Pergamon

PII: S0031-9422(98)00014-4

Phytochemistry, Vol. 49, No. 2, pp. 327–332, 1998

Printed in Great Britain
0031–9422/98/$–see front matter

GALLOTANNIN BIOSYNTHESIS: PURIFICATION OF β-GLUCOGALLIN: 1,2,3,4,6-PENTAGALLOYL-β-D-GLUCOSE GALLOYLTRANSFERASE FROM SUMAC LEAVES†

IN HONOUR OF PROFESSOR G. H. NEIL TOWERS 75TH BIRTHDAY

RUTH NIEMETZ and GEORG G. GROSS*

Universität Ulm, Abteilung Allgemeine Botanik, D-89069 Ulm, Germany

(*Received* 1 *October* 1997; *In revised form* 19 *November* 1997)

Key Word Index—*Rhus typhina*; Anacardiaceae; staghorn sumac; biosynthesis; tannins; galloyltransferase; β-glucogallin (1-*O*-galloyl-β-D-glucopyranose); 1,2,3,4,6-penta-*O*-galloyl-β-D-glucopyranose; hexagalloylglucose.

Abstract—An enzyme from leaves of staghorn sumac (*Rhus typhina*) that catalysed the galloylation of 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose to the gallotannin, 3-*O*-digalloyl-1,2,4,6-tetra-*O*-galloyl-β-D-glucose, was purified more than 500-fold to apparent homogeneity. β-Glucogallin (1-*O*-galloyl-β-D-glucopyranose) served as activated acyl donor in this conversion. For the native enzyme, a M_r value of 170,000 was determined by gel filtration, while a single polypeptide band of M_r 42,000 was detected by SDS-PAGE. The acyltransferase had pH and temperature optima of 4–4.5 and 25°, respectively, and was most stable between pH 3 and 4.5. Besides the major substrate, pentagalloylglucose, also 1,2,3,6-tetragalloylglucose and hexa- to nona-substituted gallotannins were accepted as minor substrates by this new enzyme for which the systematic name "β-glucogallin: 1,2,3,4,6-pentagalloyl-β-D-glucose (3-*O*-galloyl)-galloyltransferase" (EC 2.3.1.-) is proposed.

INTRODUCTION

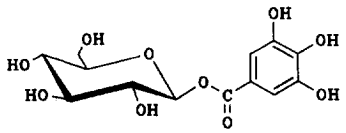
Enzyme studies with extracts from sumac (*Rhus typhina*) and oak have shown that 1,2,3,4,6-pentagalloylglucose **3**, the presumed immediate precursor of the two subclasses of hydrolysable tannins, i.e. gallotannins and ellagitannins, is formed by a series of consecutive and highly position-specific galloylation steps in which β-glucogallin (1-*O*-galloyl-β-D-glucose) **1** is not only the first intermediate but is required also as the principal activated acyl donor (cf [1–3]). With crude extracts from sumac leaves it has been demonstrated that this mechanism applies also to the subsequent conversion of pentagalloylglucose to more complex gallotannins [4]. These polyphenolic compounds are characterised by additional galloyl residues which are attached to the pentagalloylglucose core via *meta*-depside linkages (cf **2** in the structure scheme). The sequential synthesis of a variety of differently substituted oligomeric gallotannins, leading to nonagalloylglucoses, and most likely even to higher substituted derivatives, has been proven in this investigation. On the basis of the different reaction rates observed for these conversions, a preliminary scheme for the preferred pathways in the biosynthesis of gallotannins has been proposed [3]. It remained completely unknown, however, whether the individual reactions of this metabolic routes were catalysed by specific enzymes, or whether one or only few unspecific galloyltransferases were involved in these pathways. As reported below, recent studies on this question led to the isolation of a new galloyltransferase that catalysed the prevalent formation of a hexagalloylglucose, 3-*O*-digalloyl-1,2,4,6-tetra-*O*-galloyl-β-D-glucose **4**.

