Purification of the major endoglucanase from *Aspergillus fumigatus* Fresenius

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Aspergillus fumigatus (Fresenius), IMI 246651, A.T.C.C. 46324, produces two β -glucosidase enzymes, cotton-solubilizing activity, xylanase and endoglucanase enzymes which can be separated by gel-filtration chromatography. The major endoglucanase does not bind to concanavalin A–Sephadex and does not stain with periodic acid/Schiff reagent. It is homogeneous on polyacrylamide isoelectric focusing ($pI = 7.1$) and has a mol.wt. of 12 500 by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The endoglucanase produces glucose and a mixture of oligosaccharides from cellulose; the purified enzyme has a small dextranase activity. It is stable at 50°C and pH 6.

Cellulase enzyme complexes from micro-organisms have been fractionated into their component enzymes by using chromatography and electrophoresis. A number of enzymes have been fully characterized and their modes of action determined, particularly their ability to act synergistically with one another. The cellulolytic enzyme complex may contain three types of enzyme activity: exoglucanase (EC 3.2.1.91), which in a number of cases has been shown to be a cellobiohydrolase (Wood & McCrae, 1972; Berghem & Pettersson, 1973; Halliwell & Griffin, 1973; Berghem *et al.*, 1975) and endoglucanase (EC 3.2.1.4) acting on the more amorphous types of cellulose in a random manner. This enzyme produces a greater decrease in the viscosity of CM-cellulose per unit increase in reducing sugar liberated than does the exoglucanase (Selby & Maitland, 1965, 1967; Wood, 1971). β -Glucosidase (EC 3.2.1.21) is the third enzyme in the complex.

King (1963) pointed out that attack on cellulose by a series of enzymes, some acting in a random manner and others in an end-wise fashion, would facilitate the degradation of cellulose chains, as the more randomly acting enzymes would increase the number of chain ends available to the end-wise-cleaving components. Wood & McCrae (1972) and Berghem *et al.* (1975) amplified this, suggesting that

the role of initiating the attack on native cellulose can be assigned to the endoglucanase enzymes and not to the 'C₁' enzymes as originally proposed by Reese *et al.* (1950).

Several glycosidase activities have been noted in culture filtrates of *Aspergillus fumigatus*, and three of these have been purified (Rudick & Elbein, 1973, 1975). We have recently investigated some of the factors influencing the production of cellulases by *A. fumigatus* (Fresenius) (Stewart & Parry, 1981) and now report on the fractionation of the enzyme components found in culture filtrates of *A. fumigatus* and the purification of the major randomly acting endoglucanase.

Materials and methods

Organism

The strain of *Aspergillus fumigatus* Fresenius, IMI 246651, A.T.C.C. 46324, has been described elsewhere (Stewart & Parry, 1981).

Growth of organism

Media for stock culture maintenance and enzyme preparation were described previously (Stewart & Parry, 1981). Incubations were carried out in 2-litre baffled conical flasks containing 1 litre of medium, in a rotary shaker at 120 rev./min, at 37°C for 4 weeks, unless otherwise stated. Medium was separated from the mycelium and unused cellulose by filtering through sintered-glass funnels (porosity 1).

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Concentration of culture filtrates

The filtrate was freeze-dried in 250 ml portions after initial freezing in liquid N₂. The dried material was dissolved in water (30 ml of water/freeze-dried litre of culture filtrate) for desalting on Bio-Gel P-6.

Chromatography on Bio-Gel P-6

The concentrated culture filtrate (30 ml) was applied to a column of Bio-Gel P-6 (350 mm × 32 mm) equilibrated in 0.1 M-citrate buffer, pH 5, and was eluted with this buffer at 11 ml/h. Fractions containing protein (A_{280}) were combined and dialysed in Visking tubing (18 mm diameter) against distilled water at 4°C overnight. Non-diffusible material was freeze-dried and redissolved in distilled water (2 ml) for chromatography on Bio-Gel P-60.

Chromatography on Bio-Gel P-60

The solution from the Bio-Gel P-6 column (2 ml), was applied to a Bio-Gel P-60 column (750 mm × 25 mm) equilibrated with 5 mM-citrate buffer, pH 5, and eluted with the same buffer at 9 ml/h. This column was calibrated with Blue Dextran (mol.wt. 2×10^6), bovine serum albumin (mol.wt. 68 000), egg albumin (mol.wt. 45 000), deoxyribonuclease (mol.wt. 31 000), α -chymotrypsinogen (mol.wt. 23 000), cytochrome *c* (mol.wt. 12 500) and pancreatic trypsin inhibitor (mol.wt. 6500) (Andrews, 1964, 1965).

