Purification and Properties of a Novel Xyloglucan-specific Endo-(1→4)-β-d-glucanase from Germinated Nasturtium Seeds (Tropaeolum majus L.)*

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Endo-(1→4)-β-d-glucanase activity has previously been detected in the cotyledons of germinated nasturtium (Tropaeolum majus) seeds, and has been linked to the hydrolysis in vivo of storage xyloglucan (amyloid) (Edwards, M., Dea, I. C. M., Bulpin, P. V., and Reid, J. S. G. (1985) Planta (Berl.) 163, 133–140). Extracts from the cotyledons of 14-day seedlings are now shown to contain a single endo-glucanase activity, and it has been purified to apparent homogeneity by sequential anion-exchange chromatography, cation-exchange chromatography, and gel filtration. The purified enzyme gave a single protein band on polyacrylamide gel electrophoresis, dodecyl sulfate gel electrophoresis, and isoelectric focusing. The isoelectric point was 5.0, the pH optimum 4.5–5.0, and the temperature optimum 40 °C. Dodecyl sulfate gel electrophoresis gave a molecular weight of 29,000; permeation methods gave lower values. The enzyme contained about 5% carbohydrate. An endo mode of action on tamarind seed xyloglucan was deduced by monitoring solution viscosity and reducing power, and by analyzing the end products of the reaction. The specificity of the enzyme toward a wide range of substrates, including celluloses, carboxymethyl and hydroxyethyl celluloses, glucomannan, galactoglucomannans, mixed-linkage (1→3 and 1→4)-β-d-glucan, laminarin, and xyloglucans from seeds and primary cell walls, was tested. Only the xyloglucans were hydrolyzed. It is concluded that the enzyme is a pure, endo-acting (1→4)-β-d-glucanase which is novel in its apparent complete specificity toward xyloglucans. It is speculated (a) that enzymes of this type may have escaped earlier detection because it is normal practice to screen for endo-(1→3)-β-d-glucanase using artificial cellulose derivatives and (b) that they may be widely involved in xyloglucan metabolism in plant cell walls.

In the early 19th century it was observed that the cell walls in the cotyledonary or endosperm tissues of some seeds could be stained blue with iodine. The material responsible for the staining reaction was assumed to be starch-like and it was named "amyloid" (Vogel and Schleiden, 1839). Amyloid-positive cell walls are present in the seeds of at least 230 dicotyledonous species (Kooiman, 1960), and the amyloids from four species—Tropaeolum majus L. (Le Dizet, 1974), Tamarindus indica (Kooiman, 1961), Impatiens balsamina (Courtois and Le Dizet, 1974) and Annona muricata (Kooiman, 1967)—have been isolated and subjected to full structural analysis. All four amyloids are xyloglucans (or more correctly, galacto-xyloglucans), based on a (1→4)-β-linked d-glucan (cellulosic) backbone which carries α-D-xylpyranosyl and β-D-galactopyranosyl-(1→2) α-D-xylpyranosyl substituents. Both types of substituent are linked (1→6) to the d-glucan backbone. Galactose/xylose/glucose ratios range from 1:2:3 in Tropaeolum and Tamarindus amyloids to 1:1:4 in that of Annona (Reid, 1985). Polysaccharides closely similar in structure to the seed amyloids are present in the primary cell walls of dicotyledonous plants (Bauer et al., 1973; Darvill et al., 1980). The primary wall xyloglucans differ from the amyloids in that they contain an additional structural feature; some 60–80% of their side chain D-galactosyl residues are further substituted by (1→2)-linked D-fucosyl residues. The fucosyl residues are probably in the α-anomeric form (Darvill et al., 1980).

Early microscopic studies suggested that amyloids disappear from seeds after germination (Heinricher, 1888; Reiss, 1889) and it has been assumed that they are reserve polysaccharides. Surprisingly, this was confirmed only very recently in the course of an investigation of xyloglucan metabolism in the cotyledons of the nasturtium seed (T. majus L.) after germination (Edwards et al., 1985). The amyloid of the nasturtium seed was shown to be mobilized completely following germination. It constituted 30% of the total substrate reserves utilized by the seed, and must therefore be classified as a major cell-wall storage polysaccharide (Meier and Reid, 1982).

