Carboxymethylcellulase and β-Glucosidase Secretion by Protoplasts of *Trichoderma reesei*

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Novozym 234, a commercially available enzyme from *Trichoderma harzianum*, has been used to prepare protoplasts from *Trichoderma reesei* QM 9414. Optimal conditions were: 20 h old mycelium, 0·9 M-KCl as the osmotic stabilizer, 0·1% (w/v) Novozym 234 and 18 h incubation at 28 °C. To prevent damage to the protoplasts during isolation the incubation mixtures were agitated by vibration, so avoiding shaking or stirring. More than 95% of the protoplasts were viable and were also metabolically intact as shown by their ability to take up [^{14}C]leucine and incorporate it into cellular protein. Freshly prepared protoplasts could be induced by sophorose to produce and secrete carboxymethylcellulase and β-glucosidase. Optimal conditions were: 0·9 M-KCl as osmotic stabilizer, phosphate buffer pH 6·0, 7 mM-sophorose and 10^7 protoplasts ml^{-1}. Separation of the secreted proteins by fast protein liquid chromatography revealed at least seven peaks of carboxymethylcellulase activity and β-glucosidase activity, indicating that some multiplicity of the forms of these enzymes is already present at the secretion stage.

INTRODUCTION

The cellulolytic enzyme system of the filamentous fungus *Trichoderma reesei* consists of three basic types of enzyme: endo-1,4-β-glucanase (EC 3.2.1.4), cellobiohydrolase (EC 3.2.1.91) and cellobiase (β-glucosidase, EC 3.2.1.21). These enzymes have been found in multiple forms differing in their physicochemical properties (Gum & Brown, 1977; Okada *et al.*, 1968; Gong *et al.*, 1979; Labudova & Farkas, 1983). At present it is not known for certain whether this multiplicity arises from multiple genes, low fidelity of translation, post-transcriptional processing, post-secretional modification, or a combination of all these. Some workers have reported evidence for modification of secreted enzymes by proteolytic attack in the culture fluid after secretion (Nakayama *et al.*, 1976; Gong *et al.*, 1979), but this has been challenged by others (Labudova & Farkas, 1983; Dunne, 1982).

Investigations on extracellular enzyme secretion by filamentous fungi are generally hampered by the fact that the enzymes remain bound to or within the cell wall for some time (Chang & Trevithick, 1972; Polacheck & Rosenbeger, 1978; Kubicek, 1981). This precludes the identification of the form of early secreted enzymes. Moreover, this release from the wall may be accompanied or caused by a modification of the protein or its accompanying carbohydrate moiety. Protoplasts should serve as a useful tool in assessing whether cellulolytic enzymes are already secreted in multiple forms. Although Benitez *et al.* (1975) and recently Picataggio *et al.* (1983) succeeded in isolating biochemically intact protoplasts from *T. reesei*, they did not investigate these protoplasts with regard to protein secretion.

In the present paper we report the preparation of biochemically intact protoplasts from *T. reesei*, which can be induced to secrete cellulases and β-glucosidases, and present a preliminary analysis of the multiplicity of the early forms of these enzymes.

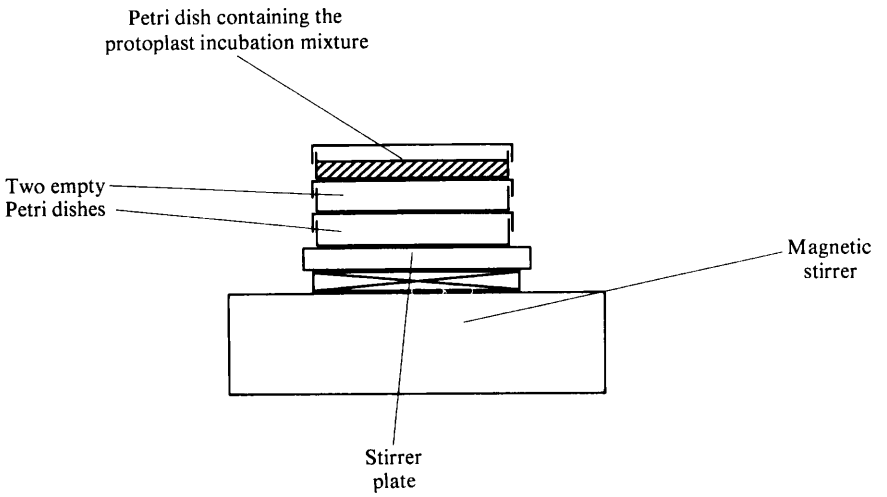

Fig. 1. Schematic drawing of the assembly for the vibration of the protoplast preparation mixture.

