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THE LIMIT DEXTRINASE OF THE BROAD BEAN (*Vicia faba* L.)*

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ABSTRACT

A limit dextrinase has been isolated from broad-bean flour and purified 800-fold. The enzyme readily hydrolysed branched α-dextrins containing maltosyl or maltotriosyl side-chains, pullulan, and amylopectin β-limit dextrin. Glycogen β-limit dextrins and amylopectin were slowly hydrolysed, but glycogens were barely susceptible to hydrolysis. There was no evidence for the presence of a second debranching enzyme in broad beans.

INTRODUCTION

Debranching enzymes, which hydrolyse the (1→6)-α-D-glucosidic inter-chain linkages in starch-type polysaccharides and in branched α-dextrins, have now been isolated from several animal, plant, and microbial sources[2]. For some enzymes, *e.g.*, muscle amylo-(1→6)-glucosidase, the preferred substrate is a polysaccharide, namely, glycogen phosphorylase limit-dextrin[2], whereas for others, *e.g.*, the cereal limit-dextrinases, enzyme action is greatest on substrates of low molecular weight[1]. The first, plant debranching-enzyme which could act on amylopectin (and its β-limit dextrin) was isolated in 1950–1951 from the potato and the broad bean[3]. Enzyme action resulted in an increase in the iodine-staining power and β-amylolysis limit of the substrates and a decrease in viscosity. With amylopectin and its β-limit dextrin, there were small increases in reducing power, equivalent to about 1 and 11% conversion into maltose, respectively. The enzyme was described, non-committally, as R-enzyme. With glycogen of normal chain-length (*i.e.*, ~12), there was little or no action, apart from a small change in the absorption spectrum of the polysaccharide–iodine complex[4]. However, with a glycogen of chain-length 18, there were small but significant increases in iodine-staining power and β-amylolysis limit (from 51 to 58%), suggesting that branched (1→4)-α-D-glucans having interior chain-lengths of a minimum of five D-glucose residues could be substrates[5].

Although normal glycogen was not hydrolysed by R-enzyme, α-amylolysis of

*Studies on Debranching Enzymes: Part IV. For Part III, see Ref. 1.

this glycogen gave a series of branched α-dextrins, which were then susceptible to R-enzyme, as shown by a substantial increase in reducing power[4]. From this and other evidence[6], it was concluded[7] that R-enzyme could act on substrates of both high and low molecular weight. During this period (1950–1958), substrates for the quantitative assay of the number of (1→6)-α-D-glucosidic linkages hydrolysed were not available, and the various experiments were carried out using preparations of unknown specific activity.

In 1959, MacWilliam and Harris[8], using alumina-column chromatography, reported the presence of two debranching enzymes in preparations from broad beans and also, from malted barley. One enzyme acted on amylopectin and its β-limit dextrin, but not on branched α-dextrins, and was referred to as R-enzyme, on the basis of the original nomenclature[3]. The second enzyme readily attacked branched α-dextrins, but apparently had no action on amylopectin, and was therefore called limit dextrinase. In view of doubts which have been expressed about the validity of these experiments[9], we have re-examined the specificity of the debranching-enzyme system from broad beans. Our results lead to the conclusion that only one enzyme is present, which can hydrolyse both α-dextrins and amylopectin. This enzymic activity will be referred to as limit dextrinase.

MATERIALS AND METHODS

Materials. — The following polysaccharides were laboratory samples prepared by standard methods: amylopectin, amylopectin β-limit dextrin, pullulan, rabbit-liver glycogen and its β-limit dextrin, and phytoglycogen A and its β-limit dextrin. Oligosaccharide substrates were prepared from mixed, salivary α-limit dextrins by paper chromatography on Whatman No. 17 paper, using the solvent system 1-propanol–ethyl acetate–water (14:2:10). All chemicals were supplied by British Drug House Chemicals Ltd., Poole, England, except the following. DEAE-cellulose (DE 52) was supplied by W. and R. Balston Ltd., Springfield Mill, Maidstone, Kent, and prepared for use as recommended by the manufacturers. Acetone was analytical grade "Pronalys" from May and Baker Ltd., Dagenham, England. 2-Mercaptoethanol was from the Sigma Chemical Company. Thin-layer plates of silica gel, coated to a thickness of 0.25 mm on aluminium sheets, were obtained from E. Merck, Darmstadt, Germany.