RESULTS AND DISCUSSION

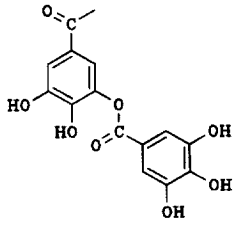
Enzyme purification

The enzyme was extracted from homogenates of sumac leaves in the presence of PVP and borate which both are known to bind inhibitory endogenous phenolics. For the same purpose, the resulting crude extract was stirred with Dowex, followed by treatment with protamine sulphate to remove residual acidic

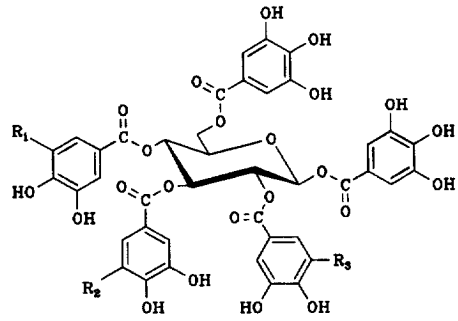
† In honour of Professor G. H. Neil Towers' 75th birthday.
* Author to whom correspondence should be addressed.



(1)



(2)



(3) R_1, R_2, R_3 = H
(4) R_1, R_3 = H, R_2 = galloyl
(5) R_1, R_2 = H, R_3 = galloyl
(6) R_2, R_3 = H, R_1 = galloyl
(7) R_1, R_3 = H, R_2 = m-digalloyl (2)
(8) R_1 = H, R_2, R_3 = galloyl
(9) R_2 = H, R_1, R_3 = galloyl
(10) R_3 = H, R_1, R_2 = galloyl

components. After a subsequent ammonium sulphate precipitation step, the enzyme was subjected to gel-filtration on Sephacryl S-300. As shown in Fig. 1A, this step revealed the existence of several galloyltransferases which all were characterised by comparatively high M_r values. The first three peaks among these were particularly active in converting pentagalloylglucose to hepta- and octagalloylglucoses as main products, in contrast to the last fraction which preferentially catalysed the formation of hexagalloylglucose. This latter fraction was rechromatographed under identical conditions, thus affording the efficient separation of the galloyltransferase from a contaminating esterase of slightly lower M_r which is known to actively degrade tannins [5]. The significant increase in measurable total enzyme activity after this step (cf Table 1) was attributed to the depletion of the enzyme solution of this counteractive protein. The most active fractions from the second gel-chromatography step (Fig. 1B) were combined and subjected to ion-exchange chromatography on DEAE cellulose, followed by hydrophobic interaction chromatography on butyl-Sepharose. After this final step the galloyltransferase had been purified more than 500-fold in 2% yield to apparent homogeneity as shown by PAGE under denaturing and non-denaturing conditions. The results of a representative purification experiment are summarised in Table 1. The pure enzyme could be stored at 0–4° for several weeks without significant loss of catalytic activity.