METHODS

Organism and growth conditions. Trichoderma reesei QM 9414 was used throughout these studies. It was kept on malt-agar slants. Inocula were prepared by harvesting 14 d old conidia in sterile tap water containing 0·1% (w/v) Tween 80. These conidia were added to the medium to a final concentration of 10^8 conidia l^{-1}. The fungus was grown in shake flasks on a rotary shaker (250 r.p.m.) at 28 °C in the medium described by Mandels & Andreotti (1978) except that glucose (0·5%, w/v) was used as the carbon source and the pH of the medium was adjusted to 5·0 with phosphate/citrate buffer as described by Labudova & Farkas (1983). Samples of this medium (200 ml) were added to 1 litre wide-mouthed Erlenmeyer flasks. The mycelia were removed after appropriate times and used for protoplast isolation as described below.

Isolation of protoplasts. All procedures were performed aseptically. Mycelia from two flasks were harvested by filtration through G1 glass sinter funnels (25 mm diameter), and were subsequently washed with 200 ml cold (4 °C) tap water and 150 ml cold (4 °C) 50 mM-phosphate buffer, pH 6·0, containing 0·9 M-KCl, 0·1% (v/v) ethanol and 100 μg chloramphenicol ml^{-1}. Care was taken that during washing the mycelium was always kept suspended in a minimum of 15 ml of liquid, since otherwise the protoplast yield was considerably reduced. After this washing, the suspension was made up to 20 ml and transferred to a sterile Petri dish. Novozym 234 (100 mg) was added, and the suspension was stirred with a plastic spatula until the enzyme was completely dissolved. The dishes were then stapled over two empty Petri dishes to provide thermal isolation, and put on a magnetic stirrer plate (see Fig. 1). The slow vibration of the stirrer plate proved the best means of agitating the mycelial digest, since stirring or shaking damaged the protoplasts within a short time. After 18 h of incubation at 28 °C the mixtures were filtered through glass wool, and then through a funnel containing (from bottom to top) a layer of milk filter, a layer of cotton and a layer of glass wool. The separation of protoplasts from mycelial debris was checked microscopically; if contaminated the protoplasts were filtered again until they were free from impurities. The protoplasts were then collected by centrifugation at 1000 *g* (10 min, 4 °C) in a swing-out rotor, and resuspended in an appropriate volume of 50 mM-phosphate buffer, pH 6·0, containing 0·9 M-KCl, to give a final concentration of about 10^7 protoplasts ml^{-1}. The viability of the protoplasts was assessed by using Trypan Blue as a vital stain: protoplasts that excluded the dye were considered viable (Hoskins *et al.*, 1956).

Protein synthesis by protoplasts. For measurements of the incorporation of labelled [^{14}C]leucine into trichloracetic acid (TCA) precipitable material, the protoplasts were suspended in 50 mM-phosphate buffer, pH 6·0, containing 0·9 M-KCl, to give a final concentration of about 0·5 to 1 × 10^7 cells ml^{-1}. To a total volume of 5 ml protoplast suspension 2·5 μCi [^{14}C]leucine (336 mCi $mmol^{-1}$, 124·3 GBq $mmol^{-1}$) was added, and the mixture incubated at 28 °C. Subsequently, at 30 min intervals 300 μl of the incubation mixture was removed and added to 2 ml of cold (4 °C) 10% (w/v) TCA and then heated in a boiling water bath for 10 min. The insoluble precipitates were collected on 0·22 μm membrane filters, washed three times with ice cold 5% (w/v) TCA, and dried. The radioactivity of the dry filters was counted.