Analytical methods. — Thin-layer plates were developed in ethyl acetate–methanol–water[10] (52:36:13). The position of sugars was detected by spraying lightly with 9M sulphuric acid and subsequently heating for 10–20 min at 110°. Reducing sugars were determined by a modified Nelson–Somogyi reagent[11], either as recommended or on a smaller scale (1/5th that recommended, *i.e.*, mini-Nelson) to increase the sensitivity of the method; absorbances were measured at 600 nm. Total carbohydrate was determined by a phenol–sulphuric acid method[12]. Protein was determined by using a slightly modified Lowry reagent[13]; column fractions were monitored at E_{280} to estimate the protein content of the fractions. In both cases, allowance had to

be made for interference by mercaptoethanol. Inorganic phosphate was measured by the method of Allen[14].

For the iodine staining of amylopectins, samples (0.1 ml) were added to M hydrochloric acid (0.1 ml) and mixed throughly, and iodine solution (0.1% of iodine in 1% potassium iodide; 0.1 ml) and water (2.0 or 3.0 ml) were then added. After mixing, the absorbances at 680 nm or over the range 400–700 nm were measured. With glycogens, the sample (0.5 ml) was added to M hydrochloric acid (0.5 ml), iodine solution (0.5 ml), and water (2.0 ml).

Assay of limit-dextrinase activity. — Debranching activity was routinely measured by incubation of a suitably diluted enzyme solution (0.1 ml) with a solution of pullulan (5 mg/ml; 0.3 ml) and Tris–hydrochloric acid buffer (0.1M, containing 25mM mercaptoethanol; 0.1 ml). After incubation at 30°, samples (0.1 ml) were removed for measurement of reducing power by the normal Nelson–Somogyi method. Substrate and enzyme controls were also prepared and analysed. One unit of activity is defined as that amount of enzyme which will release one micromole of maltotriose per minute from pullulan at 30° under the above conditions, and is the same as that used for the assay of bacterial pullulanase preparations[15]. For convenience, some activities are expressed in milliunits (mU).

Assay of phosphorylase activity. — A solution of rabbit-liver glycogen (200 mg) and D-glucopyranosyl phosphate (dipotassium salt, 372 mg) in 0.2M sodium fluoride (10 ml) was adjusted to pH 6.1 and used as substrate. Digests containing substrate (0.1 ml) and suitably diluted enzyme solution (0.1 ml) were incubated at 30° for 20 min. The reaction was terminated by adding ice-cold 10% trichloroacetic acid (TCA) (1 ml), and the digests were stored at 0° until analysed. The mixtures were centrifuged, and samples (0.5 ml) were diluted with water (0.5 ml) for analysis of inorganic phosphate.

When digests were incubated over a period of hours, toluene was present.

Polyacrylamide gel-electrophoresis. — The method used was based on that of Tombs and Akroyd[16]. 7.5% Gels were prepared using "Cyanogum 41", ammonium persulphate, and 2-(dimethylamino)ethyl cyanide as accelerator. The gel buffer was Tris–hydrochloric acid (0.1M, pH 8.9). The enzyme sample (10 μl) was applied in 10% sucrose/0.01% Bromophenol Blue. The electrode buffer was Tris–glycine (0.01M, pH 8.75). Electrophoresis was carried out using the Shandon Analytical Polyacrylamide Electrophoresis apparatus at a current of 1–2 mA per gel. The gels were stained for protein in Coomassie Blue (0.04% in 12.5% TCA) for 20 min and then destained in 10% TCA overnight.

