Carbohydrate Research, 75 (1979) 221–229

PURIFICATION AND CHARACTERISATION OF TWO ENDO-(1→3)-β-D-GLUCANASES FROM *Telescopium telescopium*

ROBERT L. CUTLER AND DAVID YELLOWLEES*

Department of Chemistry and Biochemistry, James Cook University of North Queensland, Townsville (Australia)

(Received December 5th, 1978; accepted for publication, January 31st, 1979)

ABSTRACT

The crystalline style of the gastropod *Telescopium telescopium* contains two (1→3)-β-D-glucanases and a β-D-glucosidase. The two glucanases (I and II) have been purified and shown to be endo-enzymes. Both enzymes attack laminarin, carboxymethylpachyman, and lichenin, but have no action towards carboxymethylcellulose. The main products of hydrolysis of laminarin are D-glucose and β-(1→3)-linked oligosaccharides of d.p. 2, 3, and 4. Glucanases I and II are similar to each other, although they differ in molecular weight and kinetic properties.

INTRODUCTION

The presence of (1→3)-β-D-glucanases in the crystalline style of bivalves has been reported by several workers[1,2], and the enzymes from *Spisula sachalinensis*[3,4] and *Chlamys abbidus*[5] have been purified and characterised. However, only one brief reference has been made to the occurrence of laminarinase in the crystalline style of gastropod molluscs[6]. We now report on the occurrence of two (1→3)-β-D-glucanases and a β-D-glucosidase in the style of the gastropod *Telescopium telescopium*. The two (1→3)-β-D-glucanases have been purified and some of their properties investigated.

EXPERIMENTAL

Materials. — CM-Cellulose (carboxymethylcellulose, high viscosity) was obtained from B.D.H. Chemicals Ltd., Poole, England. Laminarin, lichenin, and *p*-nitrophenyl β-D-glucopyranoside were purchased from Koch–Light Laboratories, Colnbrook, England. *Poria cocos* was kindly supplied by Shibata & Co. Ltd., Tokyo. Protein molecular-weight standards were purchased from Boehringer Mannheim. All ion-exchange and gel-chromatography media were obtained from Pharmacia South Seas.

*To whom communications should be addressed.

Substrates. — Pachyman was prepared from *Poria cocos* as described by Whelan[7]. Carboxymethylpachyman was prepared by the method of Clarke and Stone[8]. Laminarin (1 g) was reduced with sodium borohydride (100 mg) in aqueous solution (100 ml), prior to use as substrate, to eliminate blank values obtained with the Nelson–Somogyi reducing-power assay. Excess of borohydride was oxidised by addition of Amberlite IR-120(H^+) resin, and the borate removed by repeated evaporation under reduced pressure with methanol. The reduced laminarin was used as laminarin substrate unless indicated otherwise.

Periodate-oxidised laminarin was prepared by treatment of laminarin (100 mg) with 0.1M sodium metaperiodate (70 ml) at 4° in the dark for 72 h. Excess of periodate was reduced by addition of glycerol, and the resulting solution was dialysed against distilled water.

General methods. — Protein was determined by a modified Folin–Ciocalteau method[9], or by column chromatography monitored by measuring the absorbance at 280 nm. Reducing sugar was determined by a modified Nelson–Somogyi method[10] on one-half of the scale recommended. The absorbance was read at 600 nm, and D-glucose was used as the standard. Paper chromatography was performed with ethyl acetate–pyridine–water (10:4:3) as solvent in a descending manner. The alkaline silver nitrate reagents of Trevelyan *et al.*[11] were used for detection of reducing sugars.

Assay of enzyme activity. — (1→3)-β-D-Glucanase activity was measured by incubating the enzyme (0.5 ml) with laminarin (8 mg) at 37° in the presence of 33mM sodium phosphate–citric acid buffer (pH 5.8) in a total volume of 2 ml. Aliquots were removed at intervals and assayed for reducing power.