Induction of cellulolytic enzyme synthesis in protoplasts. To induce synthesis of cellulolytic enzymes sophorose was added (to give a final concentration of 7 mM) to 5 ml protoplast suspension (10^7 protoplasts ml^{-1}). After incubation at 28 °C for appropriate times, the suspensions were centrifuged in a swing-out rotor at 1000 *g* (4 °C, 10 min), and the supernatant was kept for enzyme analysis. When the induction of cellulolytic enzymes in whole

mycelia was studied, essentially the same procedure was used for comparison. Mycelia were used at a concentration of 0·5 g dry weight ml^{-1}.

Radioactive labelling of exoprotein synthesis. After induction by sophorose, exoprotein synthesis was determined by measuring the incorporation of [^{14}C]leucine or [^{14}C]mannose into TCA-precipitable extracellular material. Protoplasts (1 to 5 × 10^7 ml^{-1}) or mycelia (0·5 mg dry wt ml^{-1}) were incubated in a total volume of 5 ml 50 mM-phosphate buffer, pH 6·0, containing 0·9 M-KCl. Sophorose was added to give a final concentration of 7 mM. After 2 h incubation at 28 °C, [^{14}C]leucine or [^{14}C]mannose was added to give final activities of 2 μCi ml^{-1} (74 kBq ml^{-1}) and 6 μCi ml^{-1} (222 kBq ml^{-1}) respectively, and the incubation was continued for a further 5 h. The mixtures were then centrifuged in a swing-out rotor (1000 ***g***, 4 °C, 10 min) and the supernatants withdrawn. TCA (500 mg) was added, and the precipitates were collected on 0·22 μm membrane filters and further processed as described above.

Assay of enzyme activities. Endo-β-glucanase and aryl-β-glucosidase were assayed as described by Kubicek (1981), with carboxymethylcellulose (Serva, Heidelberg, FRG) or *p*-nitrophenyl β-D-glucoside as the substrate. The procedures cited by Picataggio *et al.* (1983) were used for the assay of the hydrolytic enzyme activities of Novozym 234, except for chitinase, which was assayed by the procedure of Monreal & Reese (1969). One unit (U) of enzyme activity is given as the release of 1 μmol glucose equivalent (or respective sugar, as appropriate) min^{-1}. Specific activities are expressed as units of activity (mg protein)$^{-1}$. Protein was estimated by the Coomassie Blue binding method (Bradford, 1976).

Separation of extracellular proteins by chromatofocusing. Chromatofocusing was done by fast protein liquid chromatography (FPLC) on a Mono P HR 5/20 prepacked column (Pharmacia) at room temperature. The column was equilibrated with 25 mM-Bis-Tris/HCl buffer pH 7·1. Samples of protein (0·5 mg) were dialysed against a 100-fold volume of Bis-Tris/NaOH buffer (25 mM), pH 9·0 (to avoid cellulolysis of the dialysis tube), for 4 h, with two changes of the tube, and finally filtered through 0·22 μm membrane filters and applied to the column. A continuous pH gradient, monitored automatically, was developed by elution with 60 ml polybuffer 74 (Pharmacia) adjusted to pH 4·0 with HCl. A flow rate of 1·0 ml min^{-1} with an accompanying back pressure of 2·5 MPa was used. Samples of 1·5 ml were collected and assayed for activity.

RESULTS

Preparation and properties of T. reesei protoplasts

Although Driselase, a hydrolytic enzyme mixture from *Irpex lateus*, has recently been recommended for protoplast preparation from *T. reesei* QM 6a, RUT-NG 14 and RUT-C 30 (Picataggio *et al.*, 1983), we were unable to isolate protoplasts from *T. reesei* QM 9414 at an acceptable yield (i.e. more than 10^5 (g wet wt)$^{-1}$. Consequently we tested other commercially available lytic enzymes for their suitability for the preparation of *T. reesei* protoplasts. Among these, Novozym 234 – a hydrolytic enzyme from *Trichoderma harzianum* – proved most successful, and could be used without any additional enzyme supplement. Virtually all additions of other enzymes had either no effect, or even a detrimental effect, on protoplast yield or viability.