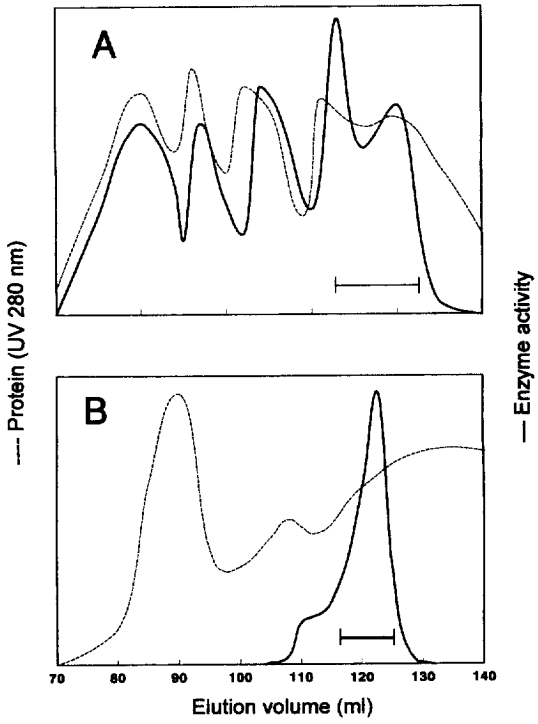


Fig. 1. Separation of β-glucogallin: pentagalloylglucose galloyltransferases by gel-filtration on Sephacryl S-300. **A**, first chromatography step; **B**, rechromatography. (– – –), UV absorption at 280 nm; (——), enzyme activity (both arbitrary units). Fractions were pooled as indicated by the inserted bars. For further details, see text.

General properties of the enzyme

Under standard assay conditions (see Experimental), the transferase reaction was linear with respect to protein concentration for about 16 μg of protein per assay, except for a pronounced "lag phase" at 0–5 μg protein. No reaction occurred with enzyme previously denatured by heat or acid. Linearity of the reaction was maintained for ca 2 h under these conditions. The enzyme was inactive below pH 2 and above pH 6.2, with a maximum at pH 4–4.5

Table 1. Purification of galloyltransferase from leaf extracts of *Rhus typhina**

Step	Total protein (mg)	Total activity (nkat)	Specific activity (nkat mg^{-1})	Purification (-fold)	Recovery (%)
Dowex filtrate	6010	513	0.085	—	100
Protamine sulfate, supernatant	4590	281	0.061	0.7	55
$(NH_4)_2SO_4$, 35–70% ppt.	178	77	0.43	5.1	15
1st Sephacryl S-300	16.8	45	2.68	31.5	9
2nd Sephacryl S-300†	1.6	235	147	1728	46
DEAE cellulose	0.90	35	38.9	458	7
Butyl-Sepharose‡	0.26	11.4	43.9	517	2

* Crude extracts were not assayed because of excess contaminants preventing HPLC analysis of reaction products.
† Enzyme from this step was used to determine its general properties.
‡ Substrate specificities and K_m values were determined with the pure enzyme.

(citrate buffer); half-maximal activities were at pH 3.2 and 5.2. After preincubation at 30° for 3 or 24 h, the transferase was found to be most stable between pH 3 and 4.5 but was rapidly inactivated above pH 6 and below pH 3. The temperature optimum of the reaction was at 25° (half-maximal activities were at 10° and 50°). The enzyme was found to be very heat labile. After preincubation for 2 h at various temperatures, it was stable only at 0–10° while it had already lost 80% of activity at 20°; complete inactivation occurred at 45° under these conditions. On the other hand, the transferase exhibited a relative enzymatic activtity of 20% even at 0°; similar cold-tolerances have been reported for galloylglucose-synthesising enzymes from oak or sumac leaves [6–8]. Between 10° and 20°, an average activation energy of 34 kJ mol^{-1} was calculated which corresponds to a Q_{10} value of 1.7. From gel-filtration experiments with a calibrated Sephacryl S-300 column [9], an apparent M_r of 170,000 was estimated for the enzyme. After denaturing SDS-PAGE the existence of only one single polypeptide band of M_r 42,000 was observed, indicating that the native enzyme existed as a tetramer of apparently four identical subunits.

Substrate specificity

The galloyltransferase exhibited normal Michaelis-Menten kinetics with the acyl donor β-glucogallin up to a maximal concentration of 4.8 mM; increasing substrate inhibition occurred above this value. The affinity of the enzyme towards a wide array of putative acceptor substrates was determined under standard assay conditions. As summarised in Table 2, the transferase was most active with 1,2,3,4,6-pentagalloylglucose **3**. The enzyme exhibited fair affinity towards 1,2,3,6-tetragalloylglucose and hexagalloylglucoses, and was still moderately active in the presence of hepta- and octagalloylglucoses. Trace activities were observed with nonagalloylglucoses as well as with β-glucogallin, 1,6-di- and 1,2,6-trigalloylglucose. No reaction occurred in the presence of α-glucogallin, 2-mono-, 6-mono-, 3,6-di-, 1,3,6-tri- and 1,2,4,6-tetragalloylglucose, i.e. compounds which do not belong to the biosynthetic route from gallic acid to gallotannins in *Rhus*.

