Volume 330, number 1, 36–40 FEBS 12926 September 1993

Purification of an UDP-glucose:flavone, 7-*O*-glucosyltransferase, from *Silene latifolia* using a specific interaction between the enzyme and phenyl–Sepharose

P. Vellekoop*, L. Lugones, J. van Brederode

University of Utrecht, Department of Plant Ecology and Evolutionary Biology section Genome Evolution, Padualaan 8, 3584 CH Utrecht, The Netherlands

Received 18 May 1993; revised version received 23 June 1993

An UDP-glucose:flavonoid, 7-*O*-glucosyltransferase, from *Silene latifolia* was isolated from petals and purified 450-fold using a combination of gel-filtration, affinity chromatography and anion-exchange chromatography. Affinity chromatography on a phenyl-Sepharose CL-4B column in combination with elution with the substrate, isovitexin (6-*C*-glucosylapigenin), was an especially effective purification step. A purification factor between 10 and 20 could be reached using this column. A possible mechanism for the specific interaction of the enzyme with the phenyl–Sepharose will be discussed. This method of purification may also be applicable to other enzymes which use aromatic compounds as substrates. On a SDS-PAGE gel a band of 54 kDa, which co-purified with enzyme activity, could be detected in the purest fraction.

Enzyme purification; Glucosyltransferase; Phenyl–Sepharose, *Silene*, Flavonoid; Affinity chromatography

1. INTRODUCTION

Flavonoids comprise a major group among plant secondary metabolites and they play important roles in interactions with the environment [1–4]. An important group of enzymes involved in the modification of flavonoids are the glycosyltransferases. The reactions which are catalyzed by these enzymes have been widely studied [5–10].

In *Silene latifolia*, a large amount of variation is present in glycosyltransferases involved in the glycosylation of the flavone, isovitexin (6-*C*-glucosylapigenin). This variation is apparent in the substrate specificity, the distribution in the plant and the time of expression during ontogeny. Six different loci (g, g1, f, O^{7G}, d^{6A} and x^{gal}) with a total of eleven alleles have so far been identified [10–13]. The loci g, g1 and f, are expressed in the petals and the vegetative parts of the plant, whereas the other loci are expressed in the vegetative parts only. Plants which are homozygous for the recessive alleles of the loci g, g1 and f, are unable to glycosylate isovitexin in the petals. These plants show an aberrant morphology due to the negative effects of the aglycone isovitexin [14].

Five different alleles have been identified for the g locus, which codes for allozymes that control the glycosylation of the 7-OH group of isovitexin: g, gX, gX′, gGm and gGd [11]. These 7-*O*-glycosyltransferases differ in substrate specificity with respect to both the sugar donor (UDP-glucose or UDP-xylose) and the flavone acceptor (isovitexin or isovitexin-2″-*O*-glycoside). The alleles gX and gX′ code for allozymes which both transfer xylose to the 7-OH group of isovitexin or its 2″-*O*-glycosides, but which differ in K_m. The allozyme encoded by gGm transfers glucose to the 7-OH group of isovitexin, but not to the 7-OH group of isovitexin-2″-*O*-rhamnoside, whereas the allozyme encoded by gGd transfers glucose only to the 7-OH group of isovitexin-2″-*O*-rhamnoside but not to the 7-OH group of isovitexin [11]. In order to elucidate the cause of these differences in substrate specificity of these closely related allozymes, it is necessary to unravel the molecular mechanisms which underlie these differences.

As a first step in the isolation of the DNA sequence for the most common allele of the g locus (gGm), the isovitexin-7-*O*-glucosyltransferase has been purified in order to raise monospecific antiserum against it. In the past purification of the enzyme was unsuccessful because of its lack of stability and loss of activity. In this paper we describe a method for the purification of the enzyme, which uses a very specific interaction between the enzyme and phenyl–Sepharose.

Future efforts will be directed at the isolation of the DNA sequences of the gGm allele from a λgt11 cDNA expression library using antiserum raised against the purest protein fraction.

*Corresponding author.

Abbreviations EG, ethylene-glycol; EGME, ethylene-glycol monoethyl ether; UDPG, uridine diphosphate glucose; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; TCA, trichloro acetic acid.

Published by Elsevier Science Publishers B.V.