In the growth medium we used *T. reesei* germinated after 13 h incubation and reached the stationary phase of growth after 35–40 h. In accordance with Picataggio *et al.* (1983), the age of the culture had a pronounced effect on the protoplast yield, the best results being obtained with 20 h cultures (early growth phase). The optimum concentration of Novozym 234 was 0·1% (w/v). The best osmoticum was 0·9 M KCl: lower concentrations led to the lysis of some protoplasts. $(NH_4)_2SO_4$ could replace KCl as an osmoticum, but the subsequent separation of protoplasts by centrifugation was more difficult.

Protoplasts were released from mycelia by extrusion from the apical regions. The mycelium also simultaneously fragmented, apparently at the septa, and protoplasts were extruded from the end of the fragments. Although appreciable formation of protoplasts had already taken place after 3 h incubation, their complete formation required 16–18 h. A purification protocol for the preparation of protoplasts is given in Table 1. The recovery of protoplasts was about 20%. The final preparation contained over 99·5% viable protoplasts, as checked by dye exclusion. The protoplasts were osmotically fragile when the buffer was diluted with water, and they were also destroyed by shearing forces. They were apparently free of cell wall material as checked by phase contrast microscopy. The protoplasts were able to take up [^{14}C]labelled leucine and to incorporate it into cellular protein (Fig. 2).

Table 1. *Recovery of protoplasts during preparation*

Protoplast numbers were determined in a counting chamber. The preparation was carried out as described in Methods. The mycelial dry weight at the beginning was 20 mg ml^{-1}.

Fraction	Volume (ml)	Protoplasts ml^{-1}	Total protoplasts
After incubation with Novozym 234	100	$4{\cdot}0 \times 10^7$	$4{\cdot}0 \times 10^9$
After filtration	260	$4{\cdot}6 \times 10^6$	$1{\cdot}2 \times 10^9$
After first centrifugation	100	$1{\cdot}1 \times 10^7$	$1{\cdot}1 \times 10^9$
After second centrifugation	50	$2{\cdot}1 \times 10^7$	$1{\cdot}0 \times 10^9$
After third centrifugation	25	$4{\cdot}0 \times 10^7$	$1{\cdot}0 \times 10^9$

Because Novozym 234, but not Driselase, was effective with *T. reesei* QM 9414 the hydrolytic enzyme activities of both enzyme preparations were compared (Table 2). The only qualitative differences observed were the presence of chitinase and protease in Novozym 234, but not in Driselase. Picataggio *et al.* (1983) have speculated on the findings that protoplast formation was successful from *T. reesei* QM 6a, RUT-NG 14 and RUT C 30 using an enzyme preparation lacking chitinase. They questioned whether the chitin skeleton might not be formed at this early stage of growth. We have not investigated whether the addition of chitinase to Driselase would induce the formation of protoplasts with *T. reesei* QM 944. We must further note that the activities of Novozym 234 as assayed in our laboratory, although qualitatively similar, were markedly lower than those reported by Hamlyn *et al.* (1981), but we cannot offer an explanation for this.

Induction of cellulolytic enzyme synthesis in T. reesei protoplasts

Cellulase and β-glucosidase biosynthesis and secretion occurred when *T. reesei* protoplasts suspended in buffer containing an osmotic stabilizer were incubated with the inducing disaccharide sophorose. Although this inducer appears to exert different effects on carboxymethylcellulase and on β-glucosidase (Sternberg & Mandels, 1980), we found that approximately the same concentration of sophorose (7 mM) was optimal for the induction of both enzymes (Fig. 3*a*). The effect of the pH of the medium and the concentration of osmoticum on the induction of cellulolytic enzymes was also examined (Fig. 3*b*,*c*). The high salt concentration apparently inhibited the synthesis of both carboxymethylcellulase and β-glucosidase; the concentration of 0·9 M-KCl thus used was a compromise between the effect of lowering of the salt concentration on protoplast lysis and on protein secretion. Other salts (e.g. ammonium sulphate) gave the same effect; polyols, however, were not used, to avoid their use as an energy source by the protoplasts. Addition of other nutrients, especially nitrogen, was not necessary for the induction, which is in accordance with the nutrient requirements of mycelia for induction (Sternberg & Mandels, 1980). Under the conditions so optimized, protoplasts were shown to synthesize and excrete carboxymethylcellulase, as well as β-glucosidase, at an approximately linear rate for at least 24 h.

