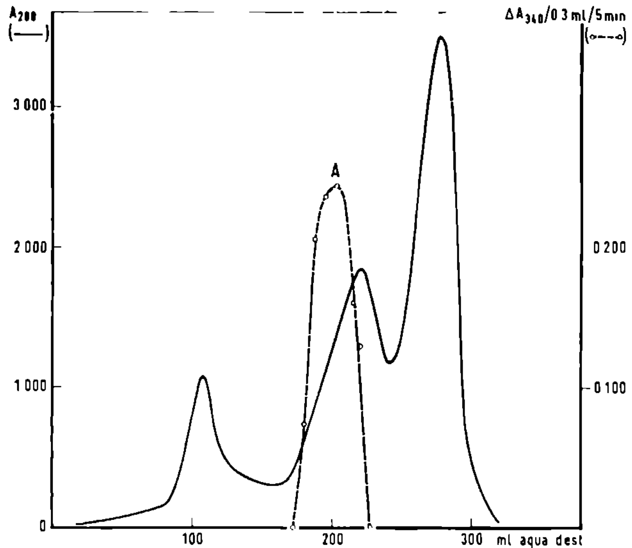

Fig 3 Fractionation of the low molecular weight fraction (substance A) on a Sephadex G 25 column

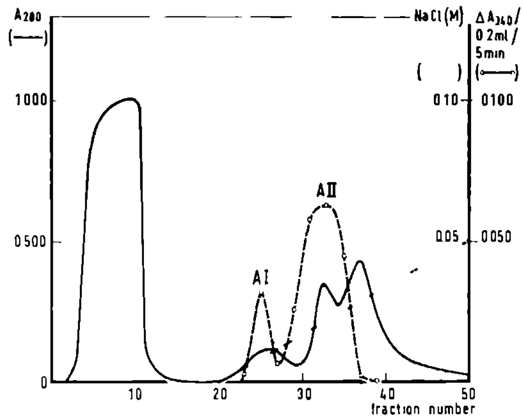

Fig 4 Fractionation of substance A obtained from the Sephadex G 25 column on a DEAE cellulose column

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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GLUTAMATE DEHYDROGENASE IN STYLES AND POLLEN OF PETUNIA HYBRIDA

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SUMMARY

Combinations of extracts from pollen and styles of Petunia hybrida caused an increase of the deamination by glutamate dehydrogenase (GDH). However, neither the combination of 54-fold purified style GDH and pollen extract, nor the combination of partly purified pollen GDH and style extract resulted in an activation of the deamination by GDH. It appeared that the increase of the GDH deamination found after combining pollen and style extract was caused by activation of a dehydrogenase which interferes with the GDH deamination assay. Although this dehydrogenase was found to be present in both pollen and styles, it was only active in the styles since it needed an aromatic amine or phenol for its catalytical action which was found in the styles, but not in the pollen.

1. INTRODUCTION

In Angiosperms there exists an interaction between the growing pollen tubes and the conducting style tissue. This interaction causes many metabolic changes, such as stimulation of the respiration, alterations of the quantities of starch, free sugars, free amino acids, growth hormones and proteins (summarized earlier by ROGGEN 1967). Changes in enzyme activities have been found by STANLEY (1958), SCHLÖSSER (1961), STANLEY & LINSKENS (1964), ROGGEN (1967), and UMEBAYASHI (1968).

After compatible pollination in *Petunia hybrida* ROGGEN (1967) found a higher activity of ketose-l-phosphate aldolase (EC 4.1.2.7), alanine aminotransferase (EC 2.6.2.1), citrate synthase (EC 4.1.3.7) and glutamate dehydrogenase (EC 1.4.1.3). In the case of glutamate dehydrogenase (GDH), which is present in *Petunia* pollen (clone T2U) and styles (clone W166K) in two different forms (isozymes), the increased activity is caused partially by a *de novo* synthesis of the GDH of the pollen and partially by an activation of the GDH in the style. The activation of the deamination by style GDH, which has also been demonstrated *in vitro* with pollen and style extracts, is caused by a high molecular weight compound, presumably a protein or RNA-like substance in the pollen. The amination could not be activated by combining pollen and style extracts (ROGGEN 1967).

The present report deals with the effect of combining pollen and style extracts on the deamination activity of GDH in styles. Combinations of pollen and style extracts, of purified style GDH and pollen extracts, and of partly purified pollen GDH and style extracts were investigated. Dialysis of pollen and style extracts was carried out since previous experiments with *Petunia* leaf extracts showed that a low molecular weight phenol or aromatic amine in the presence

of another dehydrogenase could interfere with the GDH deamination assay (BREDEMEIJER 1970). Isolation and purification of GDH from plant material has been described by several authors (*e.g.* BULEN 1956; YAKOVLEVA 1968; JOY 1969).

2. MATERIAL AND METHODS

2.1. Plant material

The self-incompatible clones W166K (incompatibility alleles S_1S_2) and T2U (S_3S_3) of *Petunia hybrida* were grown as described by ROGGEN (1967). Flowers were gathered in the bud stage just before anthesis. The styles and anthers were removed by opening the corolla with tweezers. For the experiments, styles without stigmata were used to prevent turbidity in the extracts.

2.2. Purification of the style GDH

For each experiment approximately 3000 W166K styles (9 gr. fresh weight) were homogenized in a mortar with pure quartz sand and 40 ml 0.005 M triethanolamine buffer (TRA buffer), pH 7.8, containing 0.004 M EDTA; all procedures concerning the purification took place at 4°C. The crude extract was centrifuged for 30 min at 25,000 × g. The supernatant was used for fractionation with ammonium sulphate. The proteins precipitated between 35 and 65% saturation with ammonium sulphate were collected by centrifugation at 10,000 × g for 10 min, dissolved in 3.5 ml extraction buffer and dialyzed against 300 volumes of the same buffer for 20 hours. This fraction was applied to a Sephadex G 200 column (90 cm × 2.5 cm) which was eluted with TRA buffer. Fractions of 10 ml were collected with a flow rate of 2.0 ml/cm^2/h. The fractions with the highest GDH activity were pooled and applied to a DEAE-cellulose column (10 cm × 2 cm), pretreated according to the manufacturer's instructions (DE-32 cellulose, microgranular standard, Whatman). The elution was carried out with successively TRA buffer and the same buffer containing a linear salt gradient of 0–0.2 M NaCl. Fractions of 6.7 ml were collected at a flow rate of 12.5 ml/cm^2/h.

2.3. Fractionation of pollen and style extracts on a Sephadex G 200 column

For preparing the pollen extracts 0.8 gr. fresh or cold stored (–10°C) pollen (clone W166K) were homogenized in a mortar with alcoa powder and 5 ml 0.02 M TRA buffer, pH 8.7, containing 0.004 M EDTA, at 4°C. The style extracts were prepared by homogenizing 2.9 gr. fresh styles (clone W166K) with pure quartz sand and 8 ml of the TRA buffer. The supernatants of both pollen and style extracts obtained after centrifugation at 25,000 × g for 30 min at 4°C were reduced to 4 ml by lyophilizing and fractionated on a Sephadex G 200 column (see section 2.2).

