Pergamon

PII: S0031-9422(97)00261-6

Phytochemistry, Vol. 45, No. 8, pp. 1555–1560, 1997

0031-9422/97 $17.00+0.00

A GALLOTANNIN DEGRADING ESTERASE FROM LEAVES OF PEDUNCULATE OAK

JÖRG U. NIEHAUS and GEORG G. GROSS*

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

(*Received in revised form* 14 *February* 1997)

Key Word Index—*Quercus robur* (*Q. pedunculata*); Fagaceae; pedunculate oak; tannase; esterase; depsidase; hydrolysable tannins; galloylglucose esters; defense mechanisms.

Abstract—An enzyme from leaves of pedunculate oak (*Quercus robur*, syn. *Q. pedunculata*) that catalysed the efficient hydrolysis of galloylglucose esters and related compounds was purified more than 1900-fold in 67% yield to apparent homogeneity. For the purified native enzyme, M_r values of 150 000 and 300 000, respectively, were estimated by gel filtration, while a single polypeptide band of M_r 75 000 was detected by SDS-PAGE, suggesting that the native enzyme existed both as a dimer and a tetramer of apparently identical subunits. The enzyme had a temperature optimum of 35–40°; it was inactivated above *ca* 55°, but exhibited a relative activity of 42% even at 0°. Maximal reaction rates occurred between pH 4.3 and 5.0, while no activity was observed above pH 7.8 and below pH 3.8; the enzyme was most stable at pH 5.0. The enzyme was absolutely inactive with variously substituted methyl cinnamates, 1-*O*-sinapoylglucose, substrates with nitro-substitution of the aromatic acid moiety, and with phenolic glucosides. Hydrolysis occurred with simple galloyl esters (methyl, ethyl, propyl gallate), naphthyl acetate (but not with naphthyl propionate or butyrate), mono- to hexa-substituted galloyl-β-D-glucoses, variously ring-substituted 1-*O*-benzoyl-β-D-glucoses, and with depsides like *meta*-digallic acid or chlorogenic acid. The enzyme exhibited both pronounced esterase and depsidase activities, thus closely resembling the properties of fungal tannases. By analogy to these enzymes, the esterase from oak leaves was classified as a plant 'tannase' (tannin acyl hydrolase, EC 3.1.1.20).

INTRODUCTION

Hydrolysable tannins are known as polyphenolic plant constituents which are characteristically derived from mono- to pentagalloylated β-D-glucopyranose. In the gallotannin subclass, these 'simple esters' are extended by attachment of additional galloyl residues to the phenolic galloyl-OH groups to yield *meta*-depsidic side-chains of variable length. The ellagitannin subclass, in contrast, is characterized by oxidative linkage of spatially adjacent galloyl residues of the core unit with the formation of hexahydroxydiphenoyl bridges. A series of enzyme studies with cell-free extracts from *Quercus robur*, *Q. rubra* and *Rhus typhina* has elucidated the principles of the biogenesis of gallotannins, demonstrating the particular role of β-glucogallin (1-*O*-galloyl-β-D-glucopyranose) as general acyl donor in these transformations (cf. [1–3]). In these investigations, pronounced hydrolysis of the substrate, β-glucogallin, was encountered in the *in vitro* assays. It was finally discovered that this highly disturbing interference was due to a contaminating esterase that actively degraded both galloylglucose substrates and enzymatically formed reaction products [4]. As reported below, the purification of this enzyme from leaves of pedunculate oak and its detailed characterization shows that this esterase is analogous to fungal tannases.

* Author to whom correspondence should be addressed.

RESULTS AND DISCUSSION

Enzyme purification

The enzyme was extracted from homogenates of young leaves of pedunculate oak in the presence of insoluble PVP and borate buffer both of which are known to bind inhibitory endogenous phenolic constituents. For the same purpose, the resulting crude extract was stirred with Dowex 1X4, followed by treatment with protamine sulphate to remove residual acid components. This latter step, and particularly a subsequent fractionation with ammonium sulphate, efficiently removed highly inhibitory contaminants from the enzyme solution as indicated by the sig-