Reaction products

Normal-phase HPLC of standard enzyme assays on silica gel revealed that the substrate, 1,2,3,4,6-pentagalloylglucose **3**, had been converted to hexa-, hepta- and octagalloylglucoses in a ratio of 1:0.3:0.03 (Fig. 2A). Closer analyses by reversed-phase HPLC (Fig. 2B) showed that 3-*O*-digalloyl-1,2,4,6-tetra-*O*-galloyl-β-D-glucose **4** was the predominant hexagalloylglucose, while hexagalloylglucoses **5** and **6** were formed in ca 50% lower concentrations. Among the heptagalloylglucose reaction products, approximately equal amounts of **7**–**9** were determined, in contrast to **10** which was found, like *in vivo* [10], to occur in only minute quantities (ratio ca 1:0.25). Further experiments in which the standard substrate, pentagalloylglucose, had been replaced by various hexagalloylgucoses revealed that **4**, the main product formed from pentagalloylglucose in the preceding step, was exclusively acylated to the heptagalloylglucose, 3-*O*-trigalloyl-1,2,4,6-tetra-*O*-galloyl-β-D-glucose **7**. In contrast, the side-products of the preceding step, i.e. hexagalloylglucoses **5** and **6**, preferentially yielded heptagalloylglucoses **8** and **9**. It is evident from these results that the galloyltransferase described here is specific in acylating 1,2,3,4,6-pentagalloylglucose in the 3-*O*-position to hexa- and heptagalloylgucoses, justifying the systematic name "β-glucogallin: 1,2,3,4,6-pentagalloyl-β-D-glucose (3-*O*-galloyl)-galloyltransferase" (EC 2.3.1.-) for this new enzyme.

EXPERIMENTAL

Chemicals

Chemical methods were employed for the synthesis of β-glucogallin **1** [11] and 1,2,3,4,6-pentagalloylglucose **3** [12]. α-Glucogallin, 2-*O*-galloylglucose, 6-*O*-galloylglucose and 3,6-di-*O*-gal-

Table 2. Acceptor substrate specificity of sumac leaf galloyltransferase

Substrate	Relative activity* (%)	v_{max} (nkat)	K_m (μM)	$v_{max}K_m^{-1}$ ($\times 10^{-3}$)
1-*O*-Galloyl-α-D-glucose	0	—	—	—
1-*O*-Galloyl-β-D-glucose (β-glucogallin) (**1**)	~0	18.3	3200	6
2-*O*-Galloyl-β-D-glucose	0	—	—	—
6-*O*-Galloyl-β-D-glucose	0	—	—	—
1,6-Di-*O*-galloyl-β-D-glucose	~0	6.9	650	11
3,6-Di-*O*-galloyl-β-D-glucose	0	—	—	—
1,2,6-Tri-*O*-galloyl-β-D-glucose	~0	6.8	500	14
1,3,6-Tri-*O*-galloyl-β-D-glucose	0	—	—	—
1,2,3,6-Tetra-*O*-galloyl-β-D-glucose	79	3.9	85	752
1,2,4,6-Tetra-*O*-galloyl-β-D-glucose	0	—	—	—
1,2,3,4,6-Penta-*O*-galloyl-β-D-glucose (**3**)	100	34.2	30	1141
Hexagalloyl-β-D-glucose (**4**)	51	32.3	75	431
Hexagalloyl-β-D-glucose (**5**)	81	41.8	90	464
Hexagalloyl-β-D-glucose (**6**)	70	36.1	60	602
Heptagalloyl-β-D-glucose (**7**)	27	16.3	125	130
Heptagalloyl-β-D-glucose (**8**)	11	10.7	65	164
Heptagalloyl-β-D-glucose (**9**)	15	8.0	100	80
Octagalloyl-β-D-gluccose†	41	19.0	140	136
Nonagalloyl-β-D-glucose†	7	3.4	160	21

*Determinations were performed under standard assay conditions at 0.27 mM concentration of the various substrates. Relative activities are compared to the reaction rate observed with pentagalloylglucose equal to 100%. 0, inactive; ~0, trace activity not quantifyable under standard assay conditions.