2. MATERIALS AND METHODS

2.1. *Plant materials*

Silene latifolia plants homozygous for the dominant allele, gGm, of the g locus, and homozygous for the recessive alleles of the two other loci (f and g1) which control glycosylation of isovitexin in the petals, were grown in greenhouses during the winter and in the field during the summer. Petals were harvested as soon as the plants flowered. The petals were frozen and stored at −20°C or used directly for the isolation of the 7-*O*-glucosyltransferase.

2.2. *Chemicals and column materials*

UDP-[U-^{14}C]glucose (355 mCi/mmol) was obtained from Amersham. Sephacryl S-300 superfine and phenyl-Sepharose CL-4B were obtained from Pharmacia. Cartridges for anion-exchange chromatography (Econo-Pac Q), for hydrophobic interaction chromatography (Econo-Pac HIC), and for buffer exchange (Econo-Pac P6), were obtained from Bio-Rad. Isovitexin (6-*C*-glucosylapigenin) was isolated from *Silene* [11].

2.3. *Enzyme purification*

2.3.1. Homogenization

All purification steps were carried out at 0–4°C. For the isolation of the 7-*O*-glucosyltransferase, 30 g of petals was homogenized in 150 ml of extraction buffer (20 mM mercaptoethanol, 0.1 mM PMSF, 0.1% Triton X-100, 5% PVP (M_w 44,000), 2 mM DTT in 50 mM sodium/potassium phosphate buffer pH 6.8) using an Ultra Turrax apparatus (Janke & Kunkel). The homogenate was centrifuged at 12,000 × *g* for 10 min at 4°C. Ammonium sulphate was added to the supernatant to a final concentration of 36%. After centrifugation at 17,000 × *g* for 30 min at 4°C, ammonium sulphate was added to the supernatant to a final concentration of 62%. Following centrifugation as described above, the supernatant was discarded and the pellet was resuspended in 5 ml ice-cold phosphate buffer (4 mM mercaptoethanol, 1 mM PMSF, 2 mM DTT in 10 mM sodium/potassium phosphate buffer, pH 6.8). An Econo-Pac P6 cartridge was used for desalting samples.

2.3.2. Gel filtration

The resuspended ammonium sulphate precipitate mentioned above was applied to a Sephacryl S-300 column (2.5 × 78 cm). Proteins were eluted overnight with elution buffer (4 mM mercaptoethanol, 0.1% PMSF, 2 mM DTT in 10 mM citrate buffer, pH 6.0). The flow rate was 0.5 ml/min and 5 ml fractions were collected.

2.3.3. Hydrophobic interaction chromatography

Two different resins were used for hydrophobic interaction chromatography, one with an alliphatic sidegroup (Econopac-HIC containing a methyl group) and one with an aromatic sidegroup (phenyl–Sepharose CL-4B containing a phenyl group).

An Econo-Pac HIC cartridge was equilibrated with phosphate buffer. Protein samples were loaded in phosphate buffer with 0.7 M ammonium sulphate. The eluent was collected and used in subsequent purification steps.

The sample was applied in citrate buffer on a phenyl–Sepharose CL-4B column (1 × 7 cm) equilibrated with the same buffer. The column was washed with 2 vols. of 20% ethylene glycol in citrate buffer. The enzyme was eluted with an ethylene glycol gradient (20–75% ethylene glycol in citrate buffer). The flow rate was 0.5 ml/min and 2 ml fractions were collected.

2.3.4. Affinity chromatography

Another smaller phenyl–Sepharose CL-4B column (1.5 × 2 cm) was used for elution with isovitexin. The sample containing the 7-*O*-glucosyltransferase was applied in citrate buffer with 25% ethylene glycol. The enzyme was eluted with 0.02% isovitexin in citrate buffer. The flow rate was 0.3 ml/min and 1 ml fractions were collected.

2.3.5. Anion-exchange chromatography

An Econo-Pac Q cartridge was used for anion-exchange chromatography. The cartridge was equilibrated in phosphate buffer and the sample was subsequently loaded in the same buffer. The cartridge was washed with 6 vols. of 60 mM NaCl in phosphate buffer, followed by elution of the enzyme with 150 mM NaCl in phosphate buffer. The flow rate was 1–2 ml/min.

2.4. *Enzyme assay*

The enzyme activity was determined according to the following procedure. 25 μl enzyme extract in phosphate buffer (pH 7.0) was incubated with 2 μl 0.1% isovitexin in EGME and 2 μl UDP-[U-^{14}C]glucose at 30°C. After 30 min, 5 μl of cold TCA was added and the incubation mix was subsequently extracted with 150 μl iso-amyl alcohol. Following centrifugation in an Eppendorf centrifuge, 125 μl of the iso-amyl alcohol fraction was washed with 50 μl distilled water in the same way. To 100 μl of the washed iso-amyl alcohol fraction 3 ml of Lipoluma (Lumac) was added and the radioactivity of the samples was counted in a scintillation counter. The identity of the reaction product was established by two-dimensional chromatography on Whatmann 3MM paper and subsequent identification of the product in UV-light using the different isovitexin glycosides as controls [11].

