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STUDIES ON THE STRUCTURE AND MECHANISM OF ACTION OF GLYCOSIDE HYDROLASES

PART I. PURIFICATION AND STUDY OF SOME FACTORS AFFECTING THE ACTIVITY OF *Rhizopus arrhizus* (1→3)-β-D-GLUCANASE

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ABSTRACT

The extracellular (1→3)-β-D-glucanase [(1→3)-β-D-glucan glucanohydrolase, E.C. 3.2.1.6] produced by *Rhizopus arrhizus* QM 1032 was purified 165-fold by chromatography. The purified enzyme is basic, has a molecular weight of ~10,000, and is unstable in dilute solution but may be stabilized by addition of human serum albumin. The pH–activity profile for the enzyme in the presence of serum albumin shows a peak at about pH 3.5–3.7 and a shoulder at pH 4.5–4.6, whereas in the absence of serum albumin the optimum pH is at pH 4.5–4.6, indicating the presence of two enzymic species, designated "pH 3.5 activity" and "pH 4.6 activity". In the presence of albumin the enzyme activity is resistant to inactivation by a wide range of reagents. Ammonium molybdate is, however, a powerful inhibitor of "pH 3.5 activity" although a much poorer inhibitor of "pH 4.6 activity". The enzyme activity is stable during heating at pH 3.5 in the presence of human serum albumin. Thus, 94.5 and 88.5% of "pH 3.5 activity" and "pH 4.6 activity", respectively, remained after heat treatment for 30 min at 68°. The enzyme is, however, essentially inactive at this temperature, even in the presence of albumin. To account for this finding, a temperature-dependent conformational change is proposed. The enzyme activity is not stable during heating at pH 4.6 in the presence of serum albumin. K_m values for action on laminaran are 0.54 mg/ml (pH 3.5) and 0.27 mg/ml (pH 4.6). For lichenan the corresponding values are 3.33 and 2.38 mg/ml. The V_{max} for enzyme action on lichenan is 35–40% higher than for action on laminaran at both pH values. Possible relationships between the two forms of the enzyme are briefly considered.

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INTRODUCTION

During a survey of a large number of fungi for production of extracellular polysaccharidase activities, Reese and Mandels[1] found that *Rhizopus arrhizus* QM 1032, grown on cellobiose as the carbon source, produced an endo-acting (1→3)-β-D-glucanase. Studies of the action of the enzyme on lichenan and cereal D-glucans led Perlin and co-workers[2–4] to suggest that, although classed as a (1→3)-β-D-glucanase, the enzyme also has the ability to split (1→4)-β-D-glucosidic linkages. They suggested that the specificity of the enzyme should be described in terms of the glycosyl residue that is eventually liberated by the enzyme (3-*O*-β-D-substituted glycosyl) rather than in terms of the linkage split, which may be either β-D-(1→3) or β-D-(1→4).

In view of the importance of this enzyme in the structural analysis of polysaccharides[5], and also as a possible subject for our studies on the structure and mechanism of action of glycoside hydrolases, the enzyme has been purified and some properties and aspects of its activity examined. A preliminary account of this work has been published[6].

MATERIALS AND METHODS

Growth of the fungus and preparation of crude, extracellular, enzyme mixture. — *Rhizopus arrhizus* QM 1032, kindly provided by Dr. Elwyn T. Reese, was grown in shake culture at 29° (200 ml per 1 liter flask), using the basal medium employed by Reese and Mandels, with cellobiose as the carbon source[1]. After 5 days, cells were removed by centrifugation and the culture supernatant was dialyzed against water at 2° and then freeze-dried.

Substrates. — A generous supply of cold-water insoluble laminaran, prepared from *Laminaria hyperborea* as described by Black[7], was provided by Dr. Eric T. Dewar, Inveresk Research International, Inveresk, Midlothian, Scotland, and used without further purification. This material was dissolved in warm water before incorporation into enzyme digests; it remained in solution during the incubation. Lichenan was prepared from Iceland Moss (*Cetraria islandica*) by the method of Peat *et al.*[8]

Chromatographic media. — DEAE-Cellulose (microgranular DE-52) and CM-Sephadex C-25 were from Whatman Biochemicals Ltd. and Pharmacia Fine Chemicals, respectively. These materials were treated before use as recommended by the respective manufacturers[9,10]. Bio-Gel P-60 (100–200 mesh) was obtained from Bio-Rad Laboratories (Richmond, California) and was swollen for 3 days in 1% sodium chloride at room temperature before column packing and equilibration.