† Mixture of isomeric compounds.

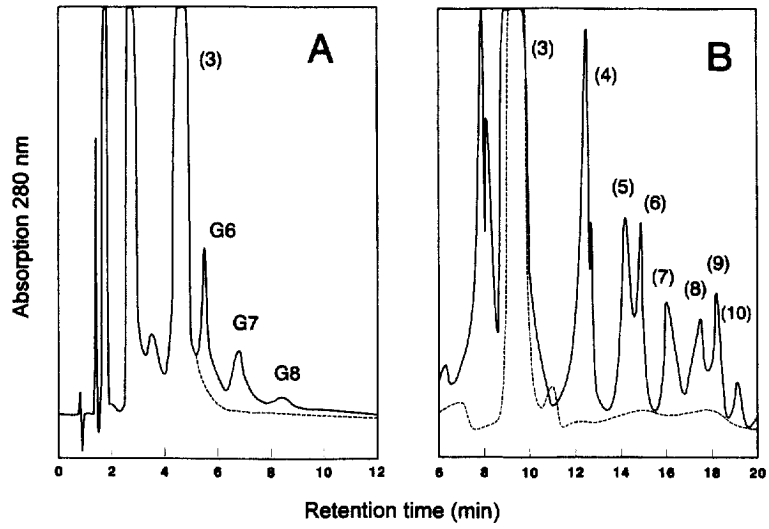


Fig. 2. HPLC analysis of galloyltransferase reaction products. **A**, normal-phase HPLC on Si 60 silica gel; **B**, reversed-phase HPLC on RP-18. (——), Standard enzyme assay; (– – –), blanks with heat-denatured enzyme. G6–G8, hexa-, hepta-, octagalloylglucose. Figures in parentheses correspond to the structures depicted in the Scheme. For further details see text.

loylglucose were provided by Dr H. Schick (Heidelberg, Germany). 1,6-Di- and 1,2,6-tri-*O*-galloylglucose were prepared enzymatically [13, 14]. 1,2,3,6-Tetra-*O*-galloylglucose was isolated from commercially available tannin (Roth GmbH, Karlsruhe, Germany) [7]. 1,3,6-Tri- and 1,2,4,6-tetra-*O*-galloyl-β-D-glucose were generous gifts of Prof. E. Haslam (Sheffield, U.K.). Hexa- to nonagalloylglucoses were isolated from leaves of *R. typhina* [1–4, and unpublished results].

Plant material. Young leaves of staghorn sumac (*R. typhina*) were collected from trees in the surroundings of the university. After washing with dist. H_2O, they were frozen in liquid N_2 and stored at $-20°$ in evacu-

ated plastic bags. Under these conditions, the plant material could be stored for more than 6 months without apparent loss of enzyme activity.

Polyacrylamide-gel electrophoresis. Anodic discontinuous PAGE with 6% separating gels (pH 8.8) and 4% stacking gels (pH 6.8) was employed for analysing the purification of the enzyme [15]; to achieve optimal separation, all samples, gels and buffers were supplemented with 0.1% Tween 20 [16]. In the case of denaturing PAGE (12.5% separating gel), this nonionic detergent was replaced by 0.1% SDS. Protein bands were detected by silver staining [17].