2.5. *Protein assay*

Total protein content of samples was measured according to Bradford et al. [15] with bovine serum albumin (BSA) as a standard. The quantity of protein in the purest fractions was determined on silver-stained SDS-PAGE gels with a densitometer (Hitachi). A concentration range of BSA (50–300 μg) was used as a standard for calibration.

2.6. *SDS-PAGE*

SDS-PAGE was performed with a Mini Protean II apparatus from Bio-Rad according to Laemmli [16]. The separation gel consisted of a running gel containing 10% acrylamide-Bis (pH 8.8), and a stacking gel which contained 4% acrylamide-Bis (pH 6.8). The running buffer consisted of 0.025 M Tris-HCl, 0.2 M glycine and 0.1% SDS. Before the samples were loaded they were mixed with an equal volume of sample buffer (0.25 M Tris-HCl, pH 6.8, 20% glycerol, 0.002% Bromophenol blue, 10% mercaptoethanol, 4% SDS) and boiled for 3 min. Protein samples that were too dilute were first concentrated using a SpeedVac (Savant SVC100H) concentrator. The gels were run for 45 min at 40 mA per gel. Silver staining was done according to Oakley et al. [17] with omission of the glutaraldehyde step.

3. RESULTS AND DISCUSSION

The 7-*O*-glucosyltransferase has been isolated from plants of the genotype gGm/gGm f/f g1/g1. They are homozygous for the dominant allele of the g locus (gGm) and homozygous for the recessive alleles of the other two loci (f and g1). Because these three loci are the only loci which are expressed in the petals, the 7-*O*-glucosyltransferase is the only enzyme in the petals of these plants which can glycosylate isovitexin. The only reaction product which can be formed during the activity test is the isovitexin-7-*O*-glucoside. The identity of the reaction product was established by two-dimensional paper chromatography [11].

After ammonium sulphate precipitation, a Sephacryl S-300 column was used to fractionate the proteins. The use of the Sephacryl column resulted in a 2- to 4-fold increase in specific activity and the removal of all the flavonoids (Fig. 1). However, the decrease in overall

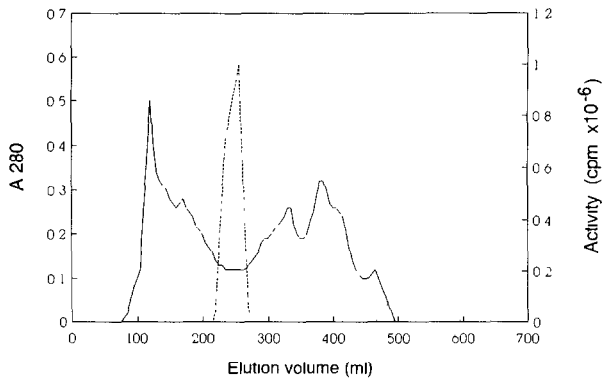

Fig. 1. Isovitexin-7-*O*-glucosyltransferase elution pattern on a Sephacryl S-300 column after application of re-dissolved ammonium sulphate precipitate to the column. (——) Protein; (---) glucosyltransferase activity; (··) flavonoids.

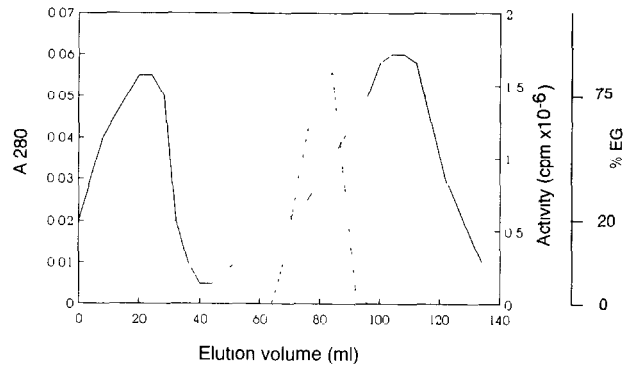

Fig. 2. Isovitexin-7-*O*-glucosyltransferase elution pattern on a phenyl–Sepharose CL-4B column with an ethylene glycol gradient The sample applied to the column consisted of pooled isovitexin-7-*O*-glucosyltransferase fractions from a Sephacryl S-300 column (——) Protein; (---) glucosyltransferase activity; (·) ethylene glycol

activity was substantial (Table I). Alternatively, a buffer exchanger (Econo-Pac P6) was used to remove the salt from the ammonium sulphate precipitate. In this case there was no decrease in overall activity, but the flavonoids remained present in the protein sample.