Table 1. Purification of 'tannase' from leaf extracts of *Quercus robur*

Step	Total protein (mg)	Total activity (nkat)	Specific activity (pkat mg $^{-1}$)	Purification (-fold)	Recovery (%)
Crude extract	437	0.6	1.4	—	100
Dowex filtrate	306	0.6	2.0	1.4	100
Protamine sulphate, supernatant	194	1.3	6.7	4.8	217
$(NH_4)_2SO_4$, 40–60% ppt.	93	4.5	48.4	34.6	750
Sephadex G-200	5.2	2.9	558	399	483
GPC-diol (gel filtration)	1.1	1.5	1360	974	250
GPC-diol (ion exchange)	0.15	0.4	2670	1910	67

nificant increase in total enzyme activity (Table 1). The esterase was further purified by two gel-filtration steps; the first, a conventional chromatography step on Sephadex G-200, was followed by HPLC on a Merck GPC-diol column. The functional groups of the latter material form complexes with borate which give the column the characteristics of a weak cation exchange resin; using this alternative for a final ion-exchange HPLC step, the enzyme could be purified more than 1900-fold to apparent homogeneity in 67% yield. The results of a typical purification experiment are summarized in Table 1. Purification was monitored by PAGE under non-denaturing and denaturing conditions. Protein bands were detected on the gels by silver staining, and esterase activities were made visible by colour reaction with naphthylacetate as chromogenic substrate (see Experimental). The purified enzyme could be stored at 0° or at −20° without significant loss: activity was maintained for several weeks in both cases.

General properties of the enzyme

Under standard assay conditions (see Experimental), the esterase reaction was linear with respect to protein concentration for *ca* 70 μg of purified enzyme per assay; no reaction occurred with protein previously denatured by heat or acid. Linearity of the reaction was maintained for *ca* 1 hr under these conditions. The enzyme was inactive below pH 3.8 and above pH 7.8, with a broad maximum between pH 4.3 and 5.0 (K-Pi/citrate buffer); half-maximal activities were at pH 4.0 and 6.3. Greatest stability of the enzyme, as determined after preincubation at 30° for 60 min or 24 hr, was found at pH 5.0. The temperature optimum of the reaction was at 35–40°; increasing heat-denaturation was observed over 50°, leading to complete inactivation at 70–80°. The esterase exhibited a relative activity of 42% even at 0°; similar cold-tolerances have been reported for galloylglucose-synthesizing enzymes from oak or sumac leaves [5–7]. Between 20° and 30°, an average activation energy of 24.3 kJ mol^{-1} was calculated, which corresponds to a Q_{10} value of 1.4.

Molecular weight

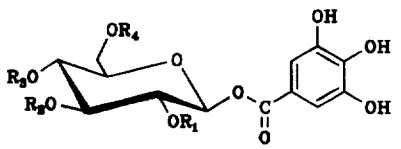
From gel-filtration experiments with a calibrated Sephadex G-200 column [8], an apparent M_r of 300 000 was estimated for the enzyme, while HPLC gel-filtration on a calibrated GPC-diol column was indicative of a M_r of only 150 000. Moreover, PAGE of native purified enzyme revealed two protein bands, as determined by silver staining, which both exhibited pronounced esterase activity upon *in situ* reaction with naphthyl acetate (cf. Experimental). After denaturing SDS-PAGE, in contrast, the existence of only one single polypeptide band of M_r 75 000 was observed. It was concluded that the native enzyme preferentially existed as a tetramer of apparently four identical subunits in slightly acidic medium, while it dissociated partially or completely into still highly reactive dimers under the more alkaline conditions employed for GPC-diol chromatography or native PAGE.