It should be noted that the increase in cellulolytic enzyme activities was not due to binding of the Novozym 234 enzymes to the protoplasts and their subsequent release, since at the beginning of the experiment, less than 5% of the final activities could be shown to be associated with the protoplast debris.

Identification of early multiple forms

Although only a small proportion of the total enzyme activity had been secreted during 8 h incubation, the mixtures were usually withdrawn after this time, and the supernatants used for enzyme separation, since sufficient enzyme had been secreted into the medium to allow its separation by chromatofocusing without prior concentration. Procedures for concentration were avoided since they might result in denaturation of the labile multiple forms which then would not be detectable. However, we were interested in detecting the earliest secreted forms of the enzymes. The chromatofocusing profile (Fig. 4*a*) showed that at this stage of cultivation there were already multiple forms of both enzymes, leading to the splitting of both

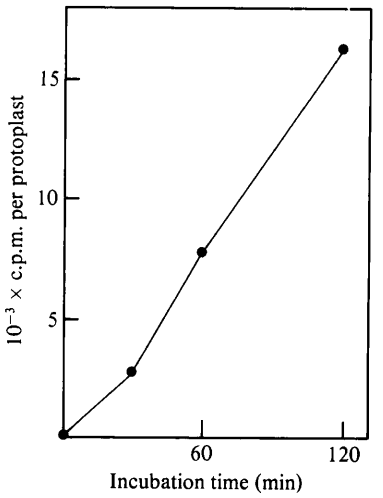

Fig. 2. Incorporation of [^{14}C]leucine into intracellular, TCA-precipitable protein. The procedures used were as described in Methods.

Table 2. *Comparison of the enzyme spectrum of Driselase and Novozym 234*

Enzyme activities were determined as described in Methods. The values for Novozym 234 given in parentheses are from Hamlyn *et al.* (1981); the value for the protease is a 'trypsin equivalent'. The values for Driselase are from Picataggio *et al.* (1983).

	Specific activity [U (mg protein)$^{-1}$]	
Enzyme	Novozym 234	Driselase
Cellulase	0·0237	0·18
Endo-β-1,4-glucanase	0·499	114·10
Cellobiase	0·653	0·270
β-1,3-Glucanase	2·86 (7000)	1·23
β-1,6-Glucanase	0·515	0·18
Chitinase	0·0544 (2250)	BDL
α-Mannanase	BDL (0)	BDL
α-Mannosidase	BDL	BDL
Protease	+ + + (300)	BDL

BDL, Below detection limit; + + +, positive result, not quantified.

carboxymethylcellulase and β-glucosidase activities into at least seven peaks within the pH range 4 to 7. With regard to a possible involvement of the cell wall in the origin of the multiple forms normally found in the culture filtrate of *T. reesei*, we initially attempted to compare the secretion profile from protoplasts (Fig. 4*a*) with that from cellulase-secreting whole mycelia. The same procedure as with the protoplasts was used, but with intact, washed mycelia. However, under these conditions both carboxymethylcellulase and β-glucosidase remained bound to the cell wall and were not released into the culture medium within 8 h (W. P. Kammel, unpublished results). We were thus forced to use a culture filtrate of *T. reesei* grown on cellulose and harvested as early as possible (i.e. after 70 h cultivation), for comparison (Fig. 4*b*). Pronounced differences were observed in some of the peaks: it was especially noted that the majority of the carboxymethylcellulase components secreted by protoplasts were found at more acid pH values than those found in a cellulose-degrading medium. However, the considerable differences in the secretion conditions between the protoplasts and the cellulolytic mycelia precludes an interpretation of these differences in multiple enzyme forms solely on the basis of modification of the enzymes during passage through the cell wall.