The post-germinative breakdown of xyloglucan in nasturtium cotyledons correlates closely with the activities of three hydrolytic enzymes: β-galactosidase (EC 3.2.1.23), α-D-xylidosidase, and endo-(1→4)-β-d-glucanase (EC 3.2.1.4) (Edwards et al., 1985). In this paper we report the purification to homogeneity of the endo-β-d-glucanase and present evidence that it is a novel enzyme with apparently complete specificity toward xyloglucans.

EXPERIMENTAL PROCEDURES

Materials—Tamarind seed xyloglucan was prepared from commercial tamarind flour as described previously (Edwards et al., 1985). Nasturtium seed xyloglucan was prepared in the same way, starting from milled seeds. Primary cell-wall xyloglucan was prepared from mung bean hypocotyls essentially according to Kato and Matsuda (1976). The 20-h extractions with cold KOH under N2 as described by these authors were replaced by 1-h extractions at 90 °C with KOH containing 0.05% (w/v) NaBH₄ to prevent alkaline oxidation of the polysaccharide (Aspinall et al., 1986). The final purification step involving incubation with endo-xylanase was omitted so that the final product was still contaminated to some extent with (1→4)-β-D-xylan. On hydrolysis by the 72–4 H₂SO₄ method (Seaman et al., 1946) it released fucose, galactose, xylose, and glucose in the molar ratio of

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0.8-1.7:11.3:10 (determined by gas-liquid chromatography of alditol acetates). The corresponding ratio for the pure xyloglucan was 1:2.5:7:10 (Kato and Matsuda, 1976), indicating that our product contained about 16% xylan. Barley mixed-linkage (1→3 and 1→4)-β-d-glucan was a gift from Prof. D. Manners (Heriot-Watt University, Edinburgh), and the galactoglucomannans from spruce wood (Meier, 1965) and Aspergillus species (Jakimow-Barras, 1973) were a gift from Professor H. Meier (Université de Fribourg, Fribourg, Switzerland).

Konjac (1→4)-β-linked d-glucanomannan was prepared by purification of commercial food-grade "Konjaku" flour as described previously (Dea et al., 1977). Laminarin was purchased from Sigma. Sodium carboxymethyl celluloses with degrees of substitution ranging from 0.4 to 1.2, and DP' values of 400, 1100, and 3200 were obtained from Hercules Ltd. The hydroxymethyl celluloses were used "Natrosol" 250 GR, (molar substitution 2.5) (Hercules Ltd.) and "Celacol" HE450 (molar substitution 2.5). Hydroxypropylmethyl celluloses were Celacol HPM 450 and 2500 (DS 1.5 (methyl) and 0.3 (hydroxypropyl)) and methyl celluloses were Celacol M 450 and 2500 (DS 16). Celacol products were obtained from I.C.I. Ltd. Phosphoric acid repertitived cellulose was prepared by dissolving cellulose (Whatman DE11) in phosphoric acid, and repertitived it by dilution (Jayne and Lang, 1963). The product was neutralized with NaHCO₃, washed free of salt, and freeze-dried. Cello-oligosaccharides were a gift from Dr. R. Sturgeon (Heriot-Watt University, Edinburgh).

Assays—In crude or partially purified enzyme preparations endo-β-d-glucanase activity was assayed, using tamarind seed xyloglucan as substrate (Edwards et al., 1985). Once purified free of other activities which were capable of releasing reducing sugars from the xyloglucan, breakdown products, the endo-glucanase was assayed, more accurately, by monitoring the release of reducing power by the direct ferricyanide method (Halliwell and Riaz, 1970) or by specifically using D-galactose dehydrogenase (Edwards et al., 1985). Activities quoted in nanokatals were calculated on the basis of glucose equivalents released.