Substrate specificity

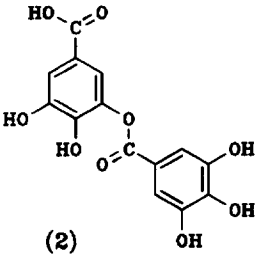
The affinity of the enzyme towards a wide array of putative substrates was determined under standard assay conditions. As summarized in Table 2, the esterase was active with numerous galloyl-β-D-glucoses, including the hexagalloylglucose **1c** as an example of a true gallotannin, but was apparently inactive with α-D-glucose derivatives as shown for 1-*O*-galloyl-α-D-glucose. The major substrate, β-glucogallin (**1a**), could be substituted by variously ring-substituted 1-*O*-benzoyl-β-D-glucoses, provided that they were devoid of nitro-groups. Lower but still significant reaction rates were observed in the presence of the 'simple' galloyl esters methyl, ethyl and propyl gallate; these compounds were characterized by extremely sigmoidal substrate saturation curves, in contrast to the other substrates which displayed normal Michaelis-Menten kinetics. Fair to excellent hydrolysis occurred with the depside substrates *meta*-digallic acid (**2**) and chlorogenic acid (**3**). No reaction was observed in the presence of a wide variety of differently ring-substituted methyl cinnamates, as well as with 1-*O*-sinapoyl-β-D-glucose or benzyl 3,5-dinitrobenzoate. *In situ* staining after native PAGE revealed that the

Table 2. Substrate specificity of oak leaf tannase

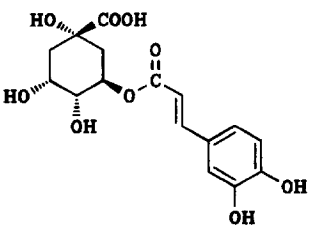
Substrate	v_{max} (pkat)	K_m (mM)	$v_{max} K_m^{-1}$ ($\times 10^{-3}$)
Galloylglucose esters			
1-*O*-Galloyl-α-D-glucose	0	—	—
1-*O*-Galloyl-β-D-glucose (β-glucogallin) (**1a**)	1.25	9.1	137
6-*O*-Galloyl-β-D-glucose	0.30	4.7	64
1,6-Di-*O*-galloyl-β-D-glucose	0.11	1.3	85
3,6-Di-*O*-galloyl-β-D-glucose	0.29	5.5	53
1,2,6-Tri-*O*-galloyl-β-D-glucose	0.17	2.3	74
1,2,3,6-Tetra-*O*-galloyl-β-D-glucose	0.59	6.9	86
1,2,3,4,6-Penta-*O*-galloyl-β-D-glucose (**1b**)	0.19	5.9	32
4-*O*-Digalloyl-1,2,3,6-tetra-*O*-galloyl-β-D-glucose (**1c**)	0.14	6.0	23
1-*O*-Benzoylglucose esters			
1-*O*-Benzoyl-β-D-glucose	0.15	2.0	75
1-*O*-*p*-Hydroxybenzoyl-β-D-glucose	0.19	2.1	90
1-*O*-Protocatechuoyl-β-D-glucose	1.7	6.0	283
1-*O*-Anisoyl-β-D-glucose	2.8	12.7	220
1-*O*-Veratroyl-β-D-glucose	2.2	4.0	550
1-*O*-Vanilloyl-β-D-glucose	8.0	17.5	457
1-*O*-Syringoyl-β-D-glucose	0.45	0.85	529
1-*O*(3,5-Dinitrobenzoyl)-β-D-glucose	0	—	—
'Simple' galloyl esters			
Methyl gallate	0.39	71.4	5.5
Ethyl gallate	0.02	2.3	8.7
Propyl gallate	0.03	2.4	12.5
Depsides			
meta-Digallic acid (**2**)	0.41	0.99	414
Chlorogenic acid (**3**)	0.08	4.1	19.5



(1a) R_{1-4} = H
(1b) R_{1-4} = galloyl
(1c) R_3 = meta-digalloyl, $R_{1,2,4}$ = galloyl



(2)



(3)

enzyme hydrolysed the chromogenic esters 1-naphthyl and 2-naphthyl acetate, but not the propionate and butyrate derivatives of 1-naphthol.

Summarizing these data, it is apparent that the nature of the alcoholic moiety of the substrate was less important than that of the acyl component. Esters with cinnamoyl residues were not accepted as substrates, e.g. methyl cinnamates and sinapoylglucose, or were hydrolysed at only low rates as shown for the depside chlorogenic acid (**3**). 1-*O*-Benzoyl (C_6C_1)-derivatives of β-D-glucose, in contrast, generally represented excellent substrates. Among these, compounds with a high proportion of methoxy groups were preferentially hydrolysed, in contrast to substrates bearing phenolic hydroxyl residues. As has been shown for differently substituted galloylglucoses, increasing molecular size of substrates significantly reduced their reactivity, a fact that must be attributed to their increasing tanning, i.e. protein denaturing and enzyme inactivating potential, and it is obvious that the enzyme described here was particularly ineffective in hydrolysing the substrates pentagalloylglucose (**1b**) and hexagalloylglucose (**1c**) which both are known to exhibit pronounced tannin properties.