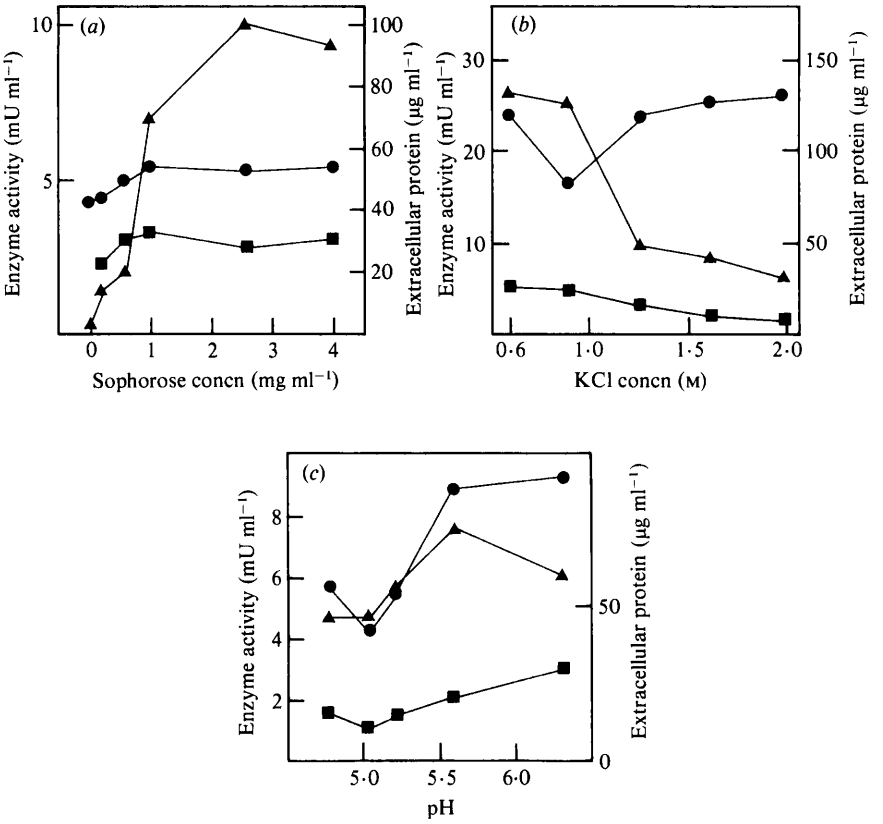

Fig. 3. Influence of sophorose concentration (*a*), osmoticum concentration (KCl) (*b*) and pH (*c*) on the induction of cellulolytic enzymes and protein in *T. reesei*. ▲, Carboxymethylcellulase; ■, β-glucosidase; ●, extracellular protein. In (*b*) and (*c*) the concentration of sophorose was 7 mM. In (*a*) and (*c*), enzyme activities were measured after 8 h incubation; in (*b*) and (*c*) they were measured after 20 h incubation. Experimental conditions were as described in Methods.

What appeared to be actively secreted cellulase and β-glucosidase could actually have been intracellular enzymes released into the extracellular medium by protoplast lysis, especially since the incubation medium was lacking a nitrogen source. We checked for the occurrence of lysis by several means. Firstly, [^{14}C]leucine and [^{14}C]mannose were added to the induction media and their incorporation into extracellular, TCA-precipitable protein was followed. The results obtained showed that the secretion of cellulases and β-glucosidases was accompanied by *de novo* synthesis and and excretion of glycoproteins. Secondly, protoplasts were harvested after 8 h induction and lysed by dilution with hypotonic buffer. The intracellular fluid was then assayed for carboxymethylcellulase and β-glucosidase. No carboxymethylcellulase could be detected (cf. Montenecourt *et al.*, 1981), but β-glucosidase was present. A separation of intracellular β-glucosidase by chromatofocusing revealed two peaks of activity, occurring at each extreme of the fractionation range (Fig. 5). Thus the five other β-glucosidase peaks, at least, could not be due to cell lysis. Finally we checked the distribution of malate dehydrogenase, an essentially intracellular enzyme, which is, however, stable (>97% recovery) at pH 6·0 for 8 h at 30 °C. The protoplast lysates had high malate dehydrogenase activity (260 units), but less than 0·060 units were detected in the extracellular fluid. We thus conclude that our protoplast system was apparently free of lysing cells, and that the multiple forms detected must represent actively secreted protein species.