β-D-Glucosidase activity was measured in a digest containing ρ-nitrophenyl β-D-glucopyranoside (0.5 mg), 50mM sodium phosphate–citric acid buffer (pH 5.2), and enzyme in a total volume of 1.5 ml. Digests were incubated at 37° before removing aliquots (0.2 ml) for addition to 3 ml of 12.5mM sodium hydroxide. The absorbance was read at 400 nm.

Extraction and DEAE-Sepharose ion-exchange chromatography of crystalline styles. — About 600 molluscs were dissected and the crystalline styles (62.9 g) washed with 20mM sodium phosphate buffer (pH 7.0). The styles were ground in a mortar with acid-washed sand at 4° in the presence of enough 20mM sodium phosphate buffer (pH 7.0) to effect solubilisation. The extract was centrifuged at 4000g for 10 min and made up to 335 ml with buffer. The viscous solution was applied to a column (38 × 2.5 cm) of DEAE-Sepharose equilibrated with the extraction buffer, and 50 ml of the same buffer were then passed through the column before applying a gradient of 0→0.5M sodium chloride in buffer (500 ml).

Purification of Glucanase I. — The Glucanase I peak (Fig. 1) obtained from the DEAE-Sepharose column was concentrated to 50 ml by using a Millipore, immersible, molecular-separation membrane, and then applied to a Sephadex G-100 column (80 × 2.6 cm) equilibrated with 15mM sodium phosphate–citric acid buffer (pH 6.1). Active fractions were combined (67 ml), and the pH was adjusted to 5.0 with 0.1M

citric acid before application to a column (12 × 0.9 cm) of CM-Sephadex C-25 equilibrated with 15mM sodium phosphate–citric acid buffer (pH 5.0). Buffer (10 ml) was passed through the column before a gradient of 0→0.5M sodium chloride in buffer (50 ml) was applied. Active fractions were combined and used for further experiments.

Purification of Glucanase II. — The Glucanase II fractions from the DEAE-Sepharose column (Fig. 1) were combined (90 ml) and applied to a column (80 × 2.6 cm) of Sephadex G-100 equilibrated with 15mM sodium phosphate–citric acid buffer (pH 6.1). Active fractions were combined and applied to a column (12 × 0.9 cm) of DEAE-Sepharose equilibrated with the same buffer. The sample was washed through the column with 10 ml of buffer followed by a 0→0.25M sodium chloride gradient in buffer (50 ml). Active fractions were combined and used for further studies.

Effect of pH on Glucanases I and II. — The respective Glucanase I and II activities towards laminarin were measured at various pH values, using 50mM sodium phosphate–citric acid buffer (pH 3.6–7.6).

Determination of molecular weight. — Molecular weights of Glucanases I and II were determined with a column of Sephadex G-75 (80 × 2.6 cm) equilibrated with 15mM sodium phosphate–citric acid buffer (pH 5.8). The column was calibrated with cytochrome C (mol. wt. 13,000), chymotrypsinogen A (25,000), pepsin (35,500), ovalbumin (45,000), and bovine serum albumin (67,000).

Action of Glucanases I and II towards polysaccharides. — Polysaccharide (5 mg) was incubated at 37° with Glucanase I (0.02 unit) or Glucanase II (0.04 unit) in the presence of 33mM sodium phosphate–citric acid buffer (pH 5.8) in a total volume of 1 ml. Samples (0.2 ml) were removed at intervals and assayed for reducing power.

Products of hydrolysis of laminarin with Glucanase I and Glucanase II. — Laminarin (2 mg) was incubated at 37° with 0.1 ml of enzyme and 0.1 ml of water. Samples were removed at intervals and examined by paper chromatography as described previously.