2.4. Glutamate dehydrogenase assay

The GDH activity was determined spectrophotometrically as described by

ROGGEN (1967). pH 8.7 was used for the deamination assay. The number of milli-units of enzyme was calculated according to the prescription for the GDH test combination given by Boehringer (see also BERGMEYER 1962).

2.5. Protein determination

The method of LOWRY *et al.* (1951) was followed. Crystalline bovine albumine, Cohn Fraction V (Calbiochem., Los Angeles, U.S.A.), was used as a standard.

3. RESULTS

3.1. Purification of style GDH

In order to study the activation of the deamination by style GDH a purification of this enzyme was carried out. The amination activity was used as the assay during the purification procedure, since this activity was higher than the deamination activity as found previously for the GDH of *Petunia* leaves (BREDEMEIJER 1970).

The purification was carried out by subsequent fractionation of the supernatant of the crude extract with ammonium sulphate, gel filtration on Sephadex G 200 of the proteins precipitated between 35 and 65% saturation with ammonium sulphate, and chromatography of the GDH containing fractions on a DEAE cellulose column (see section 2.2). This last step is shown in *fig. 1.* The GDH was eluted at 0.05 M NaCl. *Table 1* summarizes the purification procedure. A purification degree of 54-fold was obtained with a yield of 15% with respect to the activity in the crude extract.

3.2. GDH activities after combination of the purified or non-purified style GDH and pollen extract

Combinations of purified GDH from the styles (clone W166K) and pollen extracts of both clone T2U and clone W166K, at concentrations at which deamination activity was proportional to the enzyme concentration, did not result in an activation of the deamination. Conversely, combinations of non-

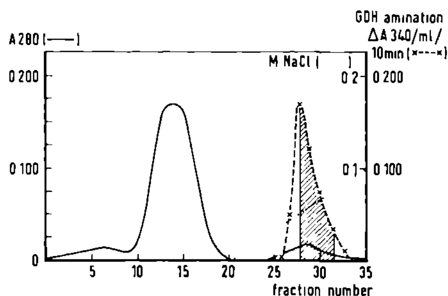

Fig. 1. Purification of the GDH obtained from the Sephadex G 200 column on a DEAE cellulose column. The fractions in the shaded area were used for the combination with pollen extract.

Table 1. Summary of the GDH-purification procedure.

Preparation	Volume, ml	Activity, Δ A340/ml/ 10 min	Milli-units	Yield, per cent	Protein, mg	Specific activity	Purification degree
Crude extract	42.0	0.460	993	100	109	0.177	1.0
Supernatant after centrifugation at 25,000 × g	98.0	0.184	926	93	63	0.287	1.6
Precipitate between 35 and 65% $(NH_4)_2SO_4$ saturation	3.5	4.880	878	88	34	0.508	2.9
Sephadex G-200 gel filtration	32.5	0.278	465	47	4 9	1.812	10.2
DEAE-cellulose chromatography	26.8	0.109	151	15	0.3	9 555	54.0

purified extracts from pollen and styles of both clones caused an activation of the GDH deamination. Essentially the same activation had been found by ROGGEN (1967). No activation was found when the style extract was dialyzed before combination with pollen extract. Dialysis of the pollen extract had no influence on the activation.

To solve the question why the purified GDH could not be activated, each step of the purification procedure was checked in order to find out where the activation capability was lost. It appeared that the GDH from the DEAE cellulose column, from the Sephadex G 200 column and from the fraction between 35 and 65% ammonium sulphate saturation could not be activated by adding pollen extract. A strong activation was obtained with the supernatant of the crude extract and the supernatant obtained after centrifugation of the fraction precipitated with 100% ammonium sulphate saturation, in which no GDH activity was found.

However, no activation was observed when the above mentioned fractions were first dialyzed against 100 volumes of 0.005 M TRA buffer, pH 7.8, containing 0.004 M EDTA for 20 hours.

3.3. Fractionation of pollen and style extracts on a Sephadex G 200 column

In the previous section it was shown that the deamination by style GDH could not be activated by adding pollen extract. Since combination of pollen extract and the supernatant obtained after saturation of the style extract with 100% ammonium sulphate caused an increase of the GDH deamination activity, it was supposed that the pollen GDH was activated by a low molecular weight substance from the styles. To check this possibility, pollen extracts were fractionated on a Sephadex G 200 column (see section 2.3.). The GDH containing fractions found with the GDH deamination assay (see *fig. 2*) were combined with the supernatant obtained after saturation of the style extract with 100% ammonium sulphate. No activation of the deamination was found. Only the last

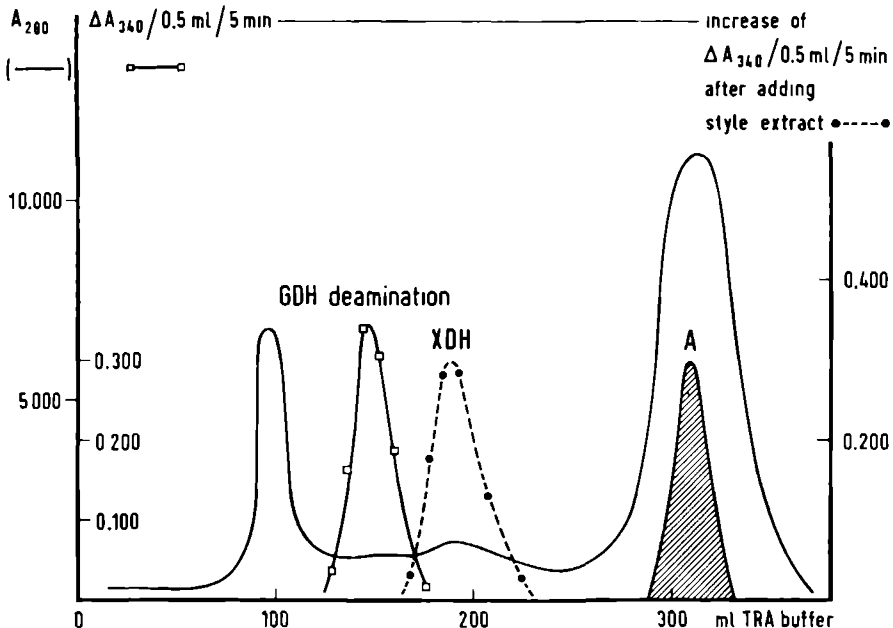

Fig. 2. Fractionation of pollen extract on a Sephadex G 200 column. The GDH, XDH and substance A were detected with the GDH deamination assay. GDH assay: 0.5 ml fraction XDH assay: 0 5 ml fraction + 0 2 ml supernatant of the style extract obtained after 100% saturation with ammonium sulphate. Peak A represents the substance from the styles which is necessary for the action of XDH. A assay: 0.5 ml fraction + 0.5 ml fraction XDH (eluted at 190 ml).

fractions of the GDH peak and a number of the following fractions (eluted between 180 and 230 ml) showed an increase of the deamination activity. This increase has been indicated as XDH (unknown dehydrogenase activity) in *fig. 2*.