Purification of the debranching enzyme. — All operations after the initial extraction were carried out at 0–5°. Apparatus was precooled before use, and column fractionations were carried out in a cold room (2–3°). The enzyme was relatively unstable during purification, but the loss of activity could be minimised if the enzyme solution was maintained in the presence of mercaptoethanol. Accordingly, at all steps of purification after the ammonium sulphate fractionation, the enzyme solution was maintained at 10mM with respect to this reagent.

Broad-bean flour was defatted by stirring in dichloromethane at −20°, followed by filtration on a Buchner funnel. The flour was washed two or three times in the funnel with the cold solvent until the filtrate was clear, and the flour was then spread out to air-dry.

The flour (200 g) was extracted by stirring in distilled water (600 ml) for 1 h at room temperature (18–20°). The creamy suspension was then quickly centrifuged at low speed (1500 *g*), and the supernatant solution was further centrifuged at a higher speed (40,000 *g*, 15 min, 3°).

The supernatant solution (*Initial extract*: volume usually ~400 ml) was stirred gently while solid ammonium sulphate was added slowly to a final concentration of ~35% saturation (209 g/litre of solution). After the addition was complete, the solution was left to stand for 15 min before centrifugation at 40,000 *g* as above. The precipitate was collected and resuspended in a small volume of ice-cold Tris–hydrochloric acid buffer (0.01M; pH 7.7) which contained sodium chloride to 0.1M and mercaptoethanol to 10mM. The solution was dialysed against 6 litres of the same buffer with efficient stirring for 3 h.

The dialysed enzyme solution (usually ~60 ml) was fractionated with acetone as follows. Cold analytical-grade acetone (at −20°) was trickled slowly, in small volumes, down the side of a flask in which the enzyme solution was stirring gently at 0°, to a final concentration of ~40% v/v. The solution was left to stand for 5 min before centrifugation at 40,000 *g*. The supernatant solution was retained and brought to a final concentration of ~70% v/v of acetone as described above, and the solution was left for 10 min before centrifugation. The relatively small precipitate was collected, resuspended in the minimum volume of buffer (as above), and dialysed overnight against 6 litres of the same buffer.

The dialysed enzyme-solution (40–70% acetone fraction; usually ~20 ml) was applied to a column of DEAE-cellulose (15 × 3 cm; previously equilibrated in the same buffer) and was well-washed through with the buffer. After elution of material not binding to the column, a continuous gradient of sodium chloride was applied in the buffer (0.1–0.5M). The debranching activity was eluted at ~0.17M sodium chloride.

The appropriate enzyme fractions were combined and concentrated in an Amicon ultrafiltration cell (Amicon Corporation, Lexington, Mass., U.S.A.) with a 30,000 molecular weight cut-off. At ~10-ml volume, a sample was usually withdrawn and kept for analyses. The solution was then further concentrated to a small volume (1–2 ml) and applied to a column (60 × 2.5 cm) of Sephadex G-150 freshly equilibrated with Tris–hydrochloric acid buffer (0.02M; pH 7.0) containing 10mM mercaptoethanol. The appropriate enzyme fractions were combined, and concentrated as above to a volume of 7–8 ml. The enzyme solution had a final specific activity of ~1 unit/mg of protein, representing a purification of ~800-fold (Table I).

Examination of the final preparation. — The enzyme preparation was free from alpha-amylase, beta-amylase, α-D-glucosidase, and D-enzyme activities, since on prolonged incubation with maltoheptaose (16 h), no change was observed either in the contents (by t.l.c.) or reducing power of the digest. It was, however, possible to detect

TABLE I

ANALYTICAL FIGURES FOR THE PURIFICATION STAGES OF THE DEBRANCHING-ENZYME PREPARATION[a]

Fraction	*Total units*	*Total protein (mg)*	*Yield (%)*	*Specific activity (Units/mg)*
1. Initial extract	30	23000	100	0.0013
2. 0–35% Ammonium Sulphate	20	4000	67	0.005
3. 40–70% Acetone	10	200	33	0.05
4. DEAE Fractions	5	25	17	0.20
5. G-150 Fractions	2.7	2.6	9	1.04