β-D-Galactosidase activity was measured by determining the release of p-nitrophenol from p-nitrophenylβ-D-galactopyranoside and by assaying the release of D-galactose from tamarind seed xyloglucan specifically using β-galactoside dehydrogenase (Edwards et al., 1985).

α-D-Xylosidase activity was assayed by determining the release of pentose from tamarind seed xyloglucan as before (Edwards et al., 1985).

D-Glucose was determined using the hexokinase/b-glucose-6-phosphate dehydrogenase procedure (Bergmeyer et al., 1974), D-galactose by using β-galactoside dehydrogenase (Wallenfels, 1974), and pentose by a modification of the p-bromoaniline procedure of Roe and Rice (1972). D-Glucose was used as standard. In both assays the substrate concentration was at pH 8.9 without stacking gels. SDS-gel electrophoresis was carried out essentially according to Laemmli (1970). Samples were denatured in the presence of SDS and mercaptoethanol. A vertical slab-gel system was used (Bio-Rad Mini-Sub) and gels (0.75 cm thickness, 8.5% acrylamide, 4% polyacrylamide (14:20), and 0.4% agarose) were run at 20 mA/gel. Proteins were localized on gels with Coomassie Blue. Molecular weight standards were cytochrome c (12,400), carbonic anhydrase (29,000), ovalbumin (44,000), and bovine serum albumin (67,000).

Isoelectric Focusing—Analytical isoelectric focusing was carried out using Ampholine PAGPlates (LKB Ltd.). They were run and stained with Coomassie Blue exactly according to the manufacturer's instructions.

Estimation of the Molecular Weight of the Native Enzyme—Both gel filtration columns mentioned earlier were calibrated and used to estimate the molecular weight of the nondenatured enzyme. The molecular weight was also determined using high performance liquid chromatography (LKB System) in conjunction with permeation chromatography columns (TSK 2000 SW and TSK 3000 SW). Molecular weight standards were cytochrome c (12,400), ribonuclease A (13,700), myoglobin (17,000), chymotrypsinogen (24,000), and ovalbumin (45,000), and bovine serum albumin (66,000).

PhOptimum and Stability—Temperature Optimum—These parameters were determined using tamarind seed xyloglucan as substrate, and assaying for reducing power by the p-hydroxybenzoic acid hydrate method. The pH optimum was determined using Mcllvaine phosphate citrate buffer covering the range pH 3.0–9.0. To determine the temperature optimum, samples were equilibrated at or near 1 h to allow the Sephadex column to run at 10 ml h⁻¹ and the Bio-Gel column at 1.6 ml h⁻¹. Fractions with high endo-β-d-glucanase activity were pooled and frozen in aliquots.

Polyacrylamide Gel Electrophoresis—Nondenaturing gels were prepared and run in tubes according to Gabriel (1971); 10% gels were used at pH 8.9 without stacking gels. SDS-gel electrophoresis was carried out essentially according to Laemmli (1970). Samples were denatured in the presence of SDS and mercaptoethanol. A vertical slab-gel system was used (Bio-Rad Mini-slab-gel) and gels (0.75 × 82 × 60 mm high, not including stacking gel) were run at 20 mA/gel. Proteins were localized on gels with Coomassie Blue. Molecular weight markers were α-lactalbumin (14,200), soybean trypsin inhibitor (20,100), trypsinogen (24,000), carbonic anhydrase (29,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), ovalbumin (45,000), and bovine serum albumin (66,000).

Mode of Action of the Enzyme—Enzyme and tamarind seed xyloglucan solutions were mixed in the same proportions as in the standard assay (final substrate concentration 10 mg ml⁻¹). Reaction progress was monitored by measuring viscometric flow time as described previously (Edwards et al., 1985) and by determining reducing power by the direct ferricyanide method. The reaction mixture was diluted after incubation, and the reducing power of the reaction mixture was determined using Mcllvaine phosphate citrate buffer covering the range pH 3.0–9.0. To determine the temperature optimum, samples were equilibrated at or near 1 h to allow the Sephadex column to run at 10 ml h⁻¹ and the Bio-Gel column at 1.6 ml h⁻¹. Fractions with high endo-β-d-glucanase activity were pooled and frozen in aliquots.