In summary, the properties of the enzyme described here reveal it to be a typical esterase that catalyses the hydrolysis of galloylglucose esters, gallotannins and related compounds. Considering its pronounced expression of both esterase and depsidase activities, i.e. catalytic properties that are traditionally ascribed to fungal tannases (tannin acyl hydrolase, EC 3.1.1.20), this new esterase represents an analog of

plant origin to these microbial enzymes. Besides one previous report on a 'tannase' from divi-divi fruit pods (*Caesalpinia coriaria*, Fabaceae) [9], this is only the second communication dealing with a 'tannase' of plant origin, and it is the first one in which a wide array of pure compounds was tested as substrates instead of rather ill-defined chemicals like 'tannin' or 'tannic acid'. The latter limitation applies particularly to our knowledge of the comparatively well-documented tannases from various microorganisms. As recently reviewed [10], such enzymes are particularly common among ascomycetous and basidiomycetous fungi but have also been observed in yeast and bacteria (e.g. as a recent example, an anaerobic ruminal species transforming tannins via gallic acid to pyrogallol [11]). It has been a matter of dispute for decades whether the esterase and depsidase activities of these microbial tannases were due to two separate enzymes or to only one enzyme catalysing both reactions. The problem can be regarded as settled by two recent reports on tannases from *Aspergillus niger* [12] and *Cryphonectria* (*Endothia*) *parasitica* [13] which were purified to apparent homogeneity and yet exhibited both esterase and depsidase activity. The basic properties of fungal tannases (e.g. M_r, pH and temperature optima) were found to be rather uniform (cf. [3, 10]), and the characteristics of the esterase from oak leaves referred to here were found to be highly consistent with these data.

Finally, the ecological significance of such a plant 'tannase' deserves discussion. Though transformation of tannins to insoluble derivatives has been discussed as major deastringency mechanism in fruit ripening [14], it is reasonable to assume that such an enzyme activity could contribute to these processes by loss of astringency via simple degradation of tannins. In green leaves, however, the role of tannase is much less apparent. It has been recognized, for instance, that condensed tannins of *Acacia nigrescens* acted as an anti-defoliating agent against browsing by giraffe [15], and it was concluded from studies with *Epilobium*, *Cornus* or *Alnus* that the soluble galloylglucoses and ellagitannins in these plants were important in the defense against ruminants [16]. A tannin-degrading enzyme in leaves would thus not make much sense. In contrast to herbivorous mammals, however, the situation with insects could be completely different. The feeding deterrent role traditionally ascribed to tannins due to their astringency, causing reduced palatability of plant parts, has been questioned; e.g. evidence has been presented that the ellagitannin geraniin preferentially acted as a protoxin that released insect growth inhibitors, particularly ellagic acid, upon hydrolytic cleavage [17].

According to this view, hydrolysable tannins could play a dual protective role in plant–herbivore interactions, not only by direct astringency-dependent deterrence of herbivorous animals, but also indirectly through their degradation products. The existence of tannase in green leaves could thus significantly contribute to the latter process. Loss of cellular compartmentation under the attack of herbivores would bring this enzyme into contact with its tannin substrates, causing the release of harmful low M_r phenolic degradation products. Analogous defence strategies, based on the hydrolysis of secondary metabolites as precursors of toxic substances, like *ortho*-coumaroyl glucosides, cyanogenic glucosides or glucosinolates, are well documented [18]. Eventually, the system tannin-'tannase' may be added to that list of chemical weapons of higher plants.