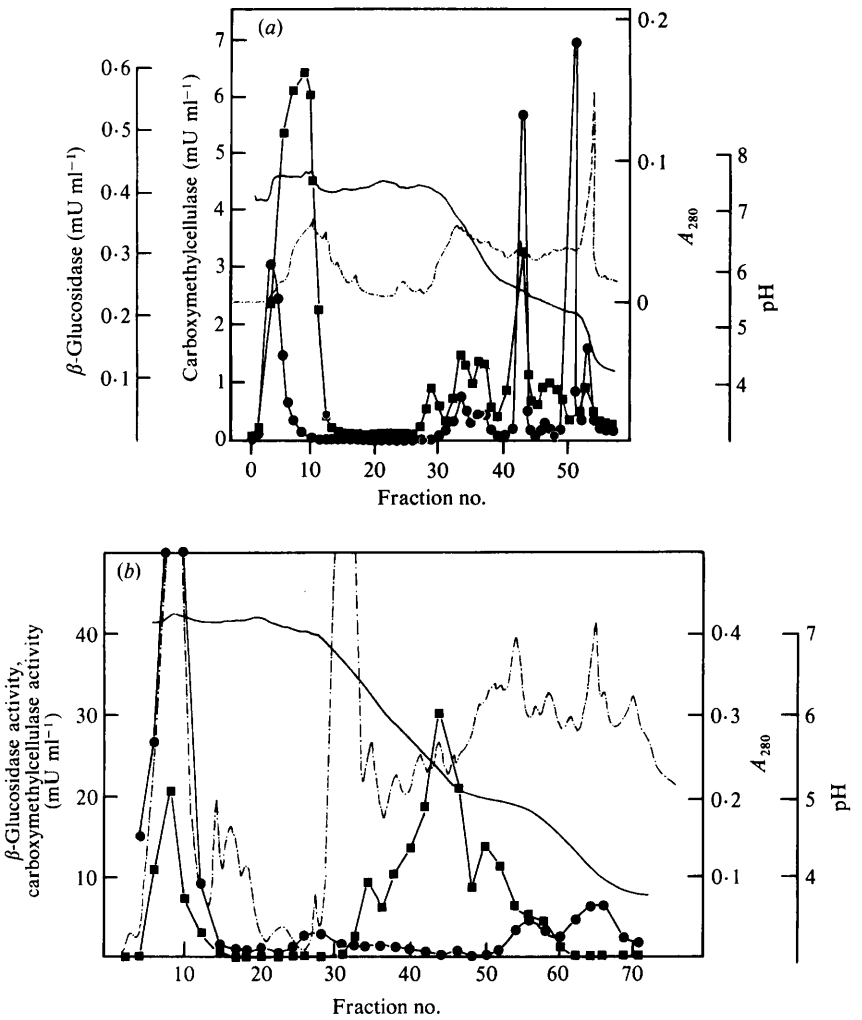

Fig. 4. Chromatofocusing of enzymes secreted by protoplasts (*a*) and whole mycelia (*b*), as described by Labudova & Farkas, 1983. ■, Carboxymethylcellulase; ●, β-glucosidase. ———, pH; ·—·—·, protein profile (A_{280}) (both determined continuously).