Other materials. — Human serum albumin was obtained from the Lister Institute of Preventive Medicine, London, and bovine serum albumin was obtained from Sigma Chemical Co.

Analyses. — Protein was determined by the method of Lowry *et al.*[11], with

bovine serum albumin as the protein standard. Total carbohydrate was estimated by the phenol–sulfuric acid method[12] with D-glucose as standard. Reducing sugars were measured by a colorimetric adaptation of the Somogyi method[13,14].

Enzyme assays. — During purification of the enzyme, activities were measured at 37° by the release of reducing sugars in digests (total volume 1.0 ml) containing substrate (insoluble laminaran, 2.5 mg), human serum albumin (250 μg), buffer (500mM acetate, pH 4.5, 0.25 ml), and enzyme. The amounts of enzyme and duration of incubation used were such that not more than 5% hydrolysis of the substrate occurred. Appropriate substrate and, where necessary, enzyme blanks were included. When necessary, dilutions of enzyme solutions were made by addition of aliquots to appropriate amounts of human serum albumin solution (0.25 mg/ml) before assay. 1 Unit of activity is defined as the amount that released 1 μmole of reducing sugars (measured as D-glucose equivalents) per min under the conditions described.

During studies on the activity of the purified enzyme, the digests used were essentially as already stated, although in some cases acetate buffer of pH 3.5 was used. The exact compositions are given in the RESULTS section.

Ultrafiltration. — This was performed at 2° in Diaflo cells (Amicon Corporation, Lexington, Massachusetts, U.S.A.) fitted with UM-10 membranes.

RESULTS

Purification of Rhizopus arrhizus *(1→3)-β-D-glucanase.* — All operations were performed at 2°. *Column chromatography on DEAE-cellulose.* Freeze-dried culture filtrate (300 mg containing 124 mg of protein) was dissolved in 25mM citrate–phosphate buffer (pH 8.0) and applied to a column (9.0 × 3.0 cm) of DEAE-cellulose, which was eluted with a gradient of 0→1.0M sodium chloride in the same buffer. Assays showed the activity to be present in the unadsorbed peak (Fig. 1).

Column chromatography on CM-Sephadex. Fractions from the DEAE-cellulose column containing the enzyme activity were combined, concentrated by ultrafiltration, dialyzed against 25mM acetate buffer (pH 5.0), and then chromatographed on a column (6.0 × 3.0 cm) of CM-Sephadex. The activity was recovered by elution with a gradient (0→1.0M) of sodium chloride in the same buffer, a salt concentration of 0.1–0.2M being required for desorption. The activity was, in this instance, well separated from the major portion of the protein present in the effluent (Fig. 2).

Column chromatography on Bio-Gel P-60. The combined enzyme fractions from the CM-Sephadex column were concentrated by ultrafiltration, and then applied to a column (95 × 2.5 cm) of Bio-Gel P-60, which was eluted with 25mM acetate buffer (pH 5.0), containing 1% of calcium chloride. The activity recovered from the column was associated with an amount of protein below the level detectable by u.v. measurements (Fig. 3). The active fractions were combined, concentrated by ultrafiltration, and dialyzed against water.

The data from a typical purification are given in Table I. The enzyme solution prepared in this way is referred to as concentrated enzyme. For most of the experi-