Substrate Specificity of the Enzyme—Soluble and insoluble sub-
strates were usually dissolved or suspended in McIlvaine phosphate-citrate buffer (pH strength), pH 5.0, and mixed with enzyme (0.06 nkat/ml, final volume, determined using tamarind xyloglucan as substrate) to give a final concentration in the assay of 10 mg ml⁻¹. Over a period of 24 h viscometric flow times were measured if appropriate and reducing power was determined on aliquots using the p-hydroxybenzoic acid hydrazide method. A control incubation was carried out for each substrate. Those substrates which gave inconveniently high viscosities at 10 mg ml⁻¹ were used at lower concentrations. The action of the enzyme on cello-oligosaccharides was determined by examining the incubation mixture by TLC.

RESULTS

Purification of the Enzyme—Nasturtium (T. majus L.) seedlings were harvested 14 days after sowing, when endo-β-D-glucanase, α-D-xylosidase, and β-D-galactosidase activities were all at or near their maximum levels in the cotyledons (Edwards et al., 1985). The endo-β-D-glucanase was purified from cotyledon extracts by a combination of DEAE-cellulose chromatography, CM-cellulose chromatography, and gel filtration (Table I and Fig. 1). Fig. 1 shows that only one form of endo-β-D-glucanase was present, whereas both β-galactosidase and α-xylosidase were multiple activities. The purified enzyme migrated as a single band on polyacrylamide gel electrophoresis (PAGE), SDS-PAGE (Fig. 2), and isoelectric focusing (Fig. 3). When concentrated, purified enzyme preparations were used to completely depolymerize tamarind seed xyloglucan, no galactose, pentose, or glucose could be detected in the final mixture of products, confirming the absence of even trace amounts of β-galactosidase, α-xylosidase, or β-glucosidase.

The purification data in Table I suggest that an approximately 9.5-fold purification results in a homogeneous enzyme. This indicates that the enzyme constituted about 10% of the protein in the cotyledon extract after ammonium sulfate precipitation and dialysis. However it is clear from the electrophoretic separation pattern (Fig. 2) that it represented a precipitation and dialysis. However it is clear from the electrophoretic separation pattern (Fig. 2) that it represented a

| TABLE 1  |
| --- |
| Purification of endo-β-D-glucanase from T. majus cotyledons |
| The enzyme was assayed viscometrically (Edwards et al., 1985). |
| Parameter | Value |
| Total volume | Total protein | Total activity | Specific activity | Recovery |
| (ml) | (mg) | (viscometric units) | (units mg⁻¹) | (%) |
| Extract | 66 | 209 | 19,388 | 92.8 | 100 |
| DEAE-cellulose | 119 | 27 | 6,694 | 248 | 35 |
| CM-cellulose | 9.0 | 3.9 | 2,966 | 761 | 15.3 |
| Sephachex G-200 | 46 | 2.3 | 2,024 | 880 | 10.4 |

* Ammonium-sulfate concentrated. Dialyzed against 106 mM NaCl and 20 mM Tris-HCl, pH 7.5.
* After concentration of pooled fractions by rotary evaporation.

chain, of molecular weight 29,000. The lower values obtained for the nonnucleated enzyme suggest that it has a compact tertiary structure.

With 1.0% tamarind seed xyloglucan as substrate, the pH optimum was at pH 4.5-5.0 (stable from pH 4–8). The temperature optimum was 40 °C. The isoelectric point of the molecule was pH 5.0 (Fig. 3).