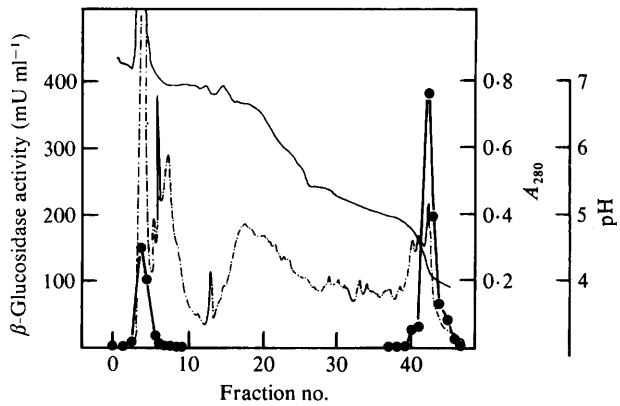

Fig. 5. Chromatofocusing of intracellular β-glucosidase from *T. reesei*. For symbols see Fig. 4(*a*) legend.

DISCUSSION

Since many secretory proteins of fungi remain bound to the cell wall, protoplasts have been valuable for studying secretion by yeasts (cf. Liras & Gascon, 1971; Sanchez *et al.*, 1982; Novick & Scheckman, 1979; Esmon *et al.*, 1984). In contrast, little use has been made of protoplasts of filamentous fungi in such studies; a paper by Trevithick & Metzenberg (1964), describing invertase secretion by *Neurospora crassa* protoplasts, appears to be the only exception of this generalization. This is surprising since the preparation of protoplasts from filamentous fungi is feasible using commercially available enzymes (Hamlyn *et al.*, 1981). At the beginning of these investigations we screened a number of available preparations, among which Novozym 234 proved to be best suited for isolating a large number of viable protoplasts from *T. reesei* QM 9414.

The multiplicity of forms of cellulolytic enzymes has been described several times (Gum & Brown, 1977; Okada *et al.*, 1968; Gong *et al.*, 1979; Labudova & Farkas, 1983). Our findings that the multiple forms are already found among the proteins secreted within 8 h by protoplasts supports the assumption (Labudova & Farkas, 1983; W. P. Kammel & C. P. Kubicek, unpublished work) that they do not originate by post-secretional modification, although it remains theoretically possible that this modification is completed within that time. The present results do not support the possibility that some of the multiple forms represent enzyme molecules bound to incompletely degraded substrate (cellulose); although such a binding might occur, it seems to contribute little to the occurrence of multiple forms, since they also exist in the absence of an insoluble substrate (i.e. during induction in protoplasts). However, neither do the results obtained support the idea that some of the multiple forms arise during their traverse of the cell wall. This conclusion, however, is not completely justified since we failed to obtain extracellular protein secretion into the culture fluid from whole mycelia under comparable conditions. The occurrence of some multiple forms with different isoelectric points in the culture filtrate from cellulose-degrading *T. reesei* could also be due to the different induction potential of cellulose relative to sophorose. However, this would only be valid if it were assumed that all the multiple forms are genetically determined, which has yet to be proved. Although the present results do not allow any conclusions as to the chemical and structural basis of the multiplicity of the cellulases of *T. reesei*, one result is noteworthy: several of the multiple forms secreted by *T. reesei* protoplasts contained carboxymethylcellulase and β-glucosidase in the same fraction. This is reminiscent of the model proposed by Sprey & Lambert (1983, 1984) which suggested that multiplicity of cellulases is – at least in part – caused by formation of multienzyme aggregates. The isoelectric points of the activity peaks from the protoplast experiments were quite similar to those of Sprey & Lambert (1984). It is interesting that both the coincidences of isoelectric points as well as the concomitant appearance of carboxymethylcellulase and β-glucosidase were far less apparent in cellulose-grown culture filtrates. A theoretical explanation for this observation can be derived from the assumption that cellulolytic enzymes are secreted as multienzyme aggregates into the periplasmic space (Sprey & Lambert, 1983); because of the high affinity of some of these enzymes for some cell wall polymers (Dickerson & Baker, 1979), these enzymes might be 'filtered' out of the aggregates, thus giving rise to the new isoelectric points of the aggregates which appear in the extracellular culture fluid. We are currently studying the protein composition of the multiple cellulases secreted by protoplasts in order to check this hypothesis.

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