RESULTS

Purification of Glucanases I and II. — Initial dissolution of the crystalline style was effected at pH 7.0, as much of the structural protein is insoluble in acid and interferes with the extraction if not fully dissolved. Ion-exchange chromatography of the extract on DEAE-Sepharose (Fig. 1) indicated the presence of three enzymes capable of hydrolysing laminarin. The two major peaks were named Glucanases I and II in order of their elution from the column. The third activity forms a shoulder of the Glucanase II peak and coincides with the presence of a β-D-glucosidase activity. The gel filtration on Sephadex G-100 of Glucanase I (Fig. 2) and Glucanase II (Fig. 3) activities removed them from most of the contaminating protein and also indicated their low molecular weights. Further purification of the two enzymes was achieved by ion-exchange chromatography on CM-Sephadex C-25 at pH 5.0 (Glucanase I)

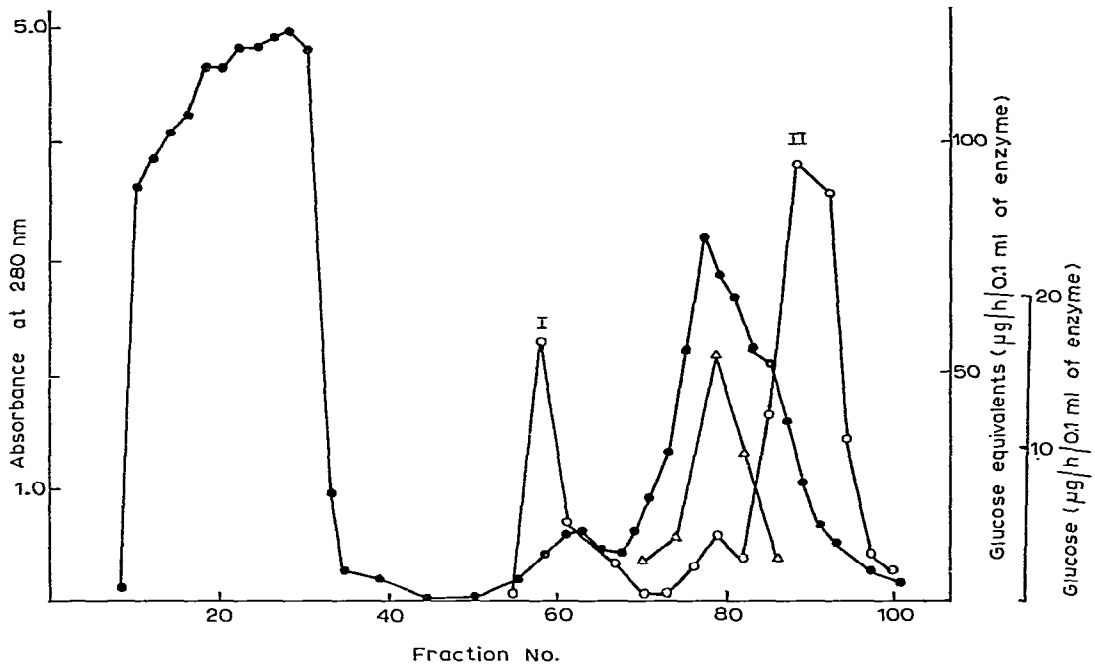

Fig. 1. Ion-exchange chromatography of *T. telescopium* crystalline-style extract on DEAE-Sepharose; —●—, absorbance at 280 nm; —○—, (1→3)-β-D-glucanase activity; —△—, β-D-glucosidase activity.