[a]Typical figures are given starting from 200 g of defatted flour. The overall purification is about 800-fold. The yield is ~9%; however, the actual yield will be slightly higher because of over-estimation of the activity in the initial extract due to the presence of α-D-glucosidase, which hydrolyses maltotriose with the production of new reducing-groups.

a trace of phosphorylase in the preparation equivalent to ~0.2% of the original phosphorylase activity. The enzyme digests used in specificity studies were therefore kept free of inorganic phosphate at all times. In this way, it was ensured that the small amount of phosphorylase present would not interfere with experimental results.

RESULTS

Properties of the enzyme

Homogeneity of the enzyme. — A concentrated sample of the final preparation in 10% sucrose was loaded on to a 7.5% gel as described in the Methods section. Electrophoresis was carried out at pH 8.75, maintaining a current of 2 mA per gel, and the gels were stained for protein. One major band appeared initially, but on destaining overnight, a faint, second band appeared.

Molecular weight. — As judged from the elution point of the enzyme from a calibrated column of Sephadex G-150, the molecular weight of the enzyme is of the order of 80,000.

pH Optimum. — The pH optimum of the enzyme was determined with both pullulan and amylopectin as substrates in the presence of citrate–phosphate buffer. Maximum activity occurred over a relatively broad range, the optimum for both substrates being pH 6.4–6.8. This result is in good agreement with that (6.5–7.0) reported by Hobson *et al.*[3], obtained by iodine-staining measurements using amylopectin β-limit dextrin.

Specificity of the enzyme

Action on polysaccharides. — The action of the debranching enzyme on amylopectin, amylopectin β-limit dextrin, and pullulan was examined under the following conditions. Digests contained substrate (5 mg/ml; 1.0 ml), buffer [Tris–hydrochloric acid (0.1M, pH 7.0) and mercaptoethanol to 50mM; 0.1 ml], and enzyme

TABLE II

ACTION OF BROAD-BEAN DEBRANCHING ENZYME ON VARIOUS POLYSACCHARIDES[a]

Substrate	*Time (h)*				
	0.33	*0.67*	*1.0*	*2.0*	*3.0*
Amylopectin	0.06	0.10	0.14	—	—
Amylopectin β-limit dextrin	0.37	0.70	1.00	—	—
Phytoglycogen A	—	—	0.03	0.10	0.15
Phytoglycogen A β-limit dextrin	—	—	0.29	0.41	0.51
Rabbit-liver glycogen	—	—	0.05	0.08	0.13
Rabbit-liver glycogen β-limit dextrin	—	—	0.22	0.37	0.52
Pullulan	0.23	0.48	0.83	—	—

[a] Results are shown as the increase in reducing power (E_{600}).