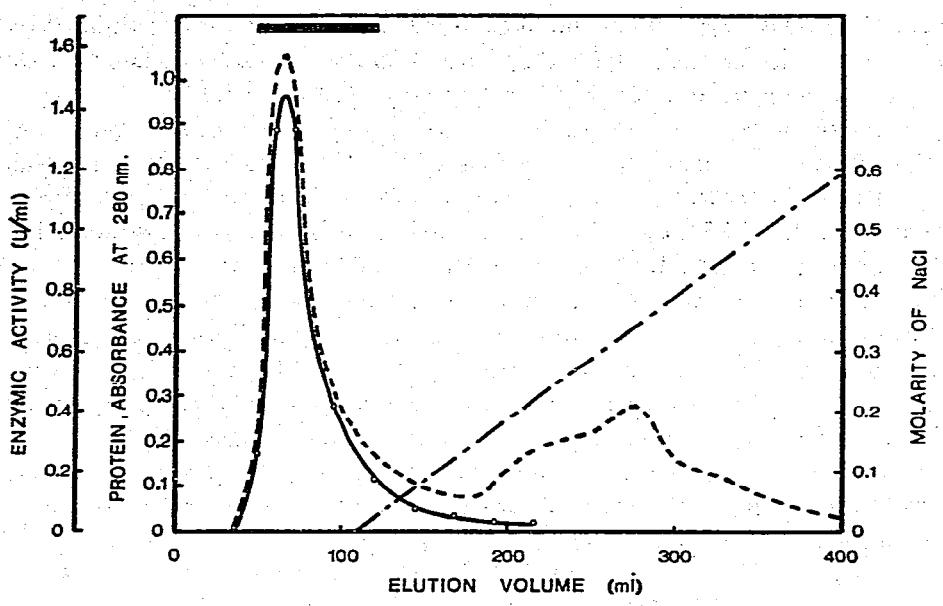


Fig. 1. Chromatography of crude *Rhizopus arrhizus* enzyme mixture on DEAE-cellulose (Whatman microgranular DE-52; for conditions, see the text); – – – – –, distribution of protein; —O—O—, (1→3)-β-D-glucanase activity; — — — — — —, sodium chloride gradient. The heavy bar shows the fractions that were combined.

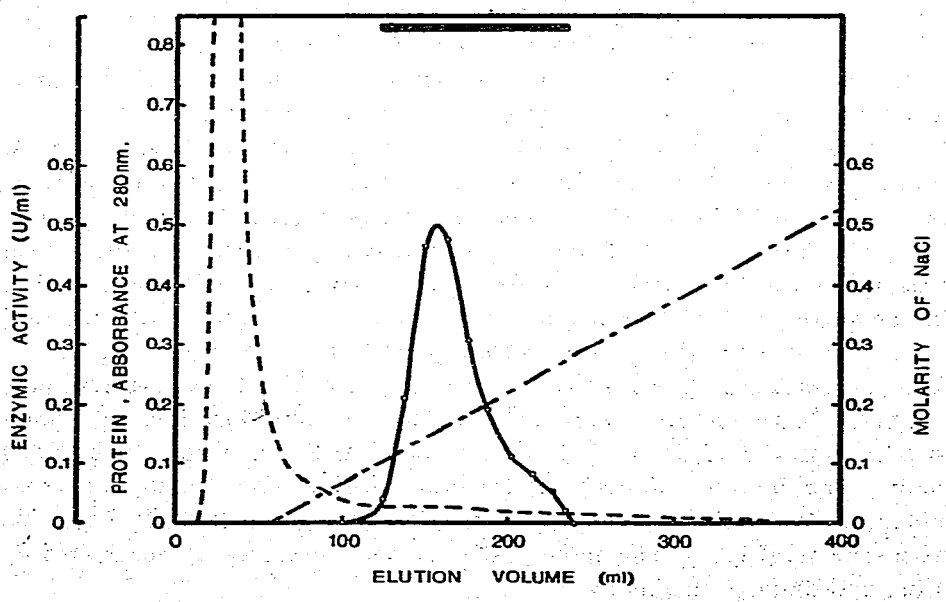


Fig. 2. Chromatography of *Rhizopus arrhizus* (1→3)-β-D-glucanase on CM-Sephadex C-25. For conditions, see the text. The symbols are as in the legend to Fig. 1.

