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 × 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 × 2.5 cm) of the substances precipitated between 35 and 65 % saturation with ammonium sulphate, and chromatography on a DEAE cellulose column (8 cm × 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.

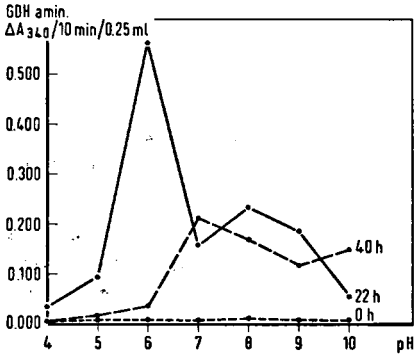
2.7. Incubation of purified GDH with maltose, starch, RNA and phytate

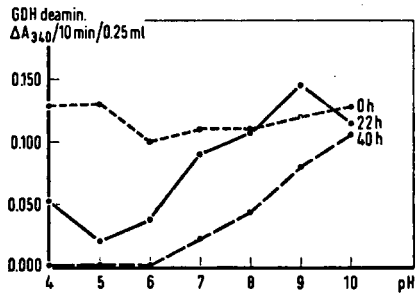
The incubation mixtures containing 1.0 ml of the fraction with purified GDH (see section 2.3), x ml maltose, starch, RNA (Sigma) or sodium phytate (Sigma) solution in 0.02 M TRA buffer, pH 7.8, (0 – 0.5 mg/ml), and 1 – x ml TRA buffer were incubated at 2 °C.

2.8. Assays

The determination of the GDH activity, the calculation of the number of milliunits 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.





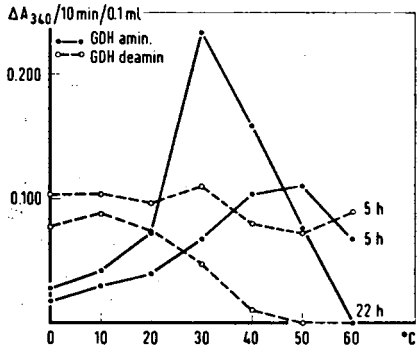


Fig. 1. GDH amination activity of pollen extract after different intervals of storage at different pH values at 30 °C.
Fig. 2. GDH deamination activity of pollen extract after different intervals of storage at different pH values at 30 °C.
Fig. 3. GDH amination and deamination activity of pollen extract 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.

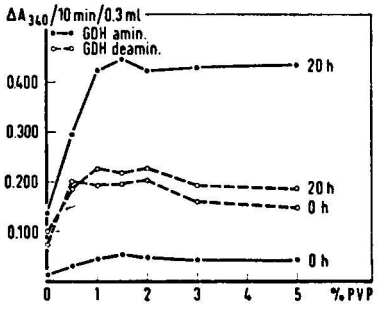


Fig. 4. GDH amination and deamination activity after 0 and 20 hrs of storage at pH 7.8 at 30 °C of extracts prepared by homogenizing portions of 60 mg pollen with an increasing percentage of PVP (w/v) in the extraction buffer.

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-

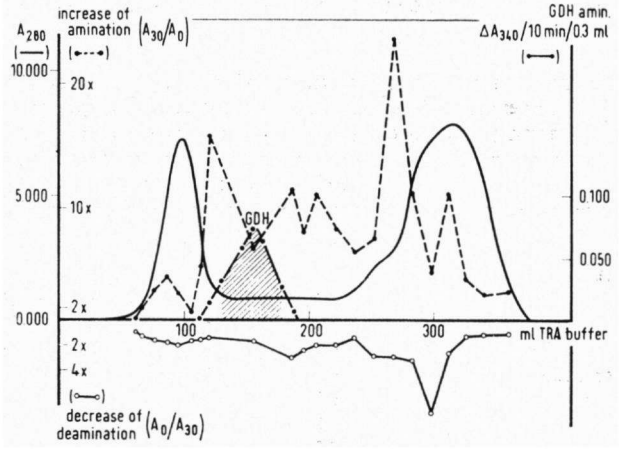


Fig. 5. Fractionation of pollen extract on a Sephadex G 200 column. The GDH containing fractions in the shaded area were pooled and divided into several portions which were incubated with samples of the other fractions during 15 hrs at pH 6.0 at 30 °C. The increase of the amination caused by the different 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/10 min	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

