GLUTAMATE DEHYDROGENASE IN STYLES AND POLLEN OF PETUNIA HYBRIDA

G. M. M. BREDEMEIJER

Botanisch Laboratorium, Universiteit, Nijmegen.

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/cm²/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/cm²/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-

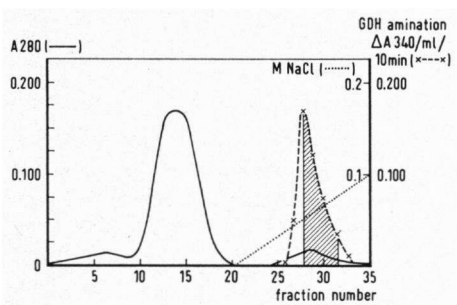

Fig. 1. Purification of the GDH obtained from the Sephadex G 200 column on a DEAE cellulose column. The fractions in the shaded area were used for the combination with pollen extract.

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

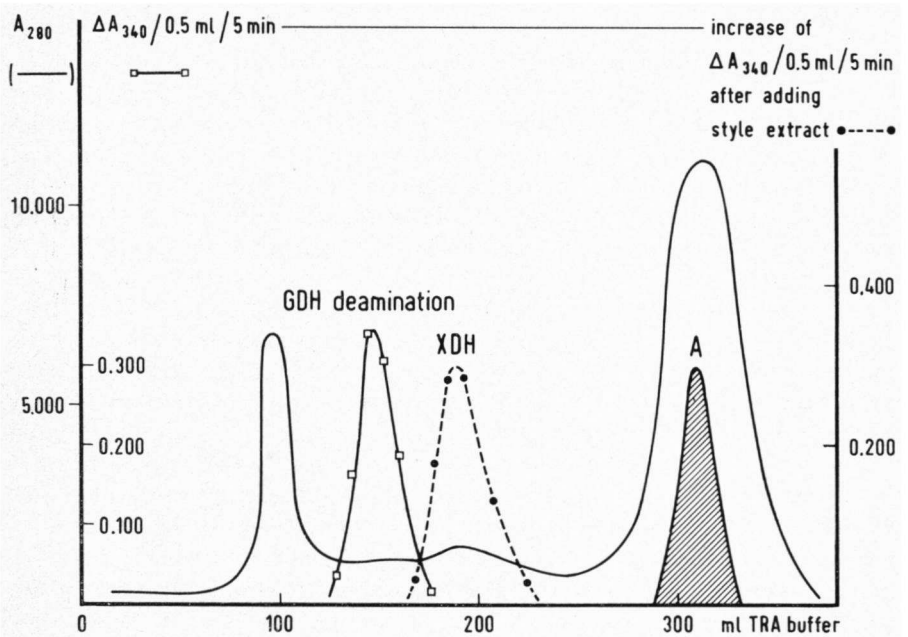

Fig. 2. Fractionation of pollen extract on a Sephadex G 200 column. The GDH, XDH and substance A were detected with the GDH deamination assay. GDH assay: 0.5 ml fraction. XDH assay: 0.5 ml fraction + 0.2 ml supernatant of the style extract obtained after 100% saturation with ammonium sulphate. Peak A 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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REFERENCES

BERGMEYER, H. A. (1962): *Methoden der enzymatischen Analyse:* 32–34. Verlag Chemie, GmbH. Weinheim.

BREDEMEIJER, G. M. M. (1970): Glutamate dehydrogenase activity in crude plant extracts. *Acta Bot. Neerl.* **19**: 445-453.

BULEN, W. A. (1956): The isolation and characterization of glutamic dehydrogenase from corn leaves. *Arch. Biochem. Biophys.* **62**: 173–183.

JOY, K. W. (1969): Nitrogen metabolism of Lemna minor. II. Enzymes of nitrate assimilation and some aspects of their regulation. *Plant Physiol.* **44**: 849–853.

LINSKENS, H. F. & K. ESSER (1959): Stoffaufnahme der Pollenschläuche aus dem Leitgewebe des Griffels. *Proc. Kon. Nederl. Akad. Wet. (Amsterdam)* **C. 62**: 150–154.

LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR & R. J. RANDALL (1961): Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193**: 265–275.

ROGGEN, H. P. J. R. (1967): Changes in enzyme activities during the progame phase in Petunia hybrida. *Acta Bot. Neerl.* **16**: 1–31.

SCHLÖSSER, K. (1961): Cytologische und cytochemische Untersuchungen über das Pollenschlauch-Wachstum selbststeriler Petunien. *Z. Bot.* **49**: 266–288.

SCHOCH-BODMER, H. & P. HUBER (1954): Auflösung und Aufnahme von Leitgewebe-Substanz durch die Pollenschläuche. *Verh. Schweiz. Naturforsch. Ges. (Zürich)* **125**: 161–162.

— & — (1947): Ernährung der Pollenschläuche durch das Leitgewebe. *Viertelj.Schr. Naturforsch. Ges. (Zürich)* **92**: 43–48.

STANLEY, R. G. (1958): Methods and concepts applied to a study of flowering in pine. The physiology of Forest Trees. *Symposium Harvard Forest* 1957, 583–599. Ronald Press: New York.

— & H. F. LINSKENS (1964): Enzyme activation in germinating Petunia pollen. *Nature (Lond.)* **203**: 542–544.

YAKOVLEVA, V. I., V. L. KRETOVICH & V. P. GORETOV (1966): Purification and properties of plant glutamate dehydrogenase. *Biokhimiya* **31**: 887–892.