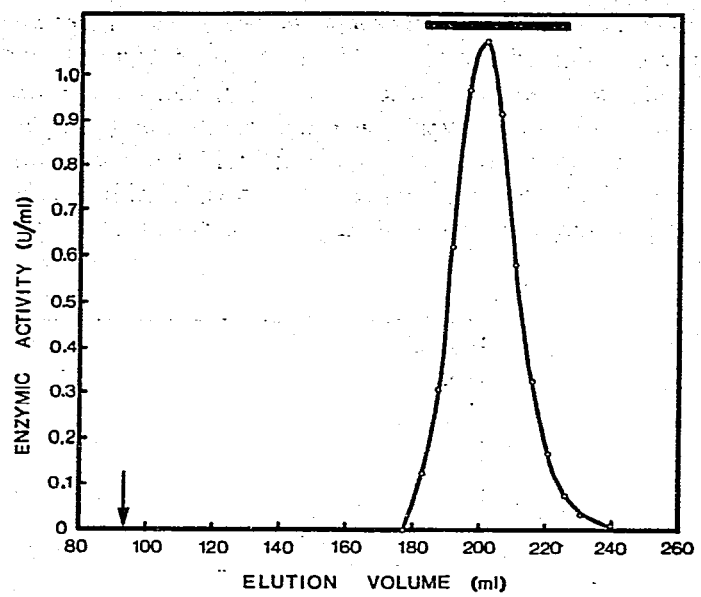


Fig. 3. Chromatography of *Rhizopus arrhizus* (1→3)-β-D-glucanase on a column of Bio-Gel P-60. For conditions see the text. The arrow indicates the void volume of the column. Protein could not be detected in the column fractions before concentration.

ments described, the concentrated enzyme solution contained 24 μg/ml of protein and initially had an activity of 2.6 U/ml. When stored at 2° in the absence of any stabilizing agent, this solution had a half-life of approximately 2 weeks. Some experiments were performed with an enzyme solution, initially of the same specific activity, but which had been freeze dried in the presence of human serum albumin (see later) when the activity had dropped to approximately 0.8 U/ml.

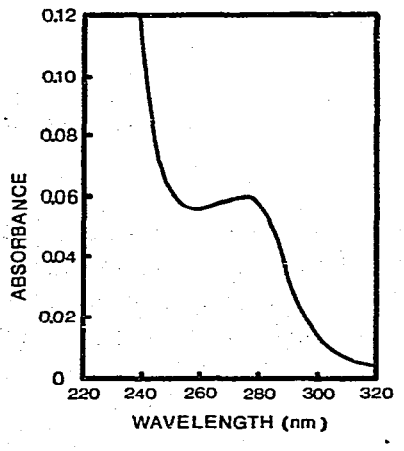
TABLE I

PURIFICATION OF *Rhizopus arrhizus* β-D-GLUCANASE

Step	*Total activity (units)*	*Total protein (mg)*	*Specific activity (U/mg)*	*Purification factor*	*Yield (%)*
1. Starting material	79.5	124	0.64	—	100
2. DEAE-Cellulose chromatography	59	61.5	0.96	1.5	74
3. CM-Sephadex chromatography	37	2.43	15.3	23.9	46.5
4. Bio-Gel P-60 chromatography	19	0.18	106	165	24

Ultra-violet absorption spectrum of purified Rhizopus arrhizus *β-glucanase.* — The u.v. spectrum (Fig. 4) was measured with a Unicam SP 1800 spectrophotometer in 1 cm cells, at a concentration of 24 μg/ml.

Molecular weight of purified Rhizopus arrhizus *β-glucanase.* — The molecular weight of the purified enzyme was estimated by molecular-sieve chromatography on a column of Bio-Gel P-60 which had been calibrated by using proteins of known molecular weight, namely ribonuclease, chymotrypsinogen, and ovalbumin (compare ref. 15). The enzyme had V_e/V_0 (ratio of elution volume to void volume of the column) of 2.15. A plot (Fig. 5) of V_e/V_0 against log (molecular weight) showed this value to correspond to a molecular weight of approximately 10,200 daltons.