Mode of Action and Substrate Specificity—The enzyme brought about a very rapid decrease in the specific viscosity of xyloglucan solutions in the early stages of the reaction, whereas the reducing power of the incubation mixture increased slowly in a linear fashion over a very much longer period (Fig. 4). This type of relationship between specific viscosity decrease and reducing power release is characteristic of endo- as opposed to exo-hydrolytic cleavage. When the hydrolysis of tamarind seed xyloglucan by the enzyme was complete, i.e. when the reducing power of the reaction mixture had reached a constant value, the products of the reaction were examined by TLC. TLC confirmed that no monosaccharides were present, and did not reveal any oligosaccharides.
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FIG. 4. Specific viscosity and reducing power of tamarind seed xyloglucan (10 mg ml⁻¹) incubated with purified endo-β-D-glucanase. See text for details.

The enzyme is clearly an endo-(1→4)-β-D-glucanase which hydrolyzes tamarind seed xyloglucan rapidly, but only to a limited extent. The final value of the reducing power released in Fig. 4 suggests a degree of hydrolysis of 9% and indicates that some 18% of the D-glucosidic links in the D-glucan backbone are hydrolyzed.

The substrate specificity of the enzyme was tested initially using soluble cellulose derivatives. Under conditions where tamarind and nasturtium seed xyloglucans were depolymerized rapidly, the enzyme brought about no change in either the viscosity or the reducing power of sodium carboxymethyl celluloses (DP 400, 1100, 3600; DS 0.4, 0.7, 1.2), hydroxyethyl celluloses, hydroxypropylmethyl celluloses, and methyl celluloses. Table II summarizes the results of further experiments in which the action of the enzyme on a wider variety of polymeric substrates containing the (1→4)-β-D-glucosidic link (plus the (1→3)-β-D-glucan laminarin) was tested. Clearly, the enzyme hydrolyzes only xyloglucans. It has no effect on cellulose (microcrystalline or phosphoric acid repre-
Substrate specificity of endo-β-D-glucanase from T. majus cotyledons

Substrates were at 10 mg ml⁻¹. Viscosity was determined at frequent intervals (see Fig. 4). Controls showed no change in viscosity or reducing power.

| Substrate | Increase in reducing power | Decrease in specific viscosity |
|-----------|---------------------------|-------------------------------|
|           | over 2 h | over 6 h | over 24 h | over 30 min | over 6 h | over 24 h |
| Nasturtium seed xyloglucan, Gal/Xyl/Glc = 4.4:6:5.10⁶ | 0.79 | 1.6 | 6.0 | 98 | 99 | 99 |
| Tamarind seed xyloglucan, Gal/Xyl/Glc = 3.1:6.9:10⁶ | 0.44 | 1.3 | 5.2 | 86 | 97 | 99 |
| Mung bean primary wall xyloglucan, Fuc/Gal/Xyl/Glc = 0.8:1.7:11.5:10.6 | 0.08 | 0.10 | 0.12 | 7.3 | 23 | 27 |
| Sodium carboxymethyl cellulose, DS 0.7; DP 1100 | 0 | 0.01 | 0.01 | 0 | 0 | 0.1 |
| Hydroxyethyl cellulose, molar substitution, 2.5 | 0 | 0.01 | 0.01 | 0 | 4.3 | 12.5 |
| Microcrystalline cellulose, Avicel | 0 | 0 | 0 | 0 | 0 | 0 |
| Phosphoric and reprecipitated cellulose | 0 | 0 | 0 | 0 | 0 | 0 |
| Barley-β-glucan, 26% (1→3) 74% (1→4) linkages | 0 | 0 | 0 | 0 | 0 | 0 |
| Konjac glucomannan, Man/Glc = 1.6:1 (Kato and Matsuda, 1973) | 0 | 0 | 0 | 3.1 | 6.9 | 0 |
| Spruce wood galactoglucomannan, Gal/Glc/Man = 1.0:6:1.65.5⁶ | 0 | 0 | 0 | 0 | 0 | 0 |
| Asparagus seed galactoglucomannan, Gal/Glc/Man = 1.0:8:0.71 | 0 | 0 | 0 | 0 | 0 | 0 |
| Cello-oligosaccharides | 0 | 0 | 0 | 0 | 0 | 0 |
| Laminarin | No hydrolysis detected⁴ | 0 | 0 | 0 | 0 | 0 |

* By analysis of actual material used.
* Our sample slightly contaminated with (1→4)-β-D-xylan. Published composition of pure polysaccharide, Fuc/Gal/Xyl/Glc = 2.5:7:11.5:10.6⁶.
* Information supplied by donors.
* No change in composition of mixture detected by TLC over 24 h.