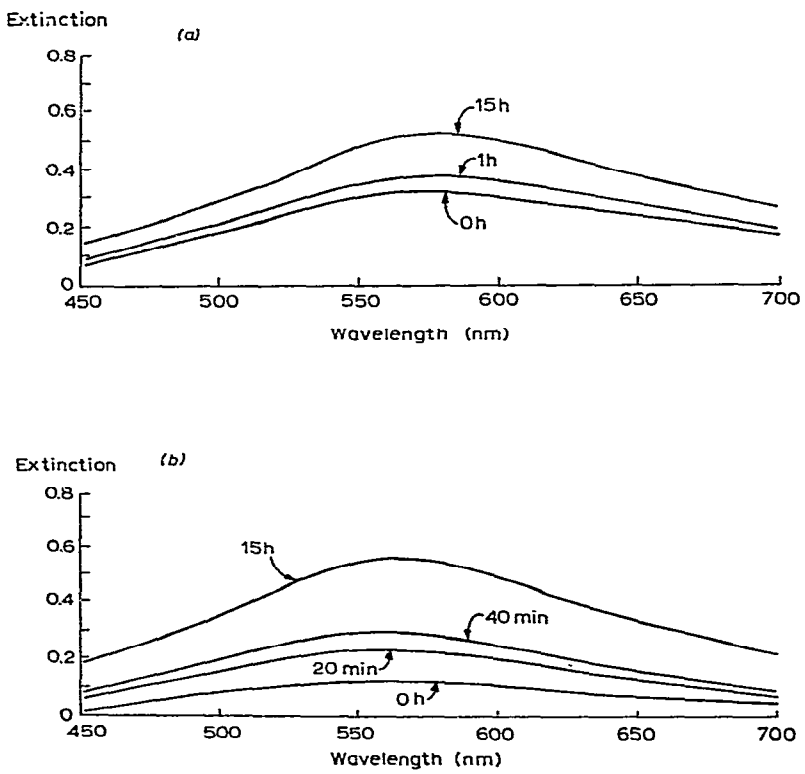

Fig. 1. Effect of broad-bean limit-dextrinase on the iodine-staining properties of (*a*) amylopectin, (*b*) amylopectin β-limit dextrin. Experimental conditions are given in the text.

solution (0.1 ml; 130 mU/ml), and these were incubated at 30°. Duplicate samples (0.1 ml) were removed at intervals for analysis. The increase in reducing power is shown in Table II, and the increase in iodine-staining power of amylopectin and its β-limit dextrin in Fig. 1. The relative, initial rate of attack on pullulan, amylopectin β-limit dextrin, and amylopectin, respectively, was approximately 100, 150, and 25. The digests were further incubated overnight (16 h) and the products examined by t.l.c. The results are given in Table III.

TABLE III

OLIGOSACCHARIDE PRODUCTS FROM DEBRANCHING OF VARIOUS SUBSTRATES

Substrate	*Oligosaccharides produced*[a]
Amylopectin	G_7–G_8 and higher
Amylopectin β-limit dextrin	G_2, G_3, (G_4), and G_6 upwards
Pullulan	G_3
6^3-α-Maltosylmaltotriose	G_2, G_3
6^3-α-Maltosylmaltotetraose	G_2, G_4, (G_3)[b]
6^3-α-Maltotriosylmaltotriose	G_3

[a]G_2, G_3, G_4 ... represent linear malto-saccharides of d.p. 2, 3, 4, etc.; minor products are shown in parenthesis. [b]The trace of G_3 came from traces of 6^3-α-maltotriosylmaltotriose which contaminated the substrate.

Enzyme action on rabbit-liver glycogen, phyto-glycogen A, and their β-limit dextrins was examined as follows. Substrate (4.0 ml; 5 mg/ml) was incubated with enzyme (0.5 ml; 130 mU/ml) and buffer (0.5 ml; as above). Duplicate samples were taken for reducing power (0.1 ml for mini-Nelson) and iodine-staining analysis (0.5 ml). Under these conditions, only limited evidence of debranching of the parent substrates was obtained, but the β-limit dextrins were readily attacked (Table II).

Action on oligosaccharides. — The action of the debranching enzyme on various oligosaccharide substrates (isomaltose, panose, 6^3-α-maltotriosylmaltotriose, 6^3-α-D-glucosylmaltotriose, 6^3-α-maltosylmaltotriose, 6^3-α-maltosylmaltotetraose, 6^3-α-maltotriosylmaltotetraose, and maltoheptaose) was studied. Digests contained substrate (0.8 ml; 2 mg/ml), buffer (0.1 ml; as above), and enzyme (0.1 ml; 240 mU/ml). Duplicate samples (0.1 ml) were taken initially and at intervals, and analysed for reducing power[11].

The oligosaccharides 6^3-α-D-glucosylmaltotriose, isomaltose, and panose, and the linear substrate maltoheptaose (included to test the purity of the preparation) were not hydrolysed. The remaining substrates were attacked at varying rates. The initial rate of attack on these substrates is given in Table IV. Thus, 6^3-α-maltotriosylmaltotetraose was the most susceptible substrate and 6^3-α-maltosylmaltotriose was the least readily attacked.