Fractionation of style extracts on the same column resulted in roughly the same elution pattern. Both GDH and XDH were detected. Moreover, the low molecular weight fractions contained a substance (A) which could be used to detect XDH with the GDH deamination assay just like the supernatant obtained after saturation of the style extract with 100% ammonium sulphate. This substance was not found after fractionation of pollen extracts. By assaying combinations of XDH from both pollen and styles with fractions containing the low molecular weight substances of the styles with the GDH deamination assay peak A was found (*fig. 2*).

A further purification of this substance by subsequent gel filtration on a Sephadex G 25 column, chromatography on a DEAE cellulose column, and thin layer chromatography on cellulose layers showed that A appeared to be identical with the aromatic amine or phenol found in *Petunia* leaf extracts (BREDEMEIJER 1970).

4. DISCUSSION

Whereas joining pollen and style extracts causes an activation of the deamination activity of the style GDH by a high molecular weight substance, presumably a protein or RNA like substance, from the pollen (ROGGEN 1967), no activation is found by combining pollen extract and 54-fold purified style GDH. Since a strong activation is found after combining pollen extract and the supernatant obtained after saturation of the style extract with 100% ammonium sulphate, or the low molecular weight fractions obtained after gel filtration of the style extract which do not show GDH activity, it is possible that the pollen GDH is activated. However, this possibility has been excluded, since partly purified pollen GDH (see section 3.3) can not be activated by adding the low molecular weight substances from the styles. It must be concluded that neither the deamination by style GDH, nor the deamination by pollen GDH is activated by combining pollen and style extract.

The increase of the GDH deamination activity found after combination of pollen and style extracts is caused by activation of a reaction which interferes with the GDH deamination assay. The unknown dehydrogenase activity (XDH) of the pollen is activated by a low molecular weight aromatic amine or phenol (A) from the style which is lacking in the pollen. Substance A of the styles and XDH of both pollen and styles are identical with the substances found in leaf extracts (BREDEMEIJER 1970). Therefore it can be concluded that the reaction which is activated by joining pollen and style extracts is the same reaction as which was found to interfere with the GDH deamination assay applied to *Petunia* leaf extracts.

Since the phenol or aromatic amine does not occur in the pollen, it is not necessary to dialyze the pollen extracts before applying the GDH deamination assay; style extracts, however, must be dialyzed like leaf extracts, because the interfering substance A is present in these extracts.

The high molecular weight substance, present in the pollen, which should activate the deamination by style GDH after combining pollen and style extract as supposed by ROGGEN (1967), must be identical with the dehydrogenase which interferes with the GDH deamination assay. Therefore it is likely to believe that the activation of the style GDH by growing pollen tubes, found after electrophoretical separation of the extracts (ROGGEN 1967), is not the same as the "activation" which takes place after combining pollen and style extracts.

It is known that many low molecular weight substances like sugars, salts, amino acids (LINSKENS & ESSER 1959), and breakdown products of the cell walls of the conducting stylar tissue (SCHOCH-BODMER & HUBER 1945 and 1947) are readily taken up by pollen tubes. Thus there is a good reason to assume that the low molecular weight aromatic amine or phenol can be taken up by pollen tubes. This should mean that a reaction is started in the pollen tubes which can use many amino acids and amines as substrate (BREDEMEIJER 1970).

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LATENT GLUTAMATE DEHYDROGENASE IN POLLEN OF PETUNIA HYBRIDA

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SUMMARY

Pollen glutamate dehydrogenase is partially present in a masked form. Removal of the masking substances was carried out by several treatments like addition of polyvinylpyrrolidone to the extraction medium, storing of pollen extracts at different pH's and temperatures, fractionation with ammonium sulphate, and incubation with several hydrolytic enzymes. Only the use of polyvinylpyrrolidone and incubation with a few enzymes caused an increase of both amination and deamination activity, whereas the other treatments caused an increase of the amination activity and a decrease of the deamination activity.

It is probable that the glutamate dehydrogenase (GDH) and the masking substances are associated through interaction of ionic groups and hydrogen bondings. Some substances may be bound by stronger links. The question whether the masked GDH is present as such *in vivo* or whether it is formed during homogenization has been discussed.

1. INTRODUCTION

Most investigations of pollen enzymes in both germinated and ungerminated pollen deal with their detection and localization (summarized by LINSKENS 1964; ROSEN 1968; LINSKENS & KROH 1970). Moreover, changes in enzyme activities during pollen germination were reported (*e.g.* HAECKEL 1951; STANLEY & LINSKENS 1964; ROGGEN 1967; LINSKENS *et al.* 1969; DICKINSON & DAVIES 1969).

The effective amount of an enzyme present at any given time is determined by the relative rates of synthesis and degradation, and by the concentrations of various kinds of inhibitors and activators (VARNER 1965). However, in the case of pollen enzymes little is known about the factors which determine the effective amount. Activators of pollen enzymes have been found by UMEBAYASHI (1968) and by DICKINSON & DAVIES (1969), whereas ROGGEN (1967) studied a *de novo* synthesis of glutamate dehydrogenase during pollen germination and pollen tube growth.

The present report deals with a number of the factors which influence the activities of glutamate dehydrogenase (GDH) in *Petunia* pollen. In previous studies it has already been shown that the activities of plant GDH are inhibited by phenols (BREDEMEIJER 1970a). Since pollen is known to contain large amounts of phenolic compounds (STANLEY & LINSKENS 1965; STROHL & SEIKEL 1965; TOGASAWA *et al.* 1966) it is likely that also the GDH activity is influenced.

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Storage of leaf extracts causes an activation of the GDH amination activity, independently of the presence of the phenols which indicates that the GDH is partly present in a latent form. The GDH deamination activity can not be studied during 24 hours after preparing the extracts since it is necessary to dialyze the extracts before they can be assayed on GDH deamination activity. Disturbing reactions are eliminated by dialysis (BREDEMEIJER 1970a). In the case of pollen extracts no dialysis is necessary since one of the reaction components of the interfering reaction is lacking in the pollen (BREDEMEIJER 1970b). Therefore it was possible to investigate the influence of storing pollen extracts on both amination and deamination activity of GDH. Moreover, the origin of the latency of GDH could be studied.

2. MATERIAL AND METHODS

2.1. Plant material

Pollen of the self-incompatible clone W166K (incompatibility alleles S_1S_2) of *Petunia hybrida* were obtained by drying the anthers which were collected on the day of anthesis. Fresh or cold stored (–10°C) pollen was used for extraction.

2.2. Preparation of pollen extracts

Pollen extracts were prepared by homogenizing the pollen at 0°C in a Potter Elvehjem homogenizer with alcoa powder and 0.02 M triethanolamine buffer (TRA), pH 7.8, containing 0.004 M EDTA. The amounts of pollen are mentioned in the sections concerned. The supernatants of the extracts obtained after centrifugation at 25,000 $\times$ g for 30 minutes at 2°C were used for incubation experiments and for further purification of the GDH.

2.3. Purification of pollen GDH

For each experiment approximately 2 g pollen were extracted as described in section 2.2. The purification of the GDH (EC 1.4.1.3) present in the extracts was carried out by subsequent fractionation with ammonium sulphate, gel filtration on a Sephadex G 200 column (90 cm $\times$ 2.5 cm) of the substances precipitated between 35 and 65% saturation with ammonium sulphate, and chromatography on a DEAE cellulose column (8 cm $\times$ 2 cm) as described for style GDH (BREDEMEIJER 1970b).