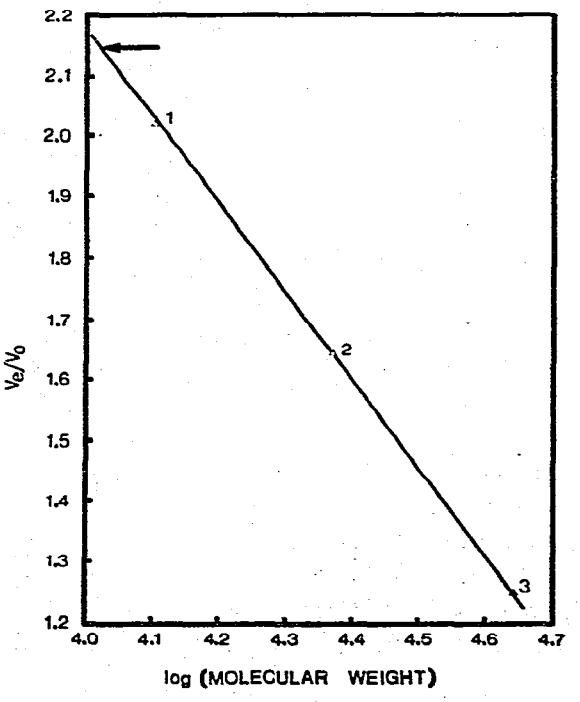


Fig. 4. Ultraviolet absorption spectrum of purified, concentrated *Rhizopus arrhizus* (1→3)-β-D-glucanase. The measurements were made in a cell of path length 1 cm and at a protein concentration of 24 μg/ml.

Fig. 5. Calibration of Bio-Gel P-60 column with proteins of known molecular weight (1 = ribonuclease, 2 = chymotrypsinogen, and 3 = ovalbumin). The arrow indicates the position of elution of *Rhizopus arrhizus* (1→3)-β-D-glucanase.

Inactivation of purified Rhizopus arrhizus *β-glucanase by dilution.* — During initial studies of the properties of the purified enzyme, it was found that activity measurements were irreproducible; apparent activities were not proportional to the amount of enzyme incorporated into enzyme digests, particularly when concentrated enzyme solution was diluted before use. These discrepancies were traced to loss of activity during dilution and rapid loss of activity in the diluted solution on storage. In view of other reports[16–18] of stabilization of highly purified carbohydrases by

addition of serum albumin, experiments were undertaken to determine whether human serum albumin affected the stability properties of the *Rhizopus* enzyme.

Loss of activity in diluted solution in the presence and absence of human serum albumin. — Samples (50 µl) of concentrated enzyme solution were diluted six-fold by addition to water and to human serum albumin solution (0.25 mg/ml) at 18°. The activities of the diluted solutions after various lengths of time at this temperature were then determined by incorporation of samples (25 µl) into digests containing laminaran (2.5 mg), buffer (500mM acetate, pH 4.5, 0.25 ml), and human serum albumin (0.25 ml, 1 mg/ml) in a total volume of 1.0 ml. The loss of activity with time in the two experiments is shown in Fig. 6.

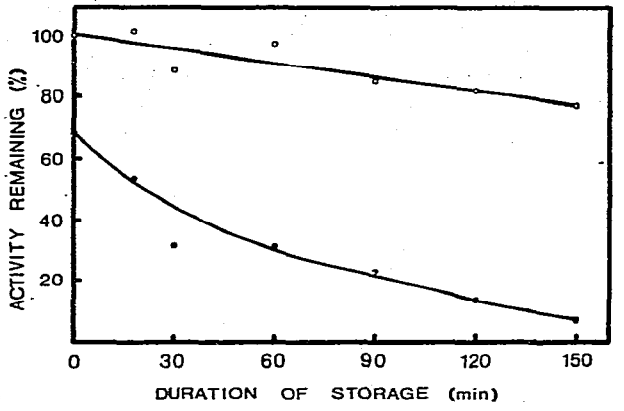


Fig. 6. Loss of activity of diluted *Rhizopus arrhizus* (1→3)-β-D-glucanase in the presence (—○—○—) and absence (—●—●—) of human serum albumin. For conditions, see the text.

Effect of human serum albumin on the loss of activity during freezing–thawing and freeze-drying. — Concentrated enzyme solution (50 µl) was added to 0.25 ml of water and to 0.25 ml of albumin solution (0.25 mg/ml). The diluted solutions were immediately frozen, stored for 2 days at −15°, and then thawed at room temperature. Duplicate samples of the diluted solutions were freeze-dried (approx. 3 h) and then redissolved in water. The activities of the frozen–thawed and freeze-dried–redissolved samples were compared with that of a solution prepared by freshly diluting concentrated enzyme 6 times with human serum albumin solution (0.25 mg/ml), by incorporation of samples (25 µl) into digests as in the last paragraph. The results are given in Table II.

Dependence of enzyme activity and stability on pH. — The activity of the purified enzyme was determined at various pH values by using laminaran as substrate, in 1.0-ml digests containing substrate (2.5 mg) and buffers (acetate, final concentration 125mM) of various pH values, both in the presence and absence of human serum albumin (250 µg). The pH–activity curve for the action of the enzyme on lichenan was determined in the same way.