Discussion

The endo-(1→4)-β-D-glucanase of the germinated nasturtium seed is novel in its apparently total specificity toward xyloglucans, and in future work we will investigate the molecular basis of this specificity. On the basis of our present data, one may propose two general hypotheses to explain the specificity of the enzyme. It may be a true xyloglucanase in the sense that it recognizes structural features in the side chains or backbone, but requires, for example, a soluble substrate with a relatively long sequence of residues which are unsubstituted at any position other than C-6. Celluloses and cello-oligosaccharides of DP > 6 are insoluble; artificial cellulose derivatives have a relatively high frequency of substitution at C-2 and C-3; soluble cello-oligosaccharides, konjac glucomannan, galactoglucomannans, and barley-β-glucan have relatively short regions of contiguous (1→4)-β-linked D-glucose residues.

Clearly, the natural substrate of the present enzyme is the storage xyloglucan in the cotyledonary cell walls of the nasturtium seed. The enzyme does, however, hydrolyze other xyloglucans including the fucose-containing primary cell-wall type. Primary cell-wall xyloglucans are now known to be major non-cellulosic polysaccharides in dicotyledonous plants (Darvill et al., 1980), and there is good evidence that they are turned over during elongation growth (Labsvith and Ray, 1974). Two cellulases (more correctly, endo-(1→4)-β-D-glucanases) have been isolated from elongating pea epicotyls (Byrne et al., 1975; Wong et al., 1977). Both hydrolyze cellulose, sodium carboxymethyl cellulose, and xyloglucan (Wong et al., 1977). The levels of both enzymes increase after auxin treatment and they may participate in the cell-wall charges associated with cell elongation. Although their natural substrate is not known, it has been suggested that it may be xyloglucan (Hayashi and Maclachlan, 1984). This is possible, but the participation of xyloglucan-specific endo-β-D-glucanases in the turnover of primary cell-wall xyloglucans must now be considered. Recently, York et al. (1984) have reported that a specific nonasaccharide product obtained by hydrolysis of a primary cell-wall type xyloglucan by Trichoderma viride endo-β-D-glucanase inhibits the auxin-stimulated elongation of pea-stem segments. This intriguing observation may indicate a wider regulatory role for those enzymes which...
catalyze the endo-hydrolysis of xyloglucan in vivo.

Although this is the first report of a xyloglucan-specific endo-(1→4)-β-D-glucanase, enzymes with a similar substrate specificity need not necessarily be uncommon in nature. The virtually universal practice of screening for endo-(1→4)-β-D-glucanase using sodium carboxymethyl cellulose or some other soluble cellulose derivative may simply have obscured their presence.

REFERENCES

Albersheim, P., Nevins, D. T., English, P. D., and Carr, A. (1967) *Carbohydr. Res.* 5, 340-345

Bauer, W. D., Talmadge, K. W., Keegstra, K., and Albersheim, P. (1973) *Plant Physiol.* (Bethesda) 51, 174-187

Aspinall, G. O., Greenwood, C. T., and Sturgeon, R. J. (1961) *J. Chem. Soc. (Lond.)* 3667-3674

Bergmeyer, H. U., Berndt, E., Schmidt, F., and Stark, H. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, U., ed) pp. 1196-1201, Verlag-Chemie, Weinheim, West Germany

Byrne, H., Christou, N. V., Verma, D. P. S., and MacLachlan, G. A. (1975) *J. Biol. Chem.* 250, 1012-1018