Chromatography on concanavalin A-Sepharose

The endoglucanase (peak V) from a single Bio-Gel P-60 column was dialysed against 50 mM-phosphate buffer, pH 7, containing 0.15 M-NaCl, 1 mM-MnCl₂, 1 mM-MgCl₂ and 1 mM-CaCl₂ (concanavalin A buffer) at 4°C overnight. Non-diffusible material applied to a column of concanavalin A-Sepharose (40 mm × 16 mm) equilibrated in concanavalin A buffer and eluted with this buffer to remove unbound protein. Bound protein was eluted with 20 mM- α -methyl mannoside in concanavalin A buffer. All elutions were at 30 ml/h. Crude culture filtrate (25 ml), dialysed against concanavalin A buffer, was applied to the concanavalin A-Sepharose column, which was eluted as described above.

Enzyme assays

Exoglucanase, endoglucanase and β -glucosidase assay methods have been described elsewhere (Stewart & Parry, 1981). Xylanase was determined by the method of Conrad (1981). Dextranase was measured by incubating 1 ml of suitably diluted enzyme with 1 ml of 1% (w/v) dextran in 0.1 M-acetate buffer, pH 5, for 15 min at 50°C and determining the reducing sugars produced by the dinitrosalicylate method of Sumner & Graham (1925).

One unit of enzyme is defined as the amount catalysing the production of 1 μ mol of reducing sugar (glucose or xylose)/h.

Protein determination

Unless otherwise stated, proteins were determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis was carried out in vertical gels (130 mm × 130 mm × 1.5 mm) prepared by the method of Laemmli (1970). Gels were developed at 50 V for 1 h and at 150 V for 3 h and, after fixing, they were stained with Coomassie Blue. Protein samples of 50–150 μ g were used. Some gels were stained with periodic acid/Schiff reagent (Segrest & Jackson, 1972).

Gels were calibrated for molecular-weight determinations with 100 μ g of a mixture ('Dalton marker'; Sigma) of lysozyme (mol.wt. 14 300), β -lactoglobulin (18 400), trypsinogen (24 000), pepsin (34 700), egg albumin (45 000) and bovine serum albumin (68 000).

Isoelectric focusing

Isoelectric focusing was on LKB PAG plates, pH range 3–10. Samples (10 μ l) containing 50 μ g of protein in 1% (w/v) glycine were applied to the gel on Whatman glass-fibre strips (5 mm × 10 mm). The gel was prefocused at 2000 V, 10 mA, 10 W for 30 min, at which time the glass-fibre strips were removed. Focusing was achieved at 2000 V, 150 mA and 30 W for 45 min. Some gels were fixed and stained with Coomassie Blue as described in the LKB handbook; others were cut into 5 mm strips, each strip being eluted with 2 ml of water at 4°C for 2 h and the pH and endoglucanase activity determined on the supernatant. Samples containing endoglucanase were combined, dialysed against distilled water and freeze-dried.

Viscometry

Suitably diluted endoglucanase (1 ml) was incubated with 5 ml of 1% (w/v) CM-cellulose and 4 ml of 1 M-acetate buffer, pH 5, at 50°C for various times. The mixture was heated to 100°C to terminate the reaction, and equilibrated to 32°C before measuring the viscosity in an Ostwald viscometer at 32°C.

Hydrolysis products

Whatman no. 1 filter paper (100 mg) was incubated with endoglucanase (2 ml; 110 units) in 0.1 M-acetate buffer, pH 5, at 50°C for 18 h. The supernatant was demineralized on columns (150 mm × 10 mm) of Dowex 50 (H⁺ form) and Dowex 2 (OH⁻ form) and the eluate was evaporated to dryness under reduced pressure at 45°C. The

dry material was dissolved in water (2ml); total carbohydrate was determined by the method of Dubois *et al.* (1956), and glucose was determined with Boehringer glucose oxidase reagent with glucose as standard. The remaining solution was chromatographed on Whatman no. 1 filter paper in ethyl acetate/pyridine/acetic acid/water (5:5:1:3, by vol.). Sugars were revealed by treatment with AgNO_3 .