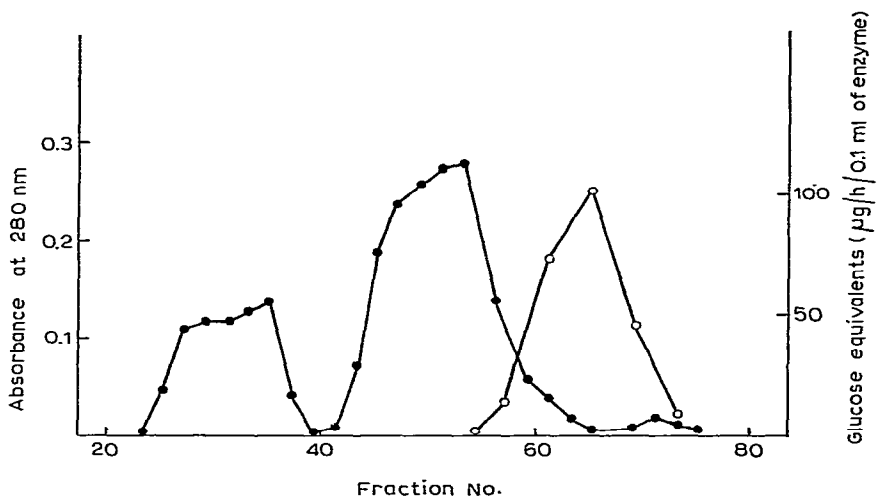

Fig. 2. Gel-permeation chromatography of Glucanase I on Sephadex G-100; —●—, absorbance at 280 nm; —○—, (1→3)-β-D-glucanase activity.

and DEAE-Sepharose at pH 6.1 (Glucanase II). Preliminary experiments showed that Glucanase I did not bind to anion or cation exchangers at pH 6.1, indicating that its isoelectric point is in that region.

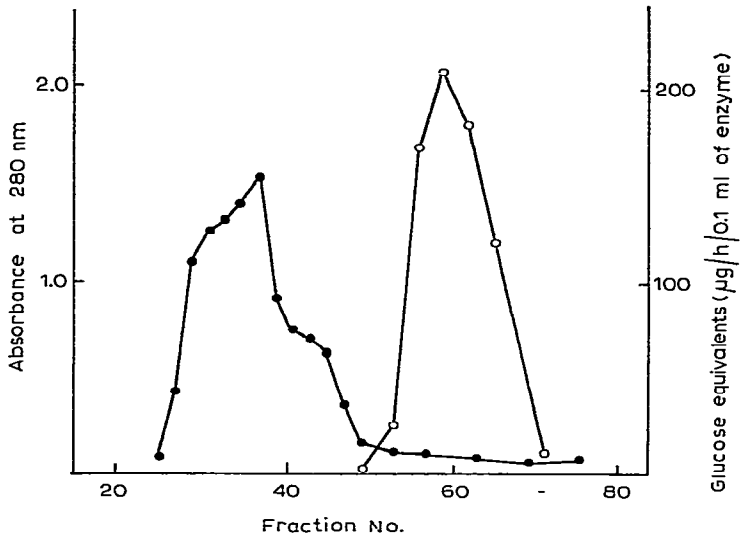

Fig. 3. Gel-permeation chromatography of Glucanase II on Sephadex G-100; —●—, absorbance at 280 nm; —○—, (1→3)-β-D-glucanase activity.

TABLE I

PURIFICATION OF GLUCANASE I

Fraction	*Volume (ml)*	*Protein (mg)*	*Total activity (U[a])*	*Specific activity (U/mg)*
Extract	335	5193	93	0.018
Eluate from DEAE-Sepharose	220	22	18.5	0.84
Eluate from Sephadex G-100	67	2.0	4.8	2.4
Eluate from CM-Sephadex C-25	18	0.33	2.5	7.6

[a]One unit is the amount of enzyme required to release 1 μmol of reducing sugar, expressed as glucose, per min.

TABLE II

PURIFICATION OF GLUCANASE II

Fraction	*Volume (ml)*	*Protein (mg)*	*Total activity (U[a])*	*Specific activity (U/mg)*
Extract	335	5193	93	0.018
Eluate from DEAE-Sepharose	90	223	40	0.18
Eluate from Sephadex G-100	103	15.4	22.5	1.46
Eluate from DEAE-Sepharose	31	4.0	6.7	1.66

[a]One unit is the amount of enzyme required to release 1 μmol of reducing sugar, expressed as glucose, per min.

A summary of the Glucanase I and II activities of the fractions obtained at each stage of the purification is given in Tables I and II, respectively.

Storage of the glucanases. — Glucanases I and II were stable when stored in solution at 4° over a pH range of 5–7 (Glucanase I) and 5.8–7.0 (Glucanase II). Glucanase I was inactivated by freezing.