For anion-exchange chromatography, an Econo-Pac Q cartridge was used in combination with a step-gradient of NaCl. The enzyme eluted at 150 mM NaCl. Just as was the case with the Sephacryl S-300, however, there was a substantial loss in activity. Flavonoids were effectively removed.

Several columns were used for hydrophobic interaction chromatography. On an Econo-Pac HIC cartridge the enzyme eluted at 700 mM ammonium sulphate; 50% of the other proteins remained bound to the column. The high salt concentration at which the enzyme eluted suggests that the enzyme is not very hydrophobic. However, on a phenyl–Sepharose CL-4B column the enzyme was tightly bound to the column in low salt conditions (10 mM citrate). When an ethylene glycol gradient was used, the enzyme eluted only at 54–57% ethylene glycol after binding to the column in citrate buffer (Fig. 2). When traces of flavonoids were present in the sample the enzyme was not bound at all. Together with the results obtained with the Econo-Pac HIC cartridge it can be concluded that the interaction between phenyl–Sepharose CL-4B and the enzyme is not a hydrophobic one, but of another nature, e.g. affinity binding. This is supported by the finding that the enzyme eluted when 0.02% isovitexin was added to the citrate buffer (Fig. 3A). This resulted in a very effective purification (10–20 times). The proteins present in the peak fractions of a phenyl–Sepharose CL-4B column in combination with elution with an ethylene glycol gradient, and the peak fractions of a subsequent phenyl–Sepharose CL-4B column in combination with elution with isovitexin, were compared on an SDS-PAGE gel. In earlier experiments the molecular weight of the native enzyme (ca. 55 kDa) had been determined on a Sephadex G-150 column [10]. From Fig. 4 it is clear that there is a marked increase

Table I

Purification of 7-*O*-glucosyltransferase from 30 g of petals from *Silene latifolia* using two different methods

Purification step	Protein (mg)	Activity (cpm)	Specific activity (cpm/mg protein)	Recovery (%)	Enrichment
1. Crude extract (pH 7.0)	240.02	3.83E + 7	1.60E + 5	100	1
2. Ammonium sulphate precipitation	69.83	2.91E + 7	4.17E + 5	76.30	2.60
Method I					
3.1. Sephacryl S-300	11 15	1.74E + 7	1.56E + 6	59.80	9.73
4.1. Phenyl–Sepharose (ethylene glycol)	0.91	5.01E + 6	5.49E + 6	17.22	34 31
5.1 Phenyl–Sepharose (isovitexin)	0.02	1.08E + 5	7.16E + 7	3 72	447.68
Method II					
3.2. Econo-Pac P6	69.83	2 91E + 7	4.17E + 5	76 30	2.60
4.2. Econo-Pac Q	7.90	1.02E + 7	1.29E + 6	26.54	8.04
5.2. Phenyl–Sepharose (ethylene glycol)	1.30	5.11E + 6	3.94E + 6	13.33	24 60
6.2 Phenyl–Sepharose (isovitexin)	0.04	2 18E + 6	5 46E + 7	5 70	341 25