EXPERIMENTAL

Chemicals. Chemical methods were employed for the synthesis of β-glucogallin (**1a**) [19] and 1,2,3,4,6-pentagalloylglucose (**1b**) [20]. α-Glucogallin, 6-*O*-galloylglucose and 3,6-di-*O*-galloylglucose were provided by Dr H. Schick (Heidelberg, Germany). 1,6-Di- and 1,2,6-tri-*O*-galloylglucose were prepared enzymatically [4, 21]. 1,2,3,6-Tetra-*O*-galloylglucose was isolated from commercially available tannin (Roth GmbH, Karlsruhe, Germany) [6], the hexagalloylglucose (**1c**) was isolated from leaves of *R. typhina* [22]. Variously substituted 1-*O*-benzoylglucoses were prepd enzymatically [23]. Differently ring-substituted methyl cinnamates were chemically synthesized according to standard procedures. 1-*O*-Sinapoyl-β-D-glucose was a gift of Prof. D. Strack (Halle, Germany). *meta*-Digallic acid (**2**) was obtained by chemical synthesis [24]. All other enzyme substrates and reference compounds were purchased from commercial suppliers.

Plant material. About 2-month-old young leaves of *Quercus robur* L. (*Q. pedunculata* Ehrh.) were collected from trees surrounding the University. After washing with dist. H_2O, they were frozen in liquid N_2 and stored at $-20°$ in evacuated 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 7% separating gels (pH 8.8) and 4% stacking gels (pH 6.8) was employed for analysing the purification of the enzyme [25]; to achieve optimal sepn, all samples, gels and buffers were supplemented with 0.1% Triton X-100 [26]. In the case of denaturing PAGE, this non-ionic detergent was replaced by 0.1% SDS. Protein bands were detected by Ag staining [27]. Esterase activities were visualized on the gels with chromogenic substrate naphthyl acetate [28].

Enzyme purification. Leaves of pedunculate oak (80 g) were frozen in liquid N_2 and ground in a precooled ultracentrifugal mill (Retsch KG, Haan, Germany). The frozen powder was mixed with 80 g prewashed PVP and stirred for 30 min with a mixt. of 120 ml of Tris–HCl buffer (1 M, pH 8.5) and 120 ml Na-borate buffer (0.1 M, pH 7.5). The homogenate was squeezed through four layers of muslin, followed by cen-

trifugation of the filtrate (20 000 *g*, 20 min). The supernatant crude extract was stirred for 15 min with 8 g Dowex 1X4 (100–200 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 2 mg protamine sulphate per 100 mg protein was reached; after stirring for 15 min, the soln was centrifuged. The supernatant was fractionated with solid $(NH_2)_2SO_4$; the 40–60% ppt. was redissolved in a minimal vol. of 50 mM Tris–HCl, pH 7.5, clarified by centrifugation (30 000 *g*, 10 min) and desalted by gel-filtration on Sephadex G-25 (PD-10 columns, Pharmacia). The filtrate was subjected to gel-filtration on Sephadex G-200 (column 30 × 2.8 cm i.d.) equilibrated in 0.2 M K-Pi/0.1 M citrate buffer, pH 5.0. The most active frs were concd by ultrafiltration (Millipore 'Centrifugal Ultrafree' kit, 30 000 *M*, exclusion limit) and subjected to HPLC by gel-permeation chromatography (GPC) on a diol-substituted column (Bio GPC-diol 250 'Superformance' glass column system, Merck, Darmstadt, Germany; 300 × 10 mm i.d.; 5 μm particle size; eluent 10 mM NaH_3PO_4 plus 300 mM NaCl, pH 7.2; flow rate 0.5 ml min^{-1}). The same column could also be used for a subsequent ion-exchange chromatography step due to the fact that it operated as a weak cation exchange resin in the presence of borate. After adsorption of the active frs of the preceding step, the pure enzyme was eluted by a linear borate/NaCl gradient (solvent A: 0.1 M borate, pH 7.5; solvent B: A plus 0.4 M NaCl; gradient: 0–2 min 100% A, 2–42 min 0–100% B; flow rate 1 ml min^{-1}).

Protein was determined turbidmetrically after pptn with CCl_3CO_2H [29], using BSA as standard. Very dilute solns were measured by UV photometry [30].