2.4. Incubation of pollen extracts at different pH values and different temperatures

Pollen extract which was prepared by homogenizing 250 mg pollen was diluted to 15 ml with TRA buffer and divided into portions of 1 ml which were adjusted to various pH values by adding 0.2 N HCl or 0.2 N NaOH. Incubation was carried out at 30°C, whereas controls were stored at 0°C. After appropriate time intervals samples of 0.25 ml were removed and used for determination of GDH activities. Incubation at different temperatures was done in the same way at pH 6.

Moreover, pollen extracts prepared with buffer containing different quantities of insoluble polyvinylpyrrolidone (Polyclar AT from General Aniline and Film Corporation, Delft, The Netherlands) were incubated at pH 7 8 at 30 °C

2 5 Fractionation of pollen extract on a Sephadex G 200 column and incubation of the GDH containing fractions with the other fractions

Pollen extract prepared by homogenizing 1 30 g pollen was fractionated on a Sephadex G 200 column (see section 2 3) The GDH containing fractions were pooled and used for preparing the incubation mixtures which contained 1 0 ml fraction with GDH and 1 5 ml from one of the other fractions After adjusting the pH to 6 with 0 2 N HCl incubation was carried out during 15 hrs at 30 °C Controls were stored at 0 °C

2 6 Incubation of pollen GDH with hydrolytic enzymes

Purified and non-purified pollen GDH was incubated with various hydrolytic enzymes which were obtained from Sigma (see *table* 1) The incubation mixtures contained 0 5 ml enzyme solution (0 5 mg per ml distilled water) and 1 0 ml fraction with GDH After being adjusted to the pH optima of the various enzymes (*table* 1) the mixtures were incubated at 30 °C In the controls the enzyme solutions were substituted by distilled water The GDH amination activity of the incubation mixture which contained GDH and acid phosphatase had to be corrected since acid phosphatase reacted also positively in the GDH amination assay

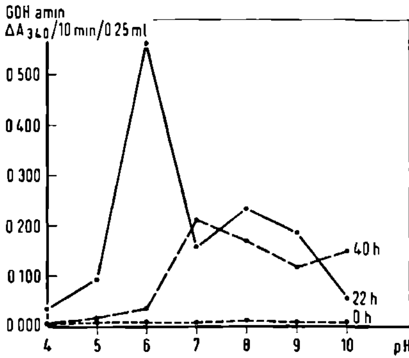
2 7 Incubation of purified GDH with maltose, starch, RNA and phytate

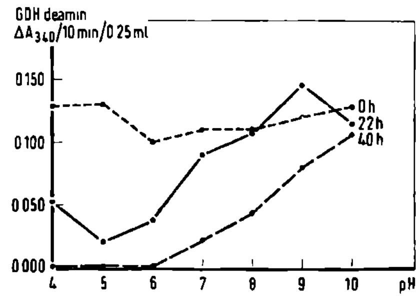
The incubation mixtures containing 1 0 ml of the fraction with purified GDH (see section 2 3), x ml maltose, starch, RNA (Sigma) or sodium phytate (Sigma) solution in 0 02 M TRA buffer, pH 7 8, (0 – 0 5 mg/ml), and 1 – x ml TRA buffer were incubated at 2 °C

2 8 Assays

The determination of the GDH activity, the calculation of the number of milli-units enzyme, and the protein determinations have been described in previous studies (BREDEMEIJER 1970a, 1970b)

The phytase activity was determined according to the procedure of PEERS (1953) The incubation mixture contained 5 ml 0 15 M acetate buffer, pH 5 15, containing 0 004 M $MgSO_4$ prewarmed to 55 °C, 1 ml of the 7 ml pollen extract prepared by extracting 100 mg pollen, and 4 ml sodium phytate solution (0 75 mg/ml) A sample of 2 ml is removed immediately and the remainder incubated at 55 °C, further 2 ml samples being removed after appropriate time intervals The 2 ml samples are added to 1 ml 10% (w/v) TCA, filtered and used for the determination of orthophosphate (ALLEN 1940) The absorbance at 830 nm was used as a measure for the amount of orthophosphate





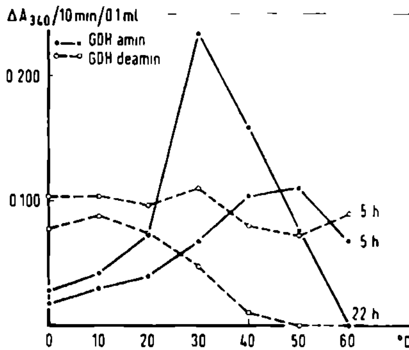


Fig. 1. GDH amination activity of pollen extract after different intervals of storage at different pH values at 30°C.

Fig. 2. GDH deamination activity of pollen extract after different intervals of storage at different pH values at 30°C.

Fig. 3. GDH amination and deamination activity of pollen extract after different intervals of storage at different temperatures at pH 6.0.

3. RESULTS

3.1. GDH activities of pollen extracts after storage at different pH values and at different temperatures

The effect of storage is shown in *figs. 1, 2* and *3*. It is observed that incubation of pollen extract at 30°C at pH values from 4 to 10 causes an increase of the GDH amination activity (*fig. 1*) and a decrease of the GDH deamination activity (*fig. 2*) compared with the activities determined immediately after preparing the extract (t = 0 h). Storage at 0°C had little effect.

The temperature optimum of the activation of the amination activity is 50°C after incubation at pH 6 during 5 hrs and 30°C after 22 hrs (*fig. 3*).

Incubation of pollen extract at pH 6 and 30°C during 22 hrs causes a 66-fold increase of the GDH amination activity, whereas the deamination activity is lowered almost 3-fold (*figs. 1* and *2*).

The GDH activities in pollen extract can be increased by addition of polyvinylpyrrolidone (PVP) to the extraction buffer as shown in *fig. 4*. At 1% PVP an increase of about 200% is observed for both amination and deamination activity. The increase of the amination activity caused by storing pollen extract remains if PVP is used in the extraction buffer. The GDH is activated by the use of PVP since inhibiting phenols are bound to the PVP.

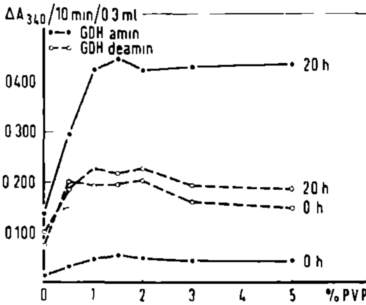


Fig. 4. GDH amination and deamination activity after 0 and 20 hrs of storage at pH 7.8 at 30 °C of extracts prepared by homogenizing portions of 60 mg pollen with an increasing percentage of PVP (w/v) in the extraction buffer.

3.2. Incubation of GDH with fractions obtained by gel filtration of pollen extract

The first step in studying the alterations of the GDH activities was to check whether the changes were caused by substances present or formed in the pollen extract, or by spontaneous events like loss of inhibitors and changes in the enzyme molecule configuration which may be due to the loss of inhibitors.