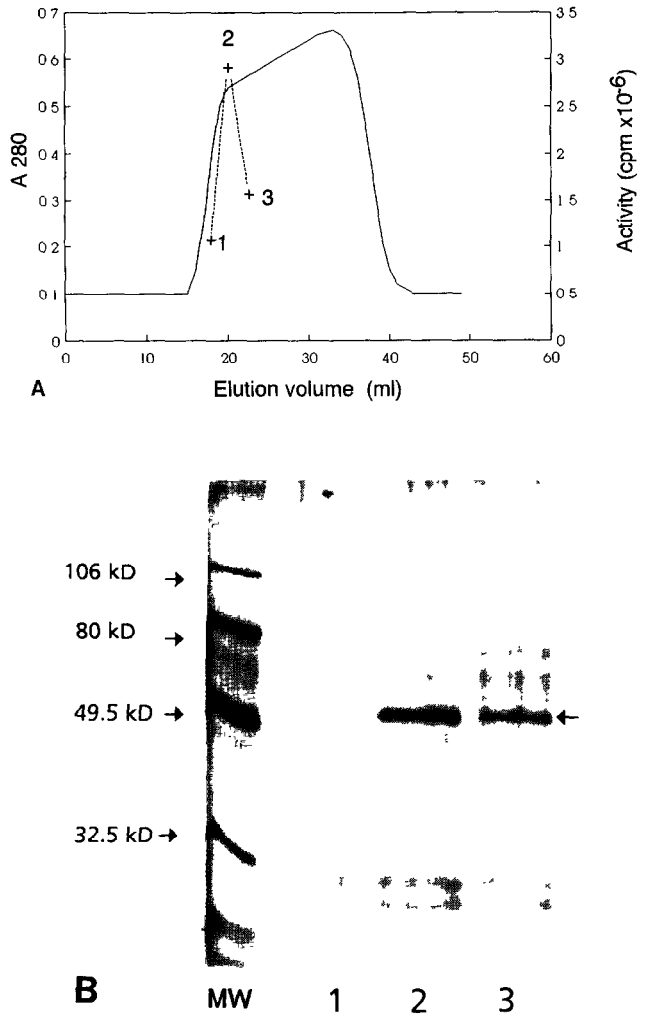

Fig. 3. (A) Isovitexin-7-*O*-glucosyltransferase elution pattern on a phenyl–Sepharose CL-4B column in combination with elution with 0.02% isovitexin. Isovitexin-7-*O*-glucosyltransferase fractions eluted from a phenyl–Sepharose CL-4B column with an ethylene glycol gradient were pooled, diluted with citrate buffer, and applied to the column. (——) Isovitexin; (---) glucosyltransferase activity. (B) SDS-PAGE gel of silver-stained proteins from the numbered fractions in (A). Fractions of 1 ml were collected and 200 μl per fraction was subsequently concentrated and applied to a mini-gel. The lane on the left (MW) contains molecular weight markers Lanes 1–3 correspond to fractions 1–3 in A. From the gel it can clearly be seen that a decrease in total 7-*O*-glucosyltransferase activity corresponds to a decrease in intensity of a 54 kDa protein band.

in the intensity of a 54 kDa band, as compared to other protein bands, which coincides with enzyme activity. As shown in Fig. 3B, fractions collected before and after elution of the peak with isovitexin showed a marked decrease in intensity of the 54 kDa band, which coincided with a marked decrease in specific activity of the fractions.

The behaviour of the 7-*O*-glucosyltransferase on the phenyl–Sepharose CL-4B suggests that the enzyme has a high affinity for the phenyl group of the phenyl-Sepharose CL-4B, perhaps because it resembles its aromatic substrate. Specific elution of the enzyme could be the result of competition between the aromatic substrate, isovitexin, and the phenyl group for the substrate binding site on the enzyme. Chromatography on a phenyl–Sepharose CL-4B column is thus more like affinity chromatography than hydrophobic interaction chromatography.

On the basis of the above mentioned results, two purification protocols for the 7-*O*-glucosyltransferase have been devised. A very important step in the purification procedure is the removal of the flavonoids, which interfere with the binding of the enzyme to the phenyl–Sepharose CL-4B. This was accomplished by using a gel filtration (Sephacryl S-300) column or an anion-exchanger (Econo-Pac Q) before a phenyl–Sepharose CL-4B column was used.

The highest purification of the 7-*O*-glucosyltransferase was obtained with the following procedure: ammonium sulphate precipitation, gel-filtration with Sephacryl S-300, and affinity chromatography using phenyl–Sepharose CL-4B, and elution with an ethylene glycol gradient and isovitexin, respectively (Table I). Although a high degree of purification could be obtained using phenyl–Sepharose CL-4B in combination with isovitexin as a last step, this fraction still contained contaminating proteins when analysed on SDS-PAGE gels. The quantity of protein in the purest fractions was too low to be determined with a Bradford [15] assay. The quantity was therefore determined on silver-stained SDS-PAGE gels with a densitometer. Although each protein may react differently onto silver staining, one may still get an indication of quantity when using a