TABLE II

EFFECT OF HUMAN SERUM ALBUMIN ON STABILITY OF *Rhizopus arrhizus* (1→3)-β-D-GLUCANASE

Activity	*Control*	*Freeze drying*		*Freezing and thawing*	
		With albumin	*Without albumin*	*With albumin*	*Without albumin*
U/ml	0.76	0.61	0.073	0.50	0.202
%	100	84	10	69	28

The stability of the enzyme at various pH values in the presence and absence of albumin was determined by using digests of the same composition, enzyme action being initiated by addition of substrate (laminaran) solution after pre-incubation of enzyme and buffer with or without albumin for 2 h at 37°.

The results are illustrated in Figs. 7a–c.

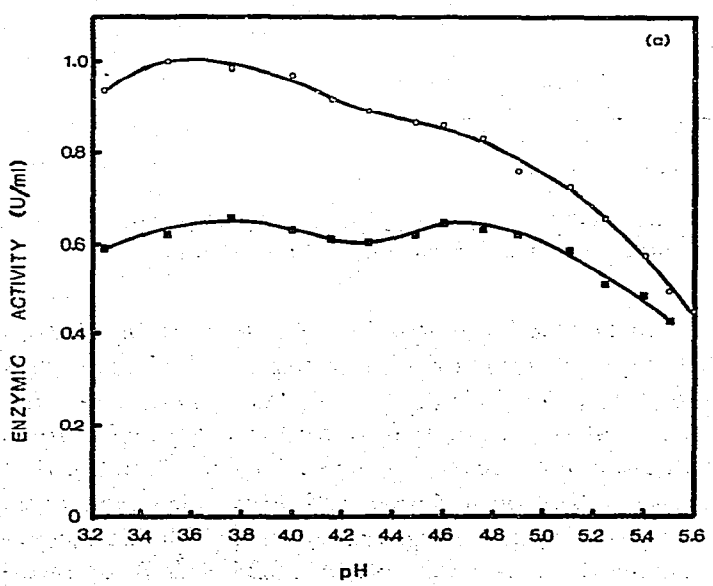
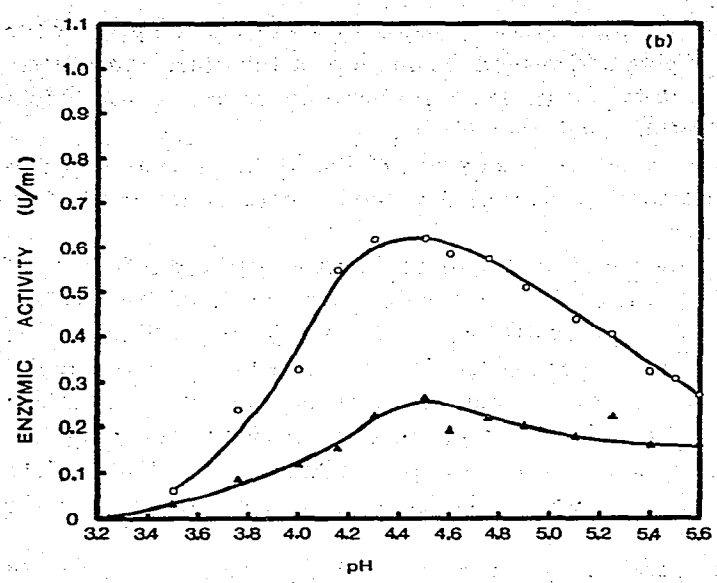
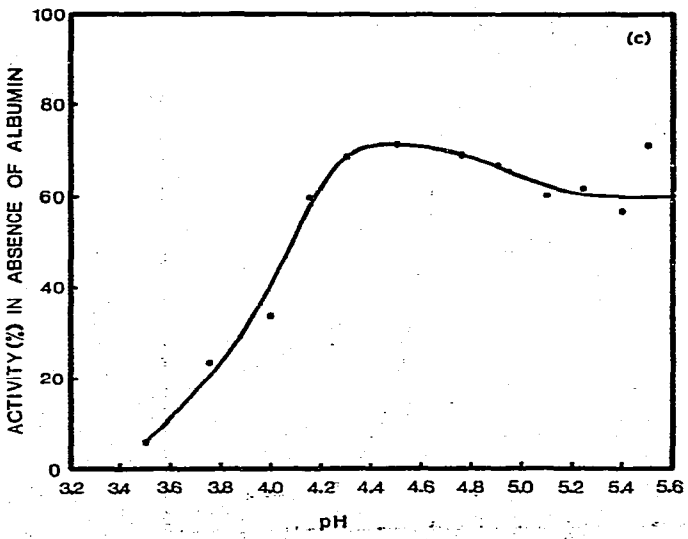