Enzyme activities were measured in standard assay mixtures (70 μl vol.) containing 25 mM citrate buffer (pH 4), 250 nmol β-glucogallin, 25 nmol pentagalloylglucose and 50 μl enzyme (for blanks, heat-denatured enzyme was used and β-glucogallin was omitted). After incubation at 30° for 2 h, the reaction was stopped by adding 10 μl 1 N HCl. Denatured protein was removed by centrifugation and the clear supernatant lyophilised in a spin-freeze dryer. The dry residue was redissolved in 50 μl abs. EtOH and aliquots (5 μl) of this solution were analysed by normal-phase HPLC on LiChrosorb Si 60 (Merck CGC glass cartridges; particle size 5 μm; column 150 × 3 mm i.d.; flow rate 1 ml min^{-1}; detection UV 280 nm). For samples with tetra- to decagalloylglucoses a solvent composed of *n*-hexane-MeOH-THF-formic acid (56:33:11:1, supplemented with 400 mg oxalic acid per liter [18]) was used, while assays with mono- to tetragalloylglucoses were analysed with a less polar modification of this solvent (63:27:9:1, plus 320 mg oxalic acid per liter [12]). This comparatively fast analytical method which, however, allowed only the separation of galloylglucoses according to their substitution degree was used for the routine measurement of enzyme activities. For the detailed determination of individual galloylglucose isomers, reversed-phase HPLC on LiChrospher 100 RP-18 (Merck LiChroCart cartridges; particle size 5 μm; column 250 × 4 mm i.d.; detection UV 280 nm) with linear 0.05% aq. H_3PO_4 (solvent A)/MeCN (solvent B) gradients was employed. Mono- to tetragalloylglucoses were separated with the following gradient: 0–2 min: 3% B, 2–4 min: 3–14%, 4–20 min: 14–23% (flow rate 1 ml min^{-1}); for penta- to heptagalloylglucoses the gradient was 0–2 min: 5% B, 2–4 min: 5–18%, 4–6 min: 18–21%, 6–26 min: 21–25% (flow rate 1.5 ml min^{-1}). Quantification of reaction products was done with a computing integrator (Merck-Hitachi D-2500) and referenced to ext. standards. A gallotannin degrading esterase ("tannase" [5]) in column eluates was determined by spot tests with the chromogenic substrate naphthyl acetate [19].

Protein was determined colorimetrically according to Bradford [20], using BSA as standard. Very dilute solns were measured by UV photometry [21].

Enzyme purification. Unless otherwise stated, all operations were carried out at 0–4° and all buffers were supplemented with 5 mM 2-mercaptoethanol. Leaves of staghorn sumac (80 g) were frozen in liquid N_2 and ground in a pre-cooled ultracentrifugal mill (Retsch KG, Haan, Germany). The frozen powder was mixed with 80 g prewashed PVP and stirred for 30 min with a mixture of 125 ml Tris-HCl buffer (1 M, pH 8) and 125 ml borate buffer (0.2 M, pH 7.5). The homogenate was squeezed through four layers of muslin, followed by centrifugation of the filtrate (35,000 *g*, 20 min). The supernatant crude extract was stirred for 15 min with 8 g Dowex 1X4 (50–100 mesh, borate form) and filtered through glass-wool. A 2% soln of protamine sulphate was added dropwise under stirring to the filtrate until a final concn of 0.2 mg per 1 mg protein was reached; after stirring for 30 min, the solution was centrifuged (20,000 *g*, 20 min). The supernatant was fractionated with solid $(NH_4)_2SO_4$; the 35–70% ppt. was redissolved in a minimal vol. of 50 mM Tris-HCl, pH 7.5 and subjected to gel-filtration on Sephacryl S-300 (column 40 × 2.4 cm i.d.) equilibrated in 50 mM Tris-HCl, pH 7.5, plus 0.1 M NaCl. The most active frs (cf Fig. 1) were concd by ultrafiltration (Filtron Macrosep, 10,000 MW exclusion limit) and rechromatographed under identical conditions. The combined active frs were adsorbed on DEAE cellulose (column 6.5 × 1 cm i.d.) in 50 mM Tris-HCl. pH 7.5, plus 0.1 M NaCl. After washing out unbound protein, the column was developed with a linear gradient of 0.1–0.5 M NaCl which eluted the transferase at ca 0.4 M NaCl. The most active frs were adjusted to pH 5, supplemented with $(NH_4)_2SO_4$ (final concn 1.25 M) and adsorbed on butyl-Sepharose (column 1 × 1.8 cm i.d.) equilibrated in 50 mM K-P_i buffer, pH 5, plus 1.25 M $(NH_4)_2SO_4$. The column was washed with 1.25 M and 0.625 M $(NH_4)_2SO_4$ in buffer, followed by elution of the enzyme with $(NH_4)_2SO_4$-free buffer.