Enzyme activities were measured in standard assay mixs (30 μl vol.) containing K-Pi (2 μmol)/citrate (1 μmol) buffer (pH 5.0), 200 nmol β-glucogallin and suitable amounts of enzyme. For substrate specificity studies, β-glucogallin was replaced by equal amounts of the examined compounds. After incubation at 30° for 45 min, the reaction was stopped by adding 10 μl 1 N HCl; blank samples were prepd by addition of acid prior to incubation. After removal of denatured protein by centrifugation, aliquots (10 μl) of the clear supernatants were analysed by reversed-phase HPLC on LiChrospher 100 RP-18 (Merck LiChroCart Cartridges; particle size 5 μm; column 125 × 4 mm i.d.; flow rate 1 ml min^{-1}; detection UV 280 nm) with linear MeCN/0.05% aq. H_3PO_4 gradients that were varied according to the different substrates and products to be analysed. Quantification of reaction products was done with a computing integrator (Merck-Hitachi D-2500) and reference to ext. standards.

Acknowledgements—Financial support by the Deutsche Forschungsgemeinschaft (Bonn), the Fonds der Chemischen Industrie (Frankfurt/M.) and by research grants of the University of Ulm 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, in press.
4. Gross, G. G., Denzel, K. and Schilling, G., *Zeitschrift für Naturforschung Ser. C*, 1990, **45**, 37.
5. Schmidt, S. W., Denzel, K., Schilling, G. and Gross, G. G., *Zeitschrift für Naturforschung Ser. C*, 1987, **42**, 87.
6. Cammann, J., Denzel, K., Schilling, G. and Gross, G. G., *Archives of Biochemistry and Biophysics*, 1989, **273**, 58.
7. Denzel, K. and Gross, G. G., *Planta*, 1991, **184**, 285.
8. Andrews, P., *Biochemical Journal*, 1965, **96**, 595.
9. Madhavakrishna, W. and Bose, S. M., *Bulletin of the Central Leather Research Institute, Madras*, 1961, **8**, 153. *Chemical Abstracts*, 1962, **56**, 11759f.
10. Scalbert, A., *Phytochemistry*, 1991, **30**, 3875.
11. Nelson, K. E., Pell, A. N., Schofield, P. and Zinder, S., *Applied and Environmental Microbiology*, 1995, **61**, 3293.
12. Bartomeuf, C., Regerat, F. and Pourrat, H., *Journal of Fermentation & Bioengineering*, 1994, **77**, 320.
13. Farias, G. M., Corbea, C., Elkins, J. R. and Griffin, G. J., *Physiological Molecular Plant Pathology*, 1994, **44**, 51.
14. Ozawa, T., Lilley, H. and Haslam, E., *Phytochemistry*, 1987, **26**, 2937.
15. Furstenburg, D. and Van Hofen, W., *Comparative Biochemistry & Physiology*, 1994, **107A**, 425.
16. Robbins, C. T., Hanley, T. A., Hagerman, A. E., Hjeljord, O., Baker, D. L., Schwartz, C. C. and Mautz, W. W., *Ecology*, 1987, **68**, 98.
17. Klocke, J. A., Van Wagenem, B. and Balandrin, M. F., *Phytochemistry*, 1986, **25**, 85.
18. Matile, P., *Naturwissenschaften*, 1984, **71**, 18.
19. Gross, G. G., *FEBS Letters*, 1982, **148**, 67.
20. Gross, G. G., *Zeitschrift für Naturforschung Ser. C*, 1983, **38**, 519.
21. Hagenah, S. and Gross, G. G., *Phytochemistry*, 1993, **32**, 637.
22. Hofmann, A. S. and Gross, G. G., *Archives of Biochemistry & Biophysics*, 1990, **283**, 530.
23. Weisemann, S., Denzel, K., Schilling, G. and Gross, G. G., *Bioorganic Chemistry*, 1988, **16**, 29.
24. Fischer, E., Bergmann, M. and Lipschitz, W., *Berichte der Deutschen Chemischen Gesellschaft*, 1918, **51**, 45.

25. Holtzhauer, A., *Biochemische Labormethoden*. Fischer, Stuttgart, 1992.
26. Dunn, M. J., *Gel Electrophoresis of Proteins*. BioScientific Publications, Oxford, 1993.
27. Blum, H., Beier, H. and Gross, H. J., *Electrophoresis*, 1987, **8**, 93.
28. Gabriel, O., *Methods in Enzymology*, 1971, **22**, 578.
29. Kunitz, M., *Journal of General Physiology*, 1952, **35**, 423.
30. Kalckar, H. M., *Journal of Biological Chemistry*, 1974, **167**, 461.