The fractions containing GDH, obtained by gel filtration of pollen extract, were pooled and incubated with the other fractions as described in section 2.5. If the incubation took place at 30 °C most fractions could increase the GDH amination activity and reduce the deamination activity (see *fig. 5*); incubation at 0 °C had little effect. It is observed that the GDH is influenced by several sub-

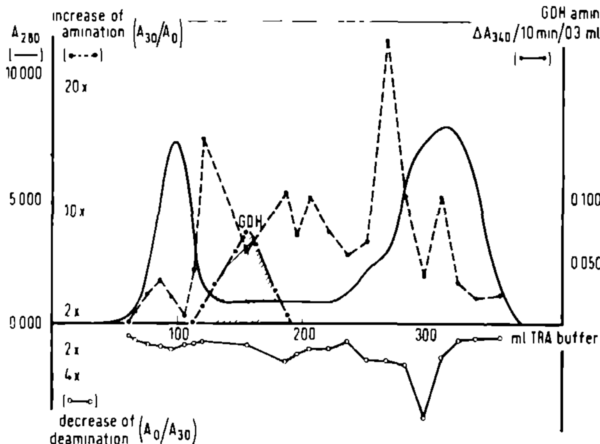


Fig. 5. Fractionation of pollen extract on a Sephadex G 200 column. The GDH containing fractions in the shaded area were pooled and divided into several portions which were incubated with samples of the other fractions during 15 hrs at pH 6.0 at 30 °C. The increase of the amination caused by the different fractions is expressed as the ratio: activity after incubation at 30 °C / activity after incubation at 0 °C (A_{30}/A_0); the decrease of the deamination activity is expressed as A_0/A_{30}.

stances with diverse molecular weights. If the incubations were carried out at pH 8, instead of at pH 6, only one peak of increased amination activity was found.

Since the influence of the different substances on the GDH is dependent on temperature, time, and pH, and since these substances have considerable molecular weights, it is possible that proteins or even enzymes are involved.

3.3. The influence of some hydrolytic enzymes on the GDH amination activity in pollen extract

The results of incubating pollen extracts with hydrolytic enzymes as described in section 2.6, are shown in *table 1*. It is obvious that the GDH amination activity is increased by many hydrolytic enzymes. Especially glycolytic enzymes like maltase, α-amylase and β-amylase have a strong effect. Moreover, galactose oxidase causes a strong increase of the GDH amination activity. In some cases like the incubation with maltase the amination activity decreases after reaching its maximum.

It is likely that the inhibitors of the GDH amination activity are hydrolyzed by the hydrolytic enzymes. Whether these inhibitors are present free in the extract or attached to the GDH molecule was studied by purification of the GDH.

3.4. Purification of pollen GDH

The purification of pollen GDH is summarized in *table 2*. Precipitation of the GDH with ammonium sulphate causes a striking increase in the amination activity and a decrease of the deamination activity. The fraction precipitated between 35 and 65% saturation with ammonium sulphate contained 2378 mU GDH (amination), whereas the starting material contained only 616 mU GDH.

Table 1. GDH amination activity of pollen extract after incubation with several hydrolytic and some other enzymes. The increase (+) or decrease (−) of activity with regard to the control activity ($\triangle A_{340}$ assay − $\triangle A_{340}$ control) was multiplied by 1000.

enzyme	pH	3 hrs	8 hrs	20 hrs	40 hrs
acid phosphatase	4.8	− 3	+ 9	+ 30	+ 28
alkaline phosphatase	10.4	− 3	+ 10	49	+ 79
lipase	7.4	− 6	+ 2	− 15	+ 30
RNase	7.0	+ 6	+ 6	+ 10	+ 31
trypsin	8.1	− 16	− 7	+ 8	+ 21
leucine aminopeptidase	8.5	− 5	− 6	− 21	19
α-glucosidase	6.8	+ 6	+ 5	+ 24	+ 20
β-glucosidase	5.2	− 18	0	+ 17	+ 33
β-galactosidase	7.2	− 13	+ 8	+ 13	+ 70
maltase	6.4	+ 102	+ 82	− 49	− 19
α-amylase	6.9	+ 9	+ 39	+ 110	+ 57
β-amylase	4.8	+ 40	+ 40	+ 32	+ 17
neuraminidase	5.0	− 12	− 42	48	+ 53
galactose oxidase	7.0	+ 104	+ 107	+ 72	+ 11
glucose oxidase	5.1	− 15	− 39	+ 38	+ 20

Table 2. Summary of the purification of pollen GDH.

preparation	volume, ml	amination activity, Δ A340/ ml/10min	milli units amin.	yield, per cent	protein, mg	amin. specific activity	purification degree amin.	deamin. specific activity	milli units deamin.	yield per cent	purification degree deamin.
supernatant after centrifugation	20.0	0.600	616	100	784	0.015	1.0	0.111	4474	100	1.0
precipitate between 35 and 65% $(NH_4)_2SO_4$ saturation	4.9	9.500	2378	386	195	0.333	22.2	0.278	1992	45	2.5
Sephadex G 200 column	56.0	0.720	2068	336	50.4	0.800	53.3	1.044	2699	60	9.4
DEAE cellulose column	80.0	0.307	1260	205	2.64	9.303	620.2	5.273	712	16	47.5

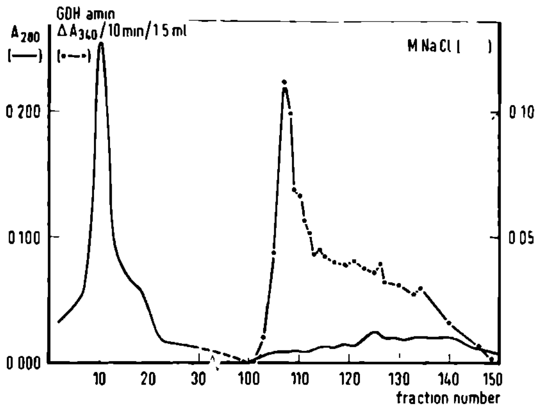


Fig. 6. Purification of pollen GDH obtained from a Sephadex G 200 column on a DEAE cellulose column. Elution was carried out with successively 0.02 M TRA buffer, pH 7.8, and the same buffer containing a linear salt gradient of 0–0.1 M NaCl. Fractions of 8 ml were collected.

The maximum purification degree was 620-fold for the amination activity and 47.5-fold for the deamination activity with yields of 205% and 16%, respectively.

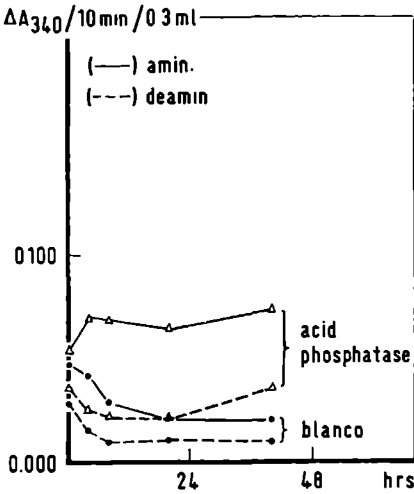
The last step of the purification, chromatography on a DEAE cellulose column, is shown in *fig. 6*. The GDH is not eluted as a homogeneous fraction; the main peak has a large tail. If the linear salt gradient was replaced by a stepwise gradient of 0–0.1 M NaCl in steps of 0.01 M, 6 GDH peaks with different ratios amination/deamination were found. However, rechromatography of a certain peak in the same way, after removing the salt by dialysis, resulted again in more than one GDH peak.