The products of digests similar to these were examined by thin-layer chromatography, and the results are given in Table III. The products were those to be expected

TABLE IV

RELATIVE, INITIAL RATE OF ATTACK ON SOME α-LIMIT DEXTRINS BY THE BEAN DEBRANCHING-ENZYME

Substrate	*Initial rate*[a]
6^3-α-Maltosylmaltotriose	75
6^3-α-Maltosylmaltotetraose	195
6^3-α-Maltotriosylmaltotriose	210
6^3-α-Maltotriosylmaltotetraose	320

[a]Relative to pullulan = 100.

from debranching, and confirm the absence of contaminating enzymes in the enzyme preparation.

Kinetic studies. — Digests contained amylopectin β-limit dextrin (0.5 ml; 5 mg/ml), buffer (0.1 ml; as previously), and enzyme and/or water to a total digest volume of 1.0 ml, representing an enzyme concentration of 0–32 mU/ml. The reducing power was determined on duplicate samples (0.1 ml) after 30 min. The result was a linear plot, indicating that, under these conditions, the rate of hydrolysis was proportional to enzyme concentration.

The kinetic values K_m and V_{max} were determined for a range of substrates. The initial rate of hydrolysis of each substrate over an appropriate range of substrate concentrations was determined, and velocity values (v) were calculated. For example, with amylopectin, digests contained substrate (0.8 ml; 0.39–12.5 mg/ml), buffer (0.1 ml; as previously), and enzyme (0.1 ml; 250 mU/ml). The digest was incubated at 30°, and duplicate samples (0.1 ml) were taken at 0, 30, 60, and 90 min for reducing-power analysis (mini-Nelson). From the Lineweaver–Burk plots, the values for K_m and V_{max} were estimated for the various substrates (Table V).

TABLE V

KINETIC PROPERTIES OF BROAD-BEAN LIMIT DEXTRINASE WITH VARIOUS SUBSTRATES

Substrate	K_m *(mg/ml)*	V_{max} *(relative units)*
Amylopectin	1.2	0.10
Amylopectin β-limit dextrin	1	0.65
Rabbit-liver glycogen β-limit dextrin	17	0.50
6^3-α-Maltotriosylmaltotetraose	0.18	—

DISCUSSION

Broad-bean flour is a rich source of both carbohydrate and reserve protein, but the presence of the latter hampers enzyme-purification studies. In the present work, acetone fractionation has provided a useful means of concentrating the debranching enzyme, and the final preparation had a specific activity which was similar to that of

limit-dextrinase preparations from ungerminated oats and rice[1]. Pullulan has again provided a useful substrate for quantitative assays.

It should be noted that the suggestion of MacWilliam and Harris[8] of the existence of two broad-bean debranching-enzymes was based on a combination of iodine-staining measurements on amylopectin and reducing-power measurements on α-dextrins. However, those enzyme fractions (obtained by alumina-column chromatography) showing limit-dextrinase activity also contained alpha-amylase, so that any conclusions on the ability of these fractions to act on amylopectin would be invalidated. For those fractions which showed neither limit-dextrinase nor alpha-amylase activity, the observed increase in iodine-staining power was relatively small, and other characteristics of debranching (*e.g.*, increase in β-amylolysis limit) were not reported. Hence, their evidence for two debranching enzymes cannot now be regarded as conclusive. On repetition of the alumina-column chromatography, we have been unable to obtain fractions which would increase the iodine-staining power of amylopectin without hydrolysing pullulan. Our results, obtained with DEAE-cellulose and Sephadex G-150 columns, gave amylase-free enzyme preparations, and suggest that only one debranching enzyme is present in broad-bean flour.

This enzyme is very similar in specificity to the cereal limit-dextrinases[1,17]. It will slowly debranch amylopectin with the liberation of maltosaccharides d.p. $\geqslant 7$, but the rate of hydrolysis is only $\sim 15\%$ of that of the corresponding β-limit dextrin. In agreement with previous results[4], glycogens are not readily attacked, although the derived β-limit dextrins are suitable substrates. Oligosaccharides containing (1→6)-linked α-D-glucosyl side-chains (*e.g.*, 6^3-α-D-glucosylmaltotriose) are not attacked, but α-dextrins with α-maltosyl or α-maltotriosyl side-chains are readily hydrolysed; the branched heptasaccharide is, in fact, hydrolysed initially at about three times the rate of pullulan.