Molecular weights of the glucanases. — The final preparations of Glucanases I and II showed single peaks of activity when chromatographed at the rate of 20 ml/h on a column of Sephadex G-75. It was determined that Glucanases I and II have molecular weights of 8,700 and 17,800, respectively.

pH Optima. — The pH optima for the two glucanases were determined by using reduced laminarin as substrate. Similar pH-profiles were obtained with each enzyme. These are illustrated in Fig. 4. Glucanase I showed a broad pH-optimum in the region of 5.0–6.5, whereas that for Glucanase II was pH 4.5–6.5.

Substrate specificity. — The relative rates of hydrolysis of various polysaccharides by Glucanases I and II were determined under the same conditions of substrate concentration and pH (Table III). Laminarin (*Laminaria hyperborea*) was hydrolysed at the greatest rate, while the reduced and periodate-oxidised derivatives are not so readily hydrolysed. CM-pachyman was attacked at 25–35% of the rate for laminarin. However, this low value may be partially due to the viscosity of the substrate solution.

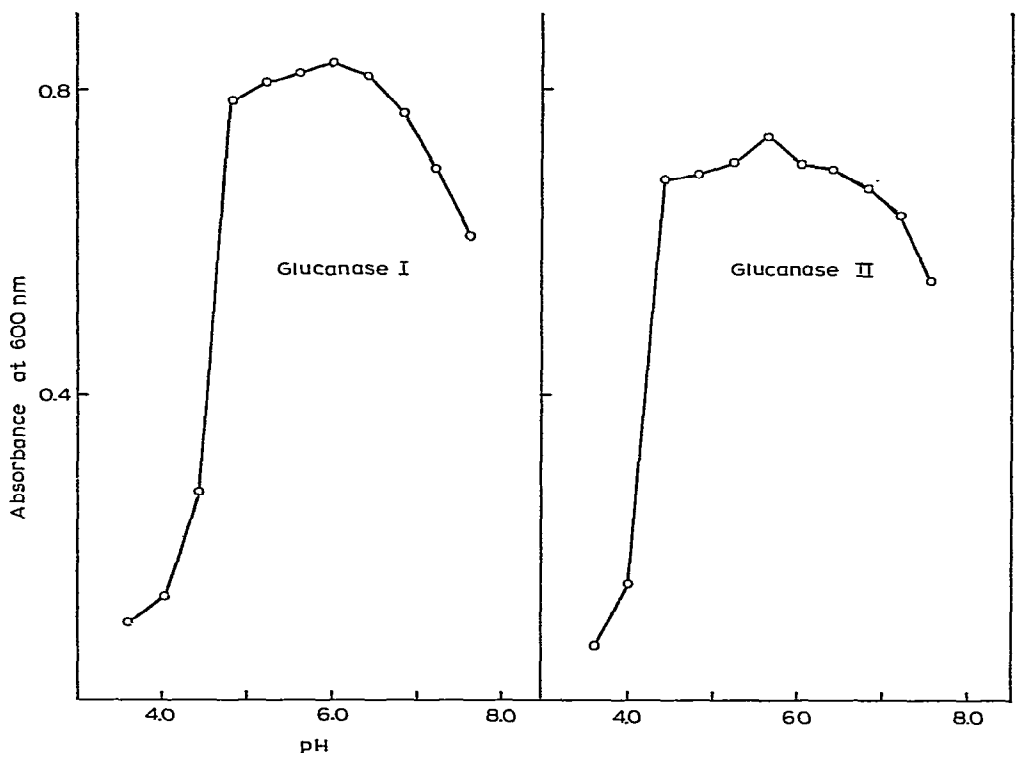

Fig. 4. pH Optima of Glucanases I and II against laminarin as substrate.