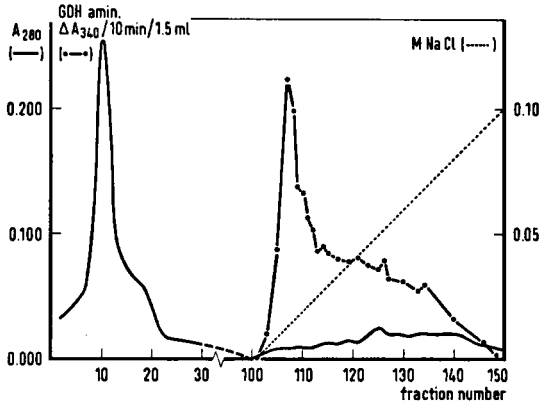


Fig. 6. Purification of pollen GDH obtained from a Sephadex G 200 column on a DEAE cellulose column. Elution was carried out with successively 0.02 M TRA buffer, pH 7.8, and the same buffer containing a linear salt gradient of 0–0.1 M NaCl. Fractions of 8 ml were collected.

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.

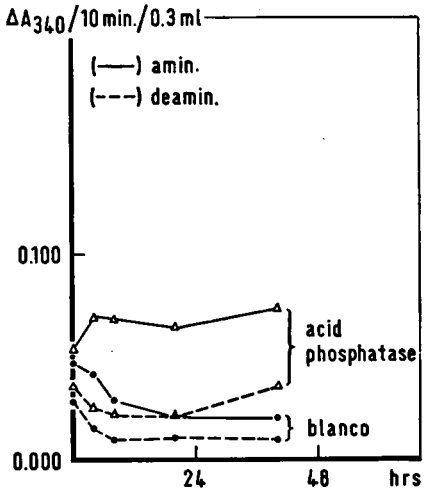
The last step of the purification, chromatography on a DEAE cellulose column, is shown in *fig. 6*. The GDH is not eluted as a homogeneous fraction; the main peak has a large tail. If the linear salt gradient was replaced by a stepwise gradient of 0–0.1 M NaCl in steps of 0.01 M, 6 GDH peaks with different 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.

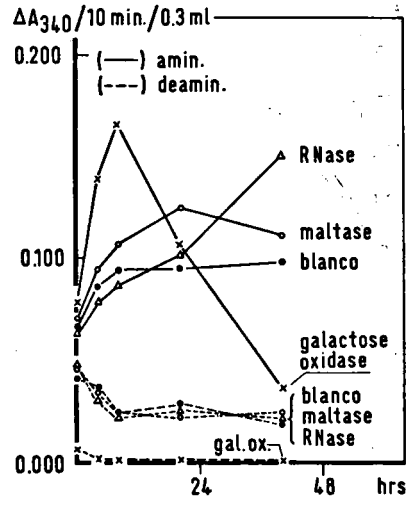
3.5. The influence of hydrolytic enzymes on purified GDH

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.





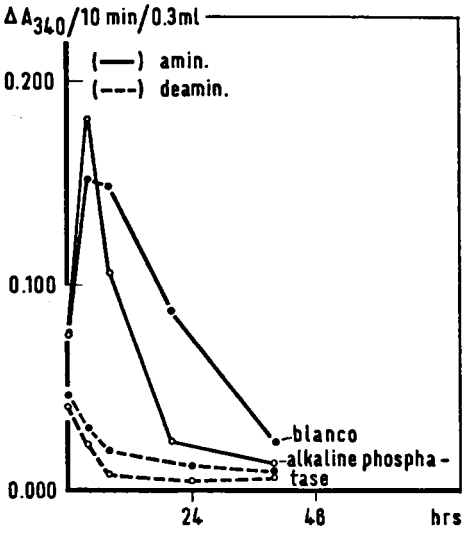


Fig. 7. GDH amination and deamination activity after incubation of purified GDH with acid phosphatase at pH 4.8. The control contained distilled water instead of acid phosphatase.

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

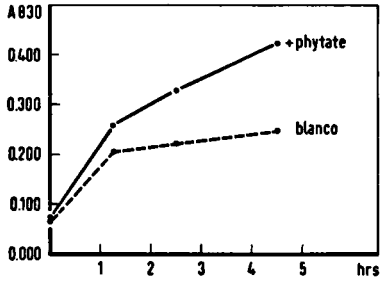


Fig. 10. The amount of orthophosphate liberated during the incubation of pollen extract at 55 °C at pH 5.15 with or without sodium phytate, expressed as absorbance at 830 nm.

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).

ACKNOWLEDGEMENTS

I wish to thank Mr. B. Weijers for technical assistance during the enzyme purification, Mr. W. Flokstra for supplying the plant material, and Dr. Ir. G. W. M. Barendse for reading the English text.

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