It now seems probable that the natural substrates for plant debranching-enzymes are α-dextrins of low molecular weight. Dunn[18] has recently proposed a model for the *in vivo* degradation of starch in which alpha-amylase plays a key role as the only enzyme capable of degrading a starch granule. The other starch-metabolising enzymes, namely, beta-amylase, α-D-glucosidase, limit dextrinase, and possibly phosphorylase, are considered to be supplementary enzymes involved in the conversion of α-dextrins into monosaccharide. According to this model, limit dextrinase would be involved in the conversion of branched α-dextrins into linear maltosaccharides, which then serve as substrates for the other enzymes.

In view of the *in vivo* role and substrate specificity of the plant debranching-enzymes, it is unfortunate that the Enzyme Nomenclature Commission[19] has recommended the name pullulan 6-glucanohydrolase (E.C. 3.2.1.41; other name, pullulanase). Although pullulan is undoubtedly a convenient substrate for the assay of these enzymes, it is not the natural substrate, and we consider that the term α-dextrin 6-glucanohydrolase (other name, limit dextrinase) more correctly describes these enzymes.

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REFERENCES

1 G. DUNN AND D. J. MANNERS, *Carbohyd. Res.*, 39 (1975) 283–293.
2 E. Y. C. LEE AND W. J. WHELAN, in P. D. BOYER (Ed.), *The Enzymes*, Vol. 5, Academic Press, New York, 3rd edition, 1971, pp. 192–234.
3 P. N. HOBSON, W. J. WHELAN, AND S. PEAT, *J. Chem. Soc.*, (1951) 1451–1459.
4 S. PEAT, W. J. WHELAN, P. N. HOBSON, AND G. J. THOMAS, *J. Chem. Soc.*, (1954) 4440–4445.
5 I. D. FLEMING AND D. J. MANNERS, *Chem. Ind. (London)*, (1958) 831–832.
6 W. J. WHELAN AND P. J. P. ROBERTS, *Nature (London)*, 170 (1952) 748–749.
7 W. J. WHELAN, *Biochem. Soc. Symp.*, No. 11 (1953) 17–26.
8 I. C. MACWILLIAM AND G. HARRIS, *Arch. Biochem. Biophys.*, 84 (1959) 442–454.
9 G. S. DRUMMOND, E. E. SMITH, AND W. J. WHELAN, *FEBS Lett.*, 9 (1970) 136–140.
10 C. N. HUBER, H. D. SCOBELL, AND HAN TAI, *Cereal Chem.*, 43 (1966) 342–346.
11 J. F. ROBYT AND W. J. WHELAN, in J. A. RADLEY (Ed.), *Starch and its Derivatives*, Chapman and Hall, London, 4th edition, 1968, pp. 432–433.
12 M. DUBOIS, K. A. GILLES, J. K. HAMILTON, P. A. REBERS, AND F. SMITH, *Anal. Chem.*, 28 (1956) 350–356
13 G. MILLER, *Anal. Chem.*, 31 (1959) 964.
14 R. J. L. ALLEN, *Biochem. J.*, 34 (1940) 858–865.
15 H. BENDER AND K. WALLENFELS, *Methods Enzymol.*, 8 (1966) 555–559.
16 M. P. TOMBS AND P. AKROYD, Shandon Instrument Applications, No. 18, 1967.
17 G. DUNN, D. G. HARDIE, AND D. J. MANNERS, *Biochem. J.*, 133 (1973) 413–416.
18 G. DUNN, *Phytochemistry*, 13 (1974) 1341–1346.
19 Enzyme Nomenclature Recommendations, Elsevier, Amsterdam, 1972, pp. 218–219.