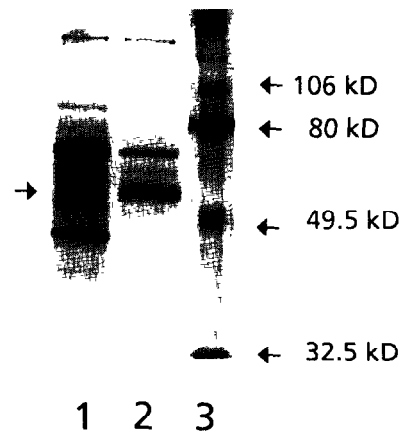

Fig. 4. SDS-PAGE gel of silver-stained proteins from fractions with the highest 7-*O*-glucosyltransferase activity after subsequent purification on two phenyl–Sepharose CL-4B columns. In lane 1 the protein content of the fractions after elution with an ethylene glycol gradient is shown. These fractions were pooled, diluted in citrate buffer, and applied to a second phenyl–Sepharose column In lane 2 the protein content of the fraction with the highest 7-*O*-glucosyltransferase activity after elution with isovitexin is shown. There is a very distinct increase in the relative intensity of a 54 kDa protein band which corresponds to the 7-*O*-glucosyltransferase. Lane 3 contains molecular weight markers.

suitable standard. BSA has a similar molecular weight as the enzyme and as such may be suitable. A range of concentrations (50–300 μg) was used for calibration. In this way, it could be shown that a total of about 20 μg enzyme in the purest fraction per 30 g of petals can be isolated. Before use as an antigen to immunize rabbits, the purest fractions will have to be run on an SDS-PAGE gel as a final purification step.

Because Econo-Pac columns can tolerate high flow rates, an alternative and shorter procedure is as follows (Table I): ammonium sulphate precipitation, Econo-Pac P6, Econo-Pac Q, phenyl–Sepharose CL-4B in combination with an ethylene glycol gradient, and phenyl–Sepharose CL-4B in combination with isovitexin. Although this resulted in a 2-fold higher yield of the enzyme, the purest fraction contained more contaminating proteins than in the first procedure, when analysed on an SDS-PAGE gel (Fig. 4, lane 2, and Fig. 3B, lane 2).

In all procedures there was a distinct discrepancy between the increase in specific activity and the decrease in total protein content. The increase in specific activity was relatively too low. It seems that the loss in total activity of the enzyme is not accompanied by loss of the enzyme protein, but the result of changes in higher order structures, such as defolding of the protein. The specific interaction between the 7-*O*-glucosyltransferase and the phenyl–Sepharose CL-4B, in combination with elution with its substrate, may also be applicable to other enzymes which use aromatic compounds as a substrate.

REFERENCES

[1] Heller, W and Forkmann, G. (1988) in: The Flavonoids: Advances in Research Since 1980 (Harborne, J.B Ed.) pp 399–425
[2] Stafford, H.A (1991) Plant Physiol. 96, 680–685.
[3] Lynn, D.G. and Chang, M. (1990) Annu. Rev Plant Physiol Plant Mol. Biol. 41, 497–526.
[4] Lamb, C.J., Lawton, M.A., Dron, M. and Dixon, R.A. (1989) Cell 56, 215–224
[5] Hoesel, W. (1981) in: The Biochemistry of Plants, vol 7 (Conn, E.E. and Stumpf, P.K. Ed.) pp 725–753, Academic Press, New York
[6] Latchinian-Sadek, L and Ibrahim, R.K. (1991) Arch Biochem. Biophys. 289, 230–236
[7] Ishikura, N and Yang, Z. (1991) Z. Naturforsch. 46c, 1003–1010
[8] Ishikura, N. and Yamamoto, K. (1990) Plant Cell Physiol. 31, 1109–1115.
[9] McIntosh, C.A., Latchinian, L and Mansell, R.L. (1990) Arch. Biochem Biophys 282, 50–57.
[10] van Brederode, J. and van Nigtevecht, G. (1973) Mol Gen. Genet. 122, 215–229
[11] Steyns, J.M. and van Brederode, J (1986) Biochem. Genet. 24, 349–367.
[12] van Brederode, J and Mastenbroek, O. (1983) Theor. Appl. Genet. 64, 151–153.
[13] van Nigtevecht, G. and van Brederode, J. (1990) Genetica 80, 67–79.
[14] van Brederode, J., Genderen, H.H. and Berendsen, W. (1982) Experientia 38, 929–931
[15] Bradford, M.M. (1976) Anal. Biochem. 72, 48–54.
[16] Laemmli, U.K. (1970) Nature 227, 680–685.
[17] Oakley, B.R., Kirsch, D R. and Morris, N.R. (1980) Anal. Biochem. 105, 361–363