TABLE III

RELATIVE RATES OF HYDROLYSIS OF VARIOUS SUBSTRATES BY GLUCANASES I AND II

Substrate	*Glucanase I*	*Glucanase II*
Laminarin (native)	100	100
Laminarin (reduced)	60	85
Laminarin (periodate-oxidised)	85	90
CM-Pachyman	25	35
Lichenin	22	22
CM-Cellulose	0	0

The mixed (1→3),(1→4)-β-D-glucan lichenin was hydrolysed by both Glucanases I and II, but no activity was shown towards the β-(1→4) linkages of CM-cellulose.

The products of hydrolysis of laminarin with both Glucanase I and Glucanase II were glucose with lesser amounts of β-(1→3)-linked oligosaccharides of d.p. 2, 3, and 4.

Determination of K_m. — The Michaelis constants were determined with laminarin as substrate, using a substrate concentration range of 0.05–0.17 mg/ml for Glucanase I and 0.05–0.5 mg/ml for Glucanase II. Considerable substrate-inhibition occurred at higher concentrations of laminarin. The K_m for Glucanase I was 8.7 mg/ml, with V_{max} = 118 μmol glucose equivalents liberated per unit of enzyme per min. Glucanase II had K_m = 1.5 mg/ml, with V_{max} = 29 μmol glucose equivalents liberated per unit of enzyme per min.

DISCUSSION

The purification procedure reported in this communication allows the separation of two (1→3)-β-D-glucanases from the crystalline style of *Telescopium telescopium*. Glucanase I was purified by a factor of 422 with a 2.7% yield, while Glucanase II was purified 92-fold with a 7.2% yield. These figures are based upon the ratio of final activity to the activity of the initial extract; as such, they are only apparent values, since the original activity of the extract represents the combined activity of Glucanases I and II and the β-D-glucosidase towards laminarin. The purification and yield for both enzymes will therefore be much greater than the cited values.

The style from *T. telescopium* is similar to that of *Spisula sachalinensis*[3], in that each contains two (1→3)-β-D-glucanases, whereas *Chlamys abbidus*[5] contains only one such enzyme. However, unlike *T. telescopium*, no β-D-glucosidase capable of hydrolysing laminarin was detected in either of the two bivalves.

The results of gel-filtration studies indicated that Glucanase II (mol. wt. 17,800) was larger than Glucanase I (8,700). However both enzymes have relatively low molecular weights, as do most (1→3)-β-D-glucanases. The glucanases of *S. sachalinensis*[3] and *C. abbidus*[5] have molecular weights of 22,000 and 20,000, respectively.

The pH optima for Glucanases I and II are similar (Fig. 4). The peak is very broad for both enzymes, and the activity drops off very quickly on the acid side of the pH optimum. This latter fact may be due to the instability of the protein at pH < 4.5. During the purification, fractions became milky when the pH was adjusted to < 4.5.

The relative rates of hydrolysis of polysaccharides were of the same magnitude for both Glucanases I and II. Both enzymes hydrolysed unmodified laminarin (*Laminaria hyperborea*) at the greatest rate, whereas the reduced and periodate-oxidised derivatives were attacked at a lower rate. Both enzymes also had significantly less activity towards the (1→3)-β-D-glucan, CM-pachyman. This substrate was relatively viscous, and it is possible that the viscosity could account for the wide difference in rates of hydrolysis of laminarin and CM-pachyman.

Both enzymes were also active against the mixed-linkage glucan, lichenin, although the rate was less than a quarter of that for laminarin. It is presumed that the activity is towards the (1→3) linkages in the glucan and not the (1→4) linkages, as neither Glucanase I nor Glucanase II had any activity towards CM-cellulose. The enzymes from *T. telescopium* are therefore similar to the glucanases from *Spisula sachalinensis*[4] and *Chlamys abbidus*[5], which showed activity towards barley glucan and lichenin, respectively.