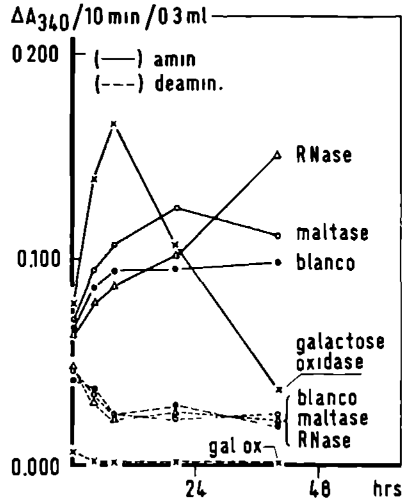
3.5. The influence of hydrolytic enzymes on purified GDH

When the fraction precipitated between 35 and 65% saturation with ammonium sulphate was incubated with the enzymes that could activate the GDH amination activity in the crude extract (see section 3.3), it appeared that only acid phosphatase, alkaline phosphatase, RNase, maltase, and galactose oxidase were able to activate the GDH.

The result of incubating these enzymes with GDH obtained from the DEAE column as described in section 2.6 is shown in *figs. 7, 8*, and *9*. The amination activity is activated by incubation with acid and alkaline phosphatase, RNase, maltase, and galactose oxidase. However, in the case of galactose oxidase and alkaline phosphatase the activation is reversed into an inhibition after 8 and 4 hrs, respectively (*figs. 8* and *9*). The deamination activity is inhibited by alkaline phosphatase and galactose oxidase, and activated by acid phosphatase; maltase and RNase have no effect.

GDH obtained from a Sephadex G 200 column without preceding ammonium sulphate fractionation could also be activated with all the enzymes that activate GDH in pollen extract.





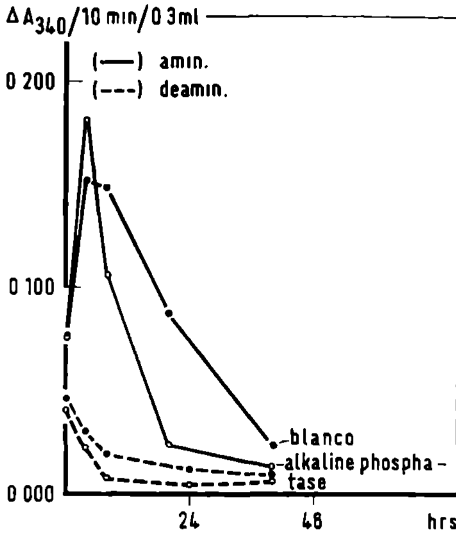


Fig. 7. GDH amination and deamination activity after incubation of purified GDH with acid phosphatase at pH 4.8. The control contained distilled water instead of acid phosphatase.

Fig. 8. GDH amination and deamination activity after incubation of purified GDH with RNase, maltase and galactose oxidase at pH 7.0. The controls contained distilled water instead of RNase, maltase and galactose oxidase.

Fig. 9. GDH amination and deamination activity after incubation with alkaline phosphatase at pH 10.4. The control contained distilled water instead of alkaline phosphatase.

3.6. The influence of maltose, starch, RNA, and phytate on purified GDH

Incubation of purified GDH with RNA, starch, and maltose during 20 hrs at 2°C had little effect on the amination activity, whereas sodium phytate (0.5 mg/ml) caused a decrease of 47% (after 3 hrs 19%).

3.7. Phytase activity in *Petunia* pollen

The amount of orthophosphate liberated during incubation of pollen extract at

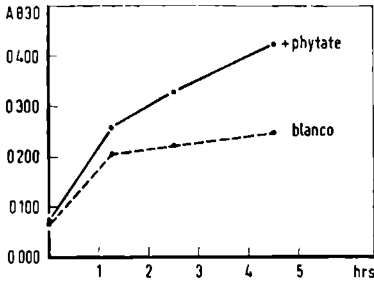


Fig. 10. The amount of orthophosphate liberated during the incubation of pollen extract at 55 °C at pH 5.15 with or without sodium phytate, expressed as absorbance at 830 nm.

55 °C is shown in *fig. 10.* If sodium phytate was added to the extract, more orthophosphate was liberated, indicating that *Petunia* pollen possesses a phytase activity. From gel filtration experiments it appeared that the phytase activity has been divided into two peaks by using Sephadex G 200 as described in section 2.5.

4. DISCUSSION

Storage of pollen extract causes an increase of the GDH amination activity and a decrease of the deamination activity. The increase of GDH amination activity was also found in leaf extracts (BREDEMEIJER 1970a). However, in leaf extracts this increase was only 2-fold, whereas in pollen extracts the increase was 66-fold.

It appeared that the GDH amination activity in crude pollen extracts could also be activated by adding various hydrolytic enzymes. Many of these enzymes are known to occur in pollen: acid phosphatase (HAECKEL 1951; GORSKA BRYLASS 1965; LINSKENS 1966; ROGGEN 1967); RNase (LINSKENS & SCHRAUWEN 1969); amylase (HAECKEL 1951; BELLARTZ 1956); β-galactosidase (LINSKENS *et al.* 1969); α-glucosidase (DICKINSON 1967) and proteinase (BELLARTZ 1956). Consequently, the presence of these enzymes in pollen can explain the increase of GDH amination activity during ageing of the extracts.

Essentially the same activation was found if GDH obtained by gel filtration of pollen extract without preceding fractionation with ammonium sulphate was used for incubation with various enzymes (see section 3.5). This means that no separation of the inhibiting substances from the GDH occurred during gel filtration on Sephadex G 200.

There are two possibilities: firstly the inhibitors are linked to the GDH molecule and, secondly, the molecular size of the inhibitors is almost identical with the size of GDH. Since it is highly improbable that the various inhibitors all have the same molecular size as GDH, it is most likely that they are bound to or associated with the GDH molecule.

Precipitation of GDH with ammonium sulphate causes an activation of the amination and a loss of the possibility to activate the GDH by treatment with most of the enzymes that activate the amination in crude extract (section 3.4 and 3.5). Therefore, it is likely that the inhibitors of the amination activity are

attached to the GDH through a weak link like hydrogen bonding or a salt linkage which can be broken by high salt concentrations. Activation of enzymes caused by precipitation with ammonium sulphate occurred, for instance, with phenolase (KENTEN 1957) and galactolipase (HELMSING 1969).