Effect of temperature

The optimum temperature for the endoglucanase assay was determined as described previously (Stewart & Parry, 1981). Temperature-stability was determined by incubating the endoglucanase in 0.1 M-acetate buffer, pH 5, at various temperatures and sampling at various times. Enzyme activity was determined as described above at 50°C.

Effect of pH

The optimum pH for the assay was measured as described previously (Stewart & Parry, 1981). pH-stability was determined by incubating the endoglucanase in 0.1 M-phosphate/citrate buffers, pH 3–8, at 50°C for 2 h or 18 h. Samples (0.2 ml) were removed, diluted to 1 ml with 0.1 M-acetate buffer, pH 5, and 1% (w/v) CM-cellulose in acetate buffer, pH 5 (1 ml), was added. After incubation at 50°C for 15 min, reducing sugar was determined by the dinitrosalicylate method.

Chemicals

CM-cellulose sodium salt (medium viscosity), *p*-nitrophenyl β -D-glucopyranoside, electrophoresis 'Dalton marker', xylan (larchwood), dextran (mol.wt. 70 000) and concanavalin A–Sephadex 4B were all from Sigma. CF11 cellulose was from Whatman, and cotton flock provided by Dr. C. Stewart of the Rowett Research Institute, Aberdeen, Scotland, U.K.

Results

Gel-filtration chromatography

Culture filtrates concentrated by freeze-drying lost activity, particularly exoglucanase (Table 1). Desalting on Bio-Gel P-6 gave a single protein peak, but desalting on Sephadex G-25 led to rapid degradation of the gel and loss of column bed volume. At buffer concentrations of less than 0.1 M on Bio-Gel P-6, absorption effects were evident, resulting in peak broadening and loss of resolution with poor recovery of activity.

Chromatography on Bio-Gel P-60 gave two major and three minor protein peaks (Fig. 1, peaks I–V). β -Glucosidase was in peaks I and II, peak I being totally excluded from the column bed. Cotton-solubilizing activity and 5% of the total endo-

Table 1. *Recovery of enzyme activities during purification of A. fumigatus cellulases*

Enzyme activities and protein concentrations are expressed as percentages of the values found in the crude culture filtrate.

Treatment	Endo-glucanase	Exo-glucanase	β -Glucosidase	Protein
Original filtrate	100	100	100	100
Freeze-drying	90	33	97	100
Bio-Gel P-6 column	38	12	65	52
Dialysis	38	6	35	55
Bio-Gel P-60 column				
Peak I	0.1	0	11	6.7
Peak II	1.5	2.2	18	32
Peak V	33	1.3	0	6.4

glucanase activity were present in peak II; peak IV had xylanase activity, but little activity against CM-cellulose. Peak V was the major endoglucanase, with 95% of the total activity. No activity has as yet been assigned to peak III.

The approximate molecular weights corresponding to peaks I–IV, estimated from the calibrated Bio-Gel P-60 column were 65 300–66 700 (peak II), 35 000–38 000 (peak III), 17 000–19 000 (peak IV) and 6500–8000 (peak V).

Effect of incubation time

Chromatography of samples harvested from 1 to 6 weeks after inoculation, at 7-day intervals, showed that all five of the above peaks were present after 7 days, the total protein in each peak increasing with the age of the culture, but no new peak appearing and no peak being lost.

Affinity chromatography on concanavalin A–Sephadex

The endoglucanase from the Bio-Gel P-60 column was not absorbed by concanavalin A–Sephadex, but elution of the lectin with α -methyl mannoside gave a second protein peak with xylanase activity, but no detectable endoglucanase activity (Fig. 2).

Chromatography of crude culture filtrate on concanavalin A–Sephadex showed the endoglucanase (peak V) to be the only major protein to be unabsorbed, the cotton-solubilizing activity, β -glucosidase and xylanase activities being bound, and eluted with α -methyl mannoside (Fig. 3).

Polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis of 4-week-old culture filtrate gave a number of bands when stained with Coomassie Blue (Fig. 4*h*), but with periodic acid/Schiff reagent some of the lower-molecular-

Fig. 1. Gel filtration of concentrated, desalted culture filtrate from a 4-week-old culture of *A. fumigatus* on a column (750 mm $\times$ 25 mm) of Bio-Gel P-60

(a) Protein distribution (○) was measured as A_{280} and endoglucanase (●) measured by the release of reducing sugars from CM-cellulose; (b) exoglucanase (△) was measured by release of soluble carbohydrates from cotton flock, β -glucosidase (▲) was determined with *p*-nitrophenyl β -D-glucopyranoside as substrate, and xylanase (□) was measured as reducing sugars released from xylan.

weight bands were unstained, notably the endoglucanase, peak V (Fig. 4g).

Polyacrylamide-gel electrophoresis of the Bio-Gel P-60 column protein peaks is shown in Figs. 4(a)–4(c). Peak I gave a number of bands, with four major components, but peak II gave an intensely staining band equivalent to the major component in the crude culture filtrate. Peak V gave a major component with an estimated molecular weight of 12 500.

Fig. 4 also shows the polyacrylamide-gel electrophoresis of samples from the concanavalin A–Sephacrose column chromatography of crude culture filtrate, the unbound material being equivalent to peak V eluted from the Bio-Gel P-60 column (Fig. 4f), the bound material giving two major components (Fig. 4e).

Isoelectric focusing

Isoelectric focusing of the endoglucanase from the lectin column gave a major component (pI 7.1) and two minor components (Fig. 5), the major com-

ponent containing 99% of the endoglucanase applied to the gel. Isoelectric focusing of crude culture filtrate showed the other components to have pI values between pH 3 and 5.

Properties of the major endoglucanase

The endoglucanase rapidly decreased the viscosity of CM-cellulose, but there was a much slower increase in the reducing sugars produced (Fig. 6). With filter-paper substrate the endoglucanase released a mixture of sugars containing 29% (w/v) glucose after 15 h.

The optimum temperature for assaying the endoglucanase was 60°C (Fig. 7b). When incubation was in buffer at pH 5 in the absence of substrate, enzyme activity was rapidly lost at 60°C and above (Fig. 7a), but 95% of the activity survived being incubated at 50°C for 18 h.

The optimum pH for assaying the endoglucanase was 4.8. Incubating the enzyme at 50°C in the absence of substrate indicated a maximum stability at pH 6 after 2 h, but after 18 h at 50°C, maximum

Fig. 2. Fractionation of pooled fractions 53–63 of a separation similar to that shown in Fig. 1 on a column (40 mm $\times$ 16 mm) of concanavalin A–Sepharose 4B. The pooled fractions were dialysed against concanavalin A buffer and a sample (5 ml) applied to the concanavalin A–Sepharose column equilibrated in the same buffer. The column was eluted at 40 ml/h with the same buffer, followed by 20 mM- α -methyl mannoside in concanavalin A buffer after fraction 18. Fractions (2.5 ml) were assayed for protein (O) by A_{280} (1–18) and by the Lowry *et al.* (1951) method (19–26). Endoglucanase (●) and xylanase (Δ) activities were assayed by the release of reducing sugars from CM-cellulose and xylan respectively.

Fig. 3. Fractionation of culture filtrate from a 4-week-old culture of *A. fumigatus* on a column (40 mm $\times$ 16 mm) of concanavalin A–Sepharose 4B.

Culture filtrate was dialysed against concanavalin A buffer and a sample (25 ml) was applied to the column equilibrated in the same buffer. The column was eluted at 30 ml/h with concanavalin A buffer and, after 11 fractions, with 20 mM- α -methyl mannoside in concanavalin A buffer. Fractions (5 ml) were assayed for protein (O) by the method of Lowry *et al.* (1951). Endoglucanase (●) was determined by the release of reducing sugars from CM-cellulose, exoglucanase (Δ) by the release of soluble carbohydrate from cotton flock, and β -glucosidase by using *p*-nitrophenyl β -D-glucopyranoside as substrate (Δ).

stability occurred at pH 5, with 90% of the activity remaining.

Discussion

We have shown that *A. fumigatus* produces a number of enzymes involved in the degradation of cellulose when grown on CF11 cellulose as substrate. The increase in enzyme concentration with the age of the culture may be due to release of enzyme absorbed on to the cellulose substrate as cellulose is degraded, or to continuous enzyme synthesis, or more probably to both.