The activity towards the periodate-oxidised laminarin, where the non-reducing end-groups are modified, indicates that the mode of action of both glucanases is by an endo- rather than exo-attack[12]. Modification of the terminal D-glucosyl units at the non-reducing ends of the glucan would inhibit attack by an exo-enzyme. Little loss in activity was in fact observed. The hydrolysis of lichenin by both glucanases is also indicative that they are endo-(1→3)-β-D-glucanases (EC 3.2.1.6), as are the enzymes isolated from both bivalves investigated[4,5].

The range of products, observed by paper chromatography, from the action of both enzymes on laminarin confirms previous evidence for an endo-mechanism. The main product for both glucanases was glucose, while smaller but significant amounts of laminaribiose, laminaritriose, and laminaritetraose were produced. Similar patterns have been obtained for other endo-(1→3)-β-D-glucanases[13,14].

The only significant differences that have been observed between Glucanase I and Glucanase II, apart from their molecular weights and mobilities on DEAE-Sepharose, are the values for K_m and V_{max} using laminarin as substrate. Glucanase II (K_m = 1.5 mg/ml) has a much lower K_m for laminarin than Glucanase I (K_m = 8.7 mg/ml), and a much lower V_{max} (29 μmol glucose equivalents liberated per unit of enzyme per min) than that (118) for Glucanase I. This might possibly indicate some difference in function, or may simply be a means of coping adequately when large amounts of (1→3)-β-D-glucan are ingested.

The diet of *T. telescopium* is principally detritus and diatoms. The detritus is made up of broken-down marine algae, in addition to leaf material from the mangrove swamps where the mollusc is found. Laminarin is widespread in algae[15], whereas diatoms contain chrysolaminarin[16], a (1→3)-β-D-glucan that contains no mannitol at the reducing end of the glucan chain. The complex of two endo-(1→3)-β-D-

glucanases and a β-D-glucosidase in the crystalline style is therefore relevant to the needs of this herbivorous mollusc for the digestion of its food material[17].

REFERENCES

1 V. V. SOVA, L. A. ELYAKOVA, AND V. E. VASKOVSKY, *Comp. Biochem. Physiol.*, 32 (1970) 459–464.
2 J. H. KRISTENSEN, *Mar. Biol.*, 14 (1972) 130–142.
3 V. V. SOVA, L. A. ELYAKOVA, AND V. E. VASKOVSKY, *Biochim. Biophys. Acta*, 212 (1970) 111–115.
4 V. V. SOVA AND L. A. ELYAKOVA, *Biochim. Biophys. Acta*, 258 (1972) 219–227.
5 N. M. PRIVALOVA AND L. A. ELYAKOVA, *Comp. Biochem. Physiol. B*, 60 (1978) 225–228.
6 S. HORIUCHI AND C. E. LANE, *Comp. Biochem. Physiol.*, 17 (1966) 1189–1197.
7 W. J. WHELAN, *Methods Carbohydr. Chem.*, 1 (1962) 330–333.
8 A. E. CLARKE AND B. A. STONE, *Phytochemistry*, 1 (1962) 175–188.
9 G. L. MILLER, *Anal. Chem.*, 31 (1959) 964.
10 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.
11 W. E. TREVELYAN, D. P. PROCTER, AND J. S. HARRISON, *Nature (London)*, 166 (1950) 444–445.
12 T. E. NELSON, J. V. SCALETTI, F. SMITH, AND S. KIRKWOOD, *Can. J. Chem.*, 41 (1963) 1671–1678.
13 G. M. BALLANCE AND D. J. MANNERS, *Phytochemistry*, 17 (1978) 1539–1543.
14 K. HORIKOSHI AND Y. ATSUKAWA, *Agric. Biol. Chem.*, 37 (1973) 1449–1456.
15 A. T. BULL AND C. G. C. CHESTERS, *Adv. Enzymol.*, 28 (1966) 325–364.
16 E. PERCIVAL, in W. PIGMAN AND D. HORTON (Eds.), *The Carbohydrates*, Vol. IIB, Academic Press, New York, 2nd Edition, 1970, pp. 541–544.
17 R. L. CUTLER AND D. YELLOWLEES, *Comp. Biochem. Physiol.*, in press.