Fig. 7a. Dependence of activity of *Rhizopus arrhizus* (1→3)-β-D-glucanase on pH, determined in the presence of human serum albumin, with laminaran (—○—○—) and lichenan (—■—■—) as substrates. For conditions, see the text.

Optimum temperature and heat stability of purified Rhizopus arrhizus *β-glucanase.* — The optimum temperature was determined by using 1.0-ml digests containing substrate (laminaran, 2.5 mg), buffer (500mM acetate, pH 3.5, 0.25 ml), human serum albumin (250 μg), and concentrated enzyme (15 μl). After incubation



b. The dependence of activity of *Rhizopus arrhizus* (1→3)-β-D-glucanase on pH (laminaran substrate) in the absence of human serum albumin (—○—○—). The stability of the enzyme as a function of pH in the absence of albumin is also shown (—▲—▲—). For conditions see the text.



c. The percentage of the activity in the presence of albumin (laminaran substrate) remaining during assay in the absence of albumin, as a function of pH. The points shown were calculated from those in Figs. 7a and 7b.

for 30 min at various temperatures, enzyme action was stopped by addition of alkaline copper reagent and the reducing sugars liberated by enzyme action were determined.

The temperature stability of the enzyme was determined by using digests of the same composition, substrate being added after incubation of the other constituents of the digest for 30 min at the stated temperature, followed by incubation for 30 min at 37° to determine the amount of activity remaining.

The same experiments were also conducted at pH 4.6. In this case, an approximately equivalent amount of enzyme, freeze-dried in the presence of serum albumin and then redissolved, was used.

As the stability experiments indicated distinct differences in the stabilities of "pH 3.5 activity" and "pH 4.6 activity", determinations were made of the effect on these two activities of the pH at which heating was performed. These were conducted essentially as for the earlier stability experiments already detailed. Duplicate samples were heated for 30 min in the presence of human serum albumin at 68° in 25mM acetate buffer (both pH 3.5 and 4.6). After heating, the activity remaining was determined at both pH values by addition of substrate in 500mM acetate buffer of the appropriate pH for assay. The measured activities were then compared with the activity of the unheated enzyme solution.

The results of the temperature-optimum and stability studies are given in Figs. 8a, 8b, and Table III.