Several glycosidase activities have been found in culture filtrates of *A. fumigatus* (Rudick & Elbein, 1973, 1975) and two β -glucosidase enzymes have been purified and characterized by those authors and found to have molecular weights of 340 000 and 40 000. The two β -glucosidase enzymes isolated in the present study may be similar to those of Rudick & Elbein (1973, 1975); the high-molecular-weight enzyme gave a number of bands on polyacrylamide-gel electrophoresis, whereas that described by Rudick & Elbein (1973, 1975) gave two polypeptide chains under similar conditions. The β -glucosidase in peak II of the Bio-Gel P-60 column has a molecular

weight of 66 000 by Bio-Gel P-60 chromatography, and this is higher than that of the enzyme of Rudick & Elbein (1973, 1975); however, the molecular weights were determined by different methods. Affinity chromatography on concanavalin A–Sepharose and polyacrylamide-gel electrophoresis with periodic acid/Schiff staining indicates that the β -glucosidase enzymes are glycoproteins, as shown by Rudick & Elbein (1973, 1975).

Peak II from the Bio-Gel P-60 column also

Fig. 4. Distribution of proteins after SDS/polyacrylamide-gel electrophoresis of freeze-dried samples from *A. fumigatus*. Proteins (50–100 µg) were run on SDS/polyacrylamide-gel plates (1.5 mm thick) at 50 V for 1 h and 150 V for 3 h. After fixing, the gels were stained with Coomassie Blue (a–f and h) or periodic acid/Schiff reagent (g). Bio-Gel P-60 column: peak I (a), peak II (b) and peak V (c); concanavalin A-Sepharose column using crude culture filtrate: culture filtrate (d), bound material eluted with α -methyl mannoside (e) and unbound material (f); crude culture filtrate stained with periodic acid/Schiff reagent (g) and re-stained with Coomassie Blue (h).

contained cotton-solubilizing activity and 5% of the total endoglucanase activity, but whether this is a true enzyme complex or a mixture of enzymes of similar molecular weight is not at present clear. Polyacrylamide-gel electrophoresis indicates a major band, and isoelectric focusing a number of acidic components, but there is no endoglucanase equivalent to peak V in either gel to indicate that the endoglucanase in peak II is a complex of cotton-solubilizing activity and the major endoglucanase (peak V).

The major endoglucanase (peak V) is not a glycoprotein, as it does not stain with periodic acid/Schiff reagent or bind to concanavalin A-Sepharose. The molecular weight determined by Bio-Gel P-60 chromatography (7500) is clearly at variance with that given by SDS/polyacrylamide-gel electrophoresis (12 500), and the enzyme shows a similar effect to that observed with *Trichoderma reesei* low-molecular-weight endoglucanase by Hakansson *et al.* (1978). Those authors suggest endoglucanases can interact with polyacrylamide

and dextran gels, and some molecular weights determined by chromatography on these materials may be low. Low-molecular-weight endoglucanases have been isolated from *T. reesei* (Hakansson *et al.*, 1978), *Botryodiplodia threobromae* (Umezurike, 1979) and *Myrothecium verucaria* (Selby & Maitland, 1967), and a number of authors (Wood & McCrae, 1972; Berghem & Pettersson, 1973; Streamer *et al.*, 1975) suggest that the endoglucanase attacks cellulose first, releasing free chain ends for subsequent attack by the exoglucanases. This suggestion indicates why endoglucanases are the first cellulases to be detected in culture filtrates of some organisms, their small size allowing them to penetrate the cellulose fibrils. Thus it should be possible to detect an increase in the free reducing ends in crystalline cellulose after treatment with the endoglucanase enzyme.

Highly purified endoglucanases of *T. reesei* (Shikita & Nisizawa, 1975) and *Irpex lactus* (Kanda *et al.*, 1976) are known to hydrolyse xylan. This is not so with the major endoglucanase of *A.*

Fig. 5. Distribution of endoglucanase activity in gel slices after polyacrylamide-gel isoelectric focusing of a solution of pooled fractions 4–10 from a suspension similar to that shown in Fig. 2.

The pooled fractions were dialysed against distilled water, freeze-dried and dissolved in 1% (w/v) glycine and applied to an LKB PAG plate (pH 3–10; 24.5 cm × 11 cm). The plate was focused at 2000 V, 150 mA and 10 W for 30 min and then at 2000 V, 150 mA and 30 W for 45 min. Part of the gel was stained with Coomassie Blue and scanned at 560 nm; the remainder was cut into 5 mm strips, which were eluted with water and assayed for pH (□) and endoglucanase activity (histogram) (determined by the release of reducing sugars from CM-cellulose).