Presumably the GDH in the pollen extract is present in a masked form, just as KENTEN (1955, 1957 and 1958) demonstrated for phenolase in leaf extracts. Phenolase which is apparently present in combination with a protein inhibitor can be activated by treatment with trypsin A similar activation occurred also with the GDH in our experiments. However, in the present study it appeared that besides carbohydrates, lipids, RNA and phosphate esters like phytate can mask the GDH. There are many examples of such masked activities, caused by interactions with RNA or proteins (SWARTZ *et al* 1956) BALTIMORE & HUANG (1970) found that RNA is bound to a heterogeneous collection of soluble proteins by ionic forces The activation of the GDH amination activity as found by WINNACKER & BARKER (1970) caused by treatment of *Clostridium* extracts with protamine sulphate is probably due to the removal of nucleic acids from the GDH, since the nucleic acid content decreases from 18 to 5% Concerning the interaction between GDH and carbohydrates it can be mentioned that in the extraction of plant proteins, soluble carbohydrates are extracted which may form complexes with proteins (STAHMANN 1963). MIKOLA *et al* (1962) suggested that when a barley grain extract is stored such carbohydrates are hydrolyzed to enhance protein resolution The same authors suggested an interaction between proteins and phytate, which occurs apparently also with GDH (section 3.6). The presence of phytase activity in *Petunia* pollen makes it possible to hydrolyze the phytate which inhibits the GDH amination activity.

Since the purified GDH can still be activated by incubation with certain enzymes it seems that some inhibitors are attached to the GDH by linkages which are not sensitive to high salt concentrations The effect of galactose oxidase and maltase may indicate that the GDH is a glycoprotein with galactose and maltose as sugar components A total purification of the GDH will be necessary to prove a glycoprotein nature Then also the influences of acid and alkaline phosphatase and RNase can be investigated further

Whatever the state of the masked GDH in the pollen extracts, the question arises whether it is present as such *in vivo* or whether it is formed during the homogenization procedure. It is almost certain that a number of phenols are attached to the GDH during the homogenization since *in vitro* experiments showed several phenols to be bound to pure dehydrogenases (FIRENZUOLI *et al.* 1969); moreover, this may have happened with phytate which alters the amination activity (section 3 6).

In dormant, metabolically almost inactive pollen the GDH may be present in a masked and inhibited state which may be overcome by the action of several enzymes Some of these enzymes, for instance acid phosphatase, amylase (HAECKEL 1951), and β-galactosidase (LINSKENS *et al.* 1969) are known to be activated during pollen germination and tube growth which means that the GDH activities can be changed. So it is possible that GDH in a normally inhib-

ited state could be a mechanism of cellular control of enzyme action, as suggested by SWARTZ *et al.* (1956). Moreover, the masking substances may protect the GDH against denaturation and proteolytic digestion during the dormant state of the pollen, like the protection of proteins by some sugars as described by MARSHALL & NEUBERGER (1968).

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1. De invloed van het mengen van pollen- en stijlextract op de GDH desamineringsactiviteit

Uit de onderzoekingen die in dit proefschrift zijn beschreven blijkt, dat de activering van het stijl GDH tijdens de progame fase niet vergeleken kan worden met de activering in het mengsel van pollen- en stijlextract. De laatstgenoemde activering is namelijk geen activering van het GDH, maar van een ander enzym dat eveneens met de GDH desamineringstest wordt bepaald[1]. De desamineringsactiviteiten van gezuiverd pollen- en gezuiverd stijl GDH nemen niet toe als respectievelijk stijl- en pollenextract worden toegevoegd[2].

Het is duidelijk dat de, in de inleiding genoemde verklaringen voor de activering van GDH niet juist zijn. Deze verklaringen zijn namelijk geheel of gedeeltelijk gebaseerd op een activering van het GDH in het mengsel van pollen- en stijlextract. Er is geen enkel verband tussen de activering van het GDH in vivo en de activering van het dehydrogenase dat met de GDH test interfereert. De activering in vivo werd namelijk gevonden door activiteiten van electroforetisch gezuiverd GDH te vergelijken[3].

2. Activering van het stijl GDH tijdens de progame fase

De GDH amineringsactiviteit in pollen- en bladextracten en vermoedelijk ook in stijlextracten, gezien de homologie tussen stijlen en bladeren, is gedeeltelijk latent[2, 4]. Deze latentie kan worden opgeheven door de extracten te bewaren. In het extract aanwezige hydrolytische enzymen breken een aantal remstoffen af die aan het GDH gehecht zijn. Deze stoffen, waaronder zetmeel, RNA, eiwitten, galactose, maltose en polyfenolen zijn voor het merendeel door waterstofbruggen en/of door interactie van geïoniseerde groepen aan het GDH gebonden en wel zodanig dat ze de catalytische werking van het GDH beïnvloeden. Gezuiverd pollen GDH[4] wordt geactiveerd door incubatie met zure- en basische fosfatase, RNase, maltase en galactose oxidase. Er zijn dus kennelijk na de zuivering nog bepaalde stoffen aan het GDH gehecht door bindingen die ongevoelig zijn voor hoge zoutconcentraties.

De stoffen die aan het GDH gehecht zijn, zijn medebepalend voor de activiteit van dit enzym. Het is mogelijk dat andere enzymen, die deze stoffen als substraat gebruiken, de GDH activiteit reguleren. Deze mogelijkheid vormt de basis van de volgende hypothese:

De activering van het stijl GDH tijdens de progame fase wordt veroor-

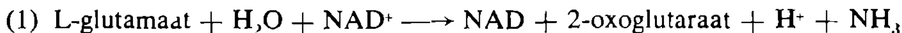
zaakt door de werking van een aantal enzymen, die door de pollen en door de pollenbuizen in de stijl worden uitgescheiden Deze enzymen hydrolyseren of veranderen de stoffen die het GDH remmen zodanig, dat ze onwerkzaam worden, met als gevolg een toename van de GDH activiteit

Deze hypothese wordt gesteund door het feit dat pollen en pollenbuizen inderdaad enzymen uitscheiden in de stijl[9, 10] Bovendien zijn een aantal van deze enzymen, zoals RNase, amylase, leucine aminopeptidase en zure fosfatase in staat de GDH aminering te activeren[4], zure fosfatase activeert bovendien de GDH desaminering

De golf van GDH activering voorafgaande aan de pollenbuisgroei, de z g „front wave"[3] kan eveneens met deze hypothese verklaard worden De enzymen die door de pollenbuizen worden uitgescheiden kunnen door diffusie, via de opgeloste matrix van de middenlamellen, de cellen van het geleidingsweefsel bereiken Als deze enzymen worden opgenomen, kunnen ze met de plasmastroom in de langgerekte cellen in dat gedeelte van de stijl komen, waar de pollenbuizen nog niet groeien

3 De reactie die met de GDH desamineringstest interfereert

Als de spectrofotometrische GDH desamineringstest, gebaseerd op de extinctieverandering door de reductie van NAD tot NADH in de aanwezigheid van L-glutamaat, wordt toegepast op ruwe extracten van plantaardig materiaal, dan worden twee reacties bepaald



$$(1)\ \text{L-glutamaat} + H_2O + NAD^+ \xrightarrow{GDH} NAD + \text{2-oxoglutaraat} + H^+ + NH_3$$

$$(2)\ \text{L-glutamaat} + \text{fenol A} + NAD^+ \xrightarrow{XDH} NADH + ?\ (\text{geen } NH_3)$$