Courtois, J. E., and Le Dizet, P. (1974) *C. R. Acad. Sci. Ser. C* 278, 81-83

Darvill, A., McNeil, M., Albersheim, P., and Delmer, D. P. (1980) in *The Biochemistry of Plants* (Tobert, N. E., ed) Vol. 1, pp. 91-129, Academic Press, New York

Dawson, R. M. C., Elliott, D. C. Elliott, W. H., and Jones, K. M. (1982) *Data for Biochemical Research*, Clarendon Press, Oxford

Dea, I. C. M., Morris, E. R., Rees, D. A., Welsh, E. J., Barnes, H. A., and Price, J. (1977) *Carbohydr. Res.* 57, 249-272

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. (1956) *Anal. Chem.* 28, 350-356

Edwards, M., Dea, I. C. M., Bulpin, P. V., and Reid, J. S. G. (1985) *Planta* (Berl.) 163, 133-140

Gabriel, O. (1971) *Methods Enzymol.* 22, 565-578

Halliwell, G., and Rizk, M. (1970) *Biochem. J.* 116, 35-42

Hayashi, T., and MacLachlan, G. (1984) in *Structure, Function, and Biosynthesis of Plant Cell Walls, Proceedings of the Seventh Annual Symposium in Botany* (Dugger, W. M., and Bartnicki-Garcia, S., eds) pp. 433-434, University of California, Riverside, CA

Heinricher, E. (1988) *Flora (Jena)* 171, 165-168

Jakimow-Barras, N. (1973) *Phytochemistry* (Oxf.) 12, 1331-1339

Jayne, G., and Lang, F. (1963) in *Methods in Carbohydrate Chemistry* (Whistler, R. L., ed) Vol. 3, pp. 75-85, Academic Press, New York

Kato, K., and Matsuda, K. (1973) *Agric. Biol. Chem.* 37, 2045-2051

Kato, Y., and Matsuda, K. (1976) *Plant Cell Physiol.* 17, 1185-1198

Kooiman, P. (1960) *Acta Bot. Neerl.* 9, 208-219

Kooiman, P. (1981) *Red. Trac. Chimi. Pays-Box Belg.* 80, 849-865

Kooiman, P. (1987) *Phytochemistry* (Oxf.) 6, 1655-1763

Kurz, G., and Wallenfels, K. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, U., ed) pp. 1279-1282, Verlag-Chemie, Weinheim, West Germany

Labavitch, J. M., and Ray, P. M. (1974) *Plant Physiol.* (Bethesda) 53, 669-675

Laemmli, U. K. (1970) *Nature* 227, 680-685

Le Dizet, P. (1972) *Carbohydr. Res.* 24, 505-509

Lever, M. (1972) *Anal. Biochem.* 47, 273-279

McClure, B. V., Taravel, F. R., and Cheetham, N. W. H. (1982) *Carbohydr. Res.* 104, 285-297

Meier, H. (1960) *Acta Chem. Scand.* 14, 749-756

Meier, H., and Reid, J. S. G. (1982) in *Encyclopedia of Plant Physiology* (Loewus, F. A., and Tanner, W. W., eds) Vol. 13A, pp. 418-471, Springer-Verlag, Berlin

Reid, J. S. G. (1985) *Adv. Bot. Res.* 11, 125-155

Reiss, R. (1889) *Landwirtsch. Jahrb. Schweiz* 18, 711-765

Roe, J. H., and Rice, E. W. (1948) *J. Biol. Chem.* 173, 507-512

Saeman, J. F., Buhl, J. L., and Harris, E. E. (1945) *Ind. Eng. Chem.* *Anal. Ed.* 17, 35-37

Sedmak, J. J., and Grossberg, S. E. (1977) *Anal. Biochem.* 79, 544-552

Vogel, T., and Schleiden, M. J. (1839) *Poggendorf’s Ann. Physik Chem.* 46, 327-339

Wong, Y.-S., Finch, G. B., and Maclachlan, G. A. (1977) *J. Biol. Chem.* 252, 1402-1407

York, W. S., Darvill, A. G., and Albersheim, P. (1984) *Plant Physiol.* (Bethesda) 75, 295-297