Fig. 6. Effect of the endoglucanase on the viscosity of CM-cellulose

Suitable diluted endoglucanase from the isoelectric-focusing gel was incubated with 1% (w/v) CM-cellulose in 0.1 M-acetate buffer, pH 5, at 50°C for the times indicated. The viscosities of the solutions were determined at 32°C in an Ostwald viscometer (□) and the reducing sugars (■) were determined by the dinitrosalicylate method.

Fig. 7. Effect of temperature on the stability of the endoglucanase

(a) Stability of the enzyme determined by incubating suitably diluted endoglucanase from the isoelectric-focusing gel in 0.1 M-acetate buffer, pH 5, at 50°C (Δ), 60°C (□), 70°C (▲) and 80°C (■) for the times indicated. In all cases the enzyme activity was determined by incubating the samples with CM-cellulose at 50°C and measuring the reducing sugars liberated; (b) the effect of temperature on the standard endoglucanase assay.

fumigatus, xylanase activity being found in one of the other protein peaks (peak IV) from the Bio-Gel P-60 chromatography. In this respect *A. fumigatus* is similar to *Phoma hibernica* (Urbanek *et al.*, 1978), *A. niger* (Conrad, 1981) and *Streptomyces griseus* (Ishaque & Kluepfel, 1981).

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References

- Andrews, P. (1964) *Biochem. J.* **91**, 222–233
 Andrews, P. (1965) *Biochem. J.* **96**, 595–606
 Berghem, L. E. R. & Pettersson, L. G. (1973) *Eur. J. Biochem.* **37**, 21–30
 Berghem, L. E. R., Pettersson, L. G. & Axio-Fredricksson, U. B. (1975) *Eur. J. Biochem.* **53**, 55–62
 Conrad, D. (1981) *Biotechnol. Lett.* **3**, 345–350
 Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. & Smith, F. (1956) *Anal. Chem.* **28**, 350–356
 Hakansson, U., Fagerstam, L., Pettersson, L. G. & Andersson, L. (1978) *Biochim. Biophys. Acta* **524**, 385–392
 Halliwell, G. & Griffin, M. (1973) *Biochem. J.* **135**, 587–594
 Ishaque, M. & Kluepfel, D. (1981) *Biotechnol. Lett.* **3**, 481–486
 Kanda, T., Wakabayashi, K. & Nisizawa, K. (1976) *J. Biochem. (Tokyo)* **79**, 977–988
 King, K. W. (1963) in *Advances in Enzymatic Hydrolysis of Cellulose and Related Materials* (Reese, E. T., ed.), pp. 159–170, Pergamon Press, Oxford, London, New York and Paris
 Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
 Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
 Reese, E. T., Siu, R. G. H. & Levinson, H. S. (1950) *J. Bacteriol.* **59**, 485–497
 Rudick, M. J. & Elbein, A. D. (1973) *J. Biol. Chem.* **248**, 6506–6513
 Rudick, M. J. & Elbein, A. D. (1975) *J. Bacteriol.* **124**, 534–541
 Segrest, J. P. & Jackson, R. L. (1972) *Methods Enzymol.* **28**, 54–63
 Selby, K. & Maitland, C. C. (1965) *Biochem. J.* **94**, 578–583
 Selby, K. & Maitland, C. C. (1967) *Biochem. J.* **104**, 716–724
 Shikita, S. & Nisizawa, K. (1975) *J. Biochem. (Tokyo)* **78**, 499–512
 Stewart, J. C. & Parry, J. B. (1981) *J. Gen. Microbiol.* **125**, 33–39
 Streamer, M., Eriksson, K.-E. & Pettersson, B. (1975) *Eur. J. Biochem.* **59**, 607–613
 Sumner, J. B. & Graham, V. A. (1925) *J. Biol. Chem.* **65**, 393–395
 Umezurike, G. B. (1979) *Biochem. J.* **177**, 9–19
 Urbanek, H., Zalewska-Sobczak, J. & Borowinska, A. (1978) *Arch. Microbiol.* **118**, 265–269
 Wood, T. M. (1971) *Biochem. J.* **121**, 353–362
 Wood, T. M. & McCrae, S. I. (1972) *Biochem. J.* **128**, 1183–1192