Het XDH is waarschijnlijk een dehydrogenase omdat het op dezelfde manier door polyvinylpyrrolidone geactiveerd wordt als GDH[1] en een aantal andere dehydrogenasen[11, 12], bovendien wordt NAD^+ omgezet in NADH De aanwezigheid van de laagmoleculaire fenolachtige stof A is noodzakelijk voor de werking van het XDH Behalve L-glutamaat kunnen ook andere aminozuren en aminen als substraat gebruikt worden, dit is niet het geval met het GDH, dat alleen L-glutamaat kan desamineren Het is mogelijk reactie (2) uit te schakelen door het ruwe extract te dialyseren De werkelijke GDH desamineringsactiviteit, bepaald na dialyse van het extract, bedraagt slechts 4% van de waarde die zonder voorafgaande dialyse wordt gevonden

Het is gebleken dat de fenolachtige stof A in alle bovengrondse delen van

Petunia hybrida aanwezig is, behalve in de pollen. Dit houdt in dat het pollen XDH onwerkzaam is en niet met de GDH desamineringstest interfereert. Het mengen van pollen- en stijlextract veroorzaakt een activering van het pollen XDH door stof A uit de stijlen. Wordt nu de GDH desamineringstest gebruikt, dan wordt een schijnbare activering van het GDH gevonden.

De mogelijkheid dat stof A tijdens de progame fase door de pollenbuizen wordt opgenomen is niet uitgesloten, omdat vele laagmoleculaire stoffen uit de stijl worden opgenomen[13-15]. Opname van stof A door de pollenbuizen maakt het mogelijk dat het pollen XDH reacties catalyseert, die in pollen en in vitro gekiemde pollen niet plaats vinden. Het ontbreken van actief XDH in pollen, die in vitro zijn gekiemd, is misschien een van de oorzaken van het achterblijven van de pollenbuisgroei in vitro ten opzichte van de groei in vivo, waarbij stof A uit de stijlen kan worden opgenomen. Uit een aantal niet gepubliceerde gegevens betreffende pollenkieming in vitro blijkt, dat toevoeging van blad- of stijlextract aan het kiemmedium (10% sucrose, 0,01% boorzuur) de pollenkieming en de pollenbuisgroei bevordert, terwijl dit niet het geval is als pollenextract wordt toegevoegd. In een kiemmedium met blad- of stijlextract kan het pollen XDH geactiveerd worden door stof A uit respectievelijk bladeren of stijlen; in een medium met pollenextract is dit onmogelijk door het ontbreken van stof A.

1. De toename van de GDH desamineringsactiviteit, veroorzaakt door het mengen van pollen- en stijlextracten van Petunia hybrida, is geen activering van het GDH. Het is een activering van een ander dehydrogenase (XDH) dat met de GDH desamineringstest interfereert.
2. Dit storende dehydrogenase is alleen actief in de aanwezigheid van de laagmoleculaire fenolachtige stof A. Stof A komt niet voor in pollen, zodat het pollen XDH inactief is. Menging van pollen- en stijlextract veroorzaakt een activering van het pollen XDH door stof A uit de stijlen, zodat een schijnbare activering van de GDH desaminering wordt gevonden.
3. De GDH desamineringsactiviteit van een ruw extract kan pas bepaald worden als de storende reactie is uitgeschakeld m.b.v. dialyse.
4. De GDH amineringsactiviteit van ruwe extracten is voor een belangrijk deel latent. Deze latentie wordt veroorzaakt door het feit dat RNA, eiwitten, zetmeel, maltose, galactose en polyfenolen door waterstofbruggen en/of interactie van geïoniseerde groepen aan het GDH gehecht zijn.
5. Pollen GDH, gezuiverd m.b.v. ammoniumsulfaat precipitatie, gel filtratie en kolomchromatografie, wordt geactiveerd door incubatie met RNase, maltase, galactose oxidase, zure- en basische fosfatase.
6. De activering van het stijl GDH tijdens de progame fase wordt vermoedelijk veroorzaakt door hydrolyse van stoffen die de GDH activiteit remmen. De hydrolyserende enzymen zijn afkomstig uit de pollenbuizen.

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* Deze artikelen maken deel uit van dit proefschrift.

STELLINGEN

1. De desamineringsactiviteit van stijl glutamaat dehydrogenase wordt niet geactiveerd door mengen van pollen- en stijlextract.

   H. P. J. R. Roggen, Acta Bot. Neerl. 16, 1-31 (1967). Dit proefschrift.

2. De spectrofotometrische test van de glutamaat dehydrogenase desamineringsactiviteit, gebaseerd op de reductie van NAD tot NADH, is niet toepasbaar op ruwe plantaardige extracten.

   Dit proefschrift.

3. De amineringsactiviteit van pollen glutamaat dehydrogenase is grotendeels latent, omdat bepaalde stoffen complexen met dit enzym vormen.

   Dit proefschrift.

4. Het ontstaan van een nieuw incompatibiliteitsallel door inteelt van Lycopersicum peruvianum kan niet worden toegeschreven aan recombinatie binnen het incompatibiliteitslocus.

   K. K. Pandey, Nature 227, 689-690 (1970).

   D. de Nettancourt, R. Ecochard, M. D G. Perquin, T. van der Drift en M Westerhof, Theoret. Appl. Genetics, in press (1971).

5. Kwantitatieve veranderingen van biochemische aard kunnen slechts dan ter verklaring van fysiologische processen gebruikt worden, als rekening wordt gehouden met veranderingen in de referentiewaarden.

   R. Bachofen en H. Neeracher, Arch. Mikrobiol. 60, 235-245 (1968).

   G. M. M Bredemeijer en W. Heinen, Acta Bot. Neerl. 17, 15-25 (1968).

6. Het is onwaarschijnlijk dat incubatie van Drosophila speekselklieren met het vervellingshormoon ecdyson een afname van een bepaalde histonfractie veroorzaakt.

   L. H. Cohen en B. V. Gotchel, Fed. Proc. 28, 600 (1969).

   P. J. Helmsing, persoonlijke mededeling.

7. Het voorkomen van metabolisch DNA in Chlorella is niet bewezen.

T. Iwamura, Proc. nucl. Acid Res. molec. Biol. 5, 133-155 (1966).

F. Wanka, H. F. P. Joosten en W. J. de Grip, Arch. Mikrobiol. 75, 25-36 (1970).

8. De afbraak van gezuiverd cutine in een groeimedium met bodembacteriën, stemt niet overeen met de afbraak van cutine door bacteriën onder natuurlijke omstandigheden.

W. Heinen en H. de Vries, Arch. Mikrobiol. 54, 331-338 (1966).

H. de Vries, G. Bredemeijer en W. Heinen, Acta Bot. Neerl. 16, 102-110 (1967).

9. Bij het onderzoek van allergieën voor huisstof componenten wordt te weinig aandacht geschonken aan effecten van de algen, die in het huisstof voorkomen.

L. Berrens, Progr. Allergy 14, 259-339 (1970).

I. L. Bernstein en R. S. Safferman, Nature 227, 851-852 (1970).

10. De wetenschap is opgebouwd uit dwalingen, maar uit zulke dwalingen, waarvan het goed is die te begaan, want zij voeren langzamerhand tot de waarheid.

Jules Verne, Voyage au centre de la terre (1864).

7 mei 1971 G. M. M. Bredemeijer