Acknowledgements—We thank Professor E. Haslam (Sheffield) and Dr H. Schick (Heidelberg) for providing rare galloylglucoses, and Mrs A. Müller for excellent technical assistance. Financial support by the Deutsche Forschungsgemeinschaft (Bonn) and the Fonds der Chemischen Industrie (Frankfurt/M.) is gratefully acknowledged.

REFERENCES

1. Gross, G. G., in *Phenolic Metabolism in Plants*, ed. H. A. Stafford and R. K. Ibrahim. *Recent Advances in Phytochemistry*, 1992, **26**, 297.
2. Gross, G. G., in *Plant Polyphenols: Synthesis, Properties, Significance*, ed. R. W. Hemingway and P. E. Laks. Plenum Press, New York, 1992, p. 43.
3. Gross, G. G., in *Comprehensive Natural Products Chemistry. Vol. 3. Carbohydrates and Their Derivatives Including Tannins, Cellulose, and the Related Lignins*, ed. B. M. Pinto. Elsevier, Oxford, 1998, in press.

4. Hofmann, A. S. and Gross, G. G., *Archives of Biochemistry and Biophysics*, 1990, **283**, 530.
5. Niehaus, J. U. and Gross, G. G., *Phytochemistry*, 1997, **45**, 1555.
6. Schmidt, S. W., Denzel, K., Schilling, G. and Gross, G. G., *Zeitschrift für Naturforschung Ser. C*, 1987, **42**, 87.
7. Cammann, J., Denzel, K., Schilling, G. and Gross, G. G., *Archives of Biochemistry and Biophysics*, 1989, **273**, 58.
8. Denzel, K. and Gross, G. G., *Planta*, 1991, **184**, 285.
9. Andrews, P., *Biochemical Journal*, 1965, **96**, 595.
10. Nishizawa, M., Yamagishi, T., Nonaka, G. and Nishioka, I., *Journal of the Chemical Society, Perkin Transactions* 1, 1982, 2963.
11. Gross, G. G., *FEBS Letters*, 1982, **148**, 67.
12. Gross, G. G., *Zeitschrift für Naturforschung Ser. C*, 1983, **38**, 519.
13. Gross, G. G., Denzel, K. and Schilling, G., *Zeitschrift für Naturforschung Ser. C*, 1990, **45**, 37.
14. Hagenah, S. and Gross, G. G., *Phytochemistry*, 1993, **32**, 637.
15. Holtzhauer, A., *Biochemische Labormethoden*. Fischer, Stuttgart, 1992.
16. Dunn, M. J., *Gel Electrophoresis of Proteins*. BioScientific Publ., Oxford, 1993.
17. Blum, H., Beier, H. and Gross, H. J., *Electrophoresis*, 1987, **8**, 93.
18. Nishizawa, M., Yamagishi, T., Nonaka, G. and Nishioka, I., *Chemical and Pharmaceutical Bulletin (Tokyo)*, 1980, **28**, 2850.
19. Gabriel, O., *Methods in Enzymology*, 1971, **22**, 578.
20. Bradford, M. M., *Analytical Biochemistry*, 1976, **72**, 248.
21. Kalckar, H. M., *Journal of Biological Chemistry*, 1974, **167**, 461.