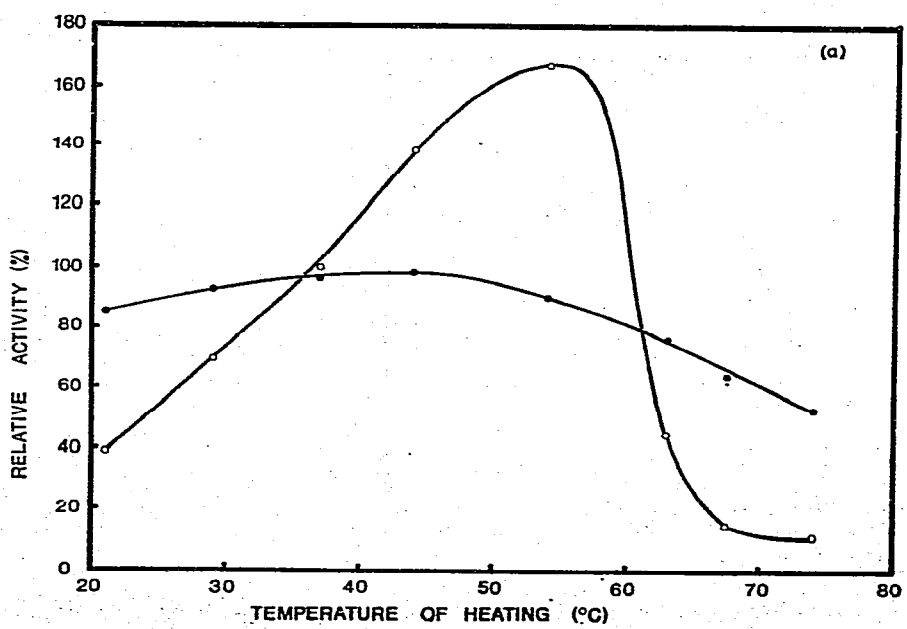
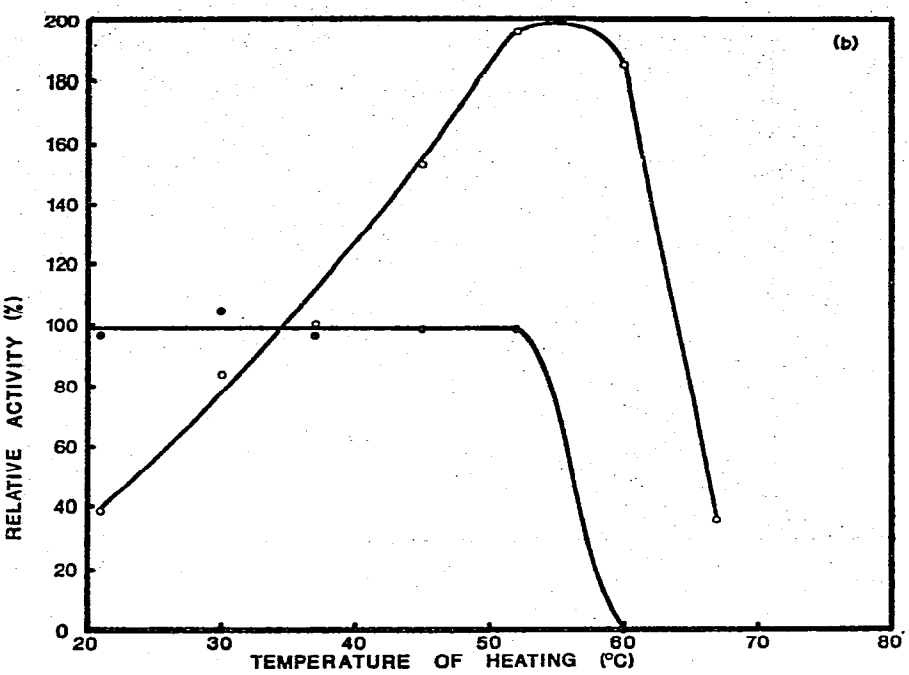


Fig. 8a. Dependence of *Rhizopus arrhizus* (1→3)-β-D-glucanase activity on temperature (—○—○—). The activity remaining after heat treatment for 30 min at various temperatures in the absence of substrate is also shown (—●—●—). All activities are expressed relative to the activity at 37° in a control digest (taken as 100%). Heat treatment and assay were conducted at pH 3.5. For further details see the text.



b. As in (a) but with heat treatment and assay conducted at pH 4.6.

TABLE III

EFFECT OF pH ON STABILITY OF *Rhizopus arrhizus* β-D-GLUCANASE ACTIVITY (pH 3.5 AND pH 4.6 ACTIVITIES) DURING HEAT TREATMENT (30 MIN, 68°)

pH of assay	*pH of heat treatment*	*Activity remaining (as % of activity in control at assay pH)*
3.5	3.5	88.5
3.5	4.6	20.0
4.6	3.5	94.5
4.6	4.6	0

Dependence of enzyme activity on substrate concentration. — Initial rates of enzyme action were determined in digests (total volume 1.0 ml) containing substrate (laminaran or lichenan, 0.05–7.5 mg), human serum albumin (250 μg), buffer (500mM acetate, pH 3.5 or 4.6, 0.25 ml), and enzyme (15 μl). The kinetic constants for the enzyme acting on the two substrates at the two pH values were determined from Lineweaver–Burk double-reciprocal plots (Figs. 9a and 9b), and are summarized in Table IV.

Effect of various reagents on enzyme activity. — The effect of various ions and other reagents on the purified (1→3)-β-D-glucanase was determined by incorporation of the species under test into digests containing substrate (laminaran, 2.5 mg), human serum albumin (250 μg), and buffer (500mM acetate, pH 3.5, 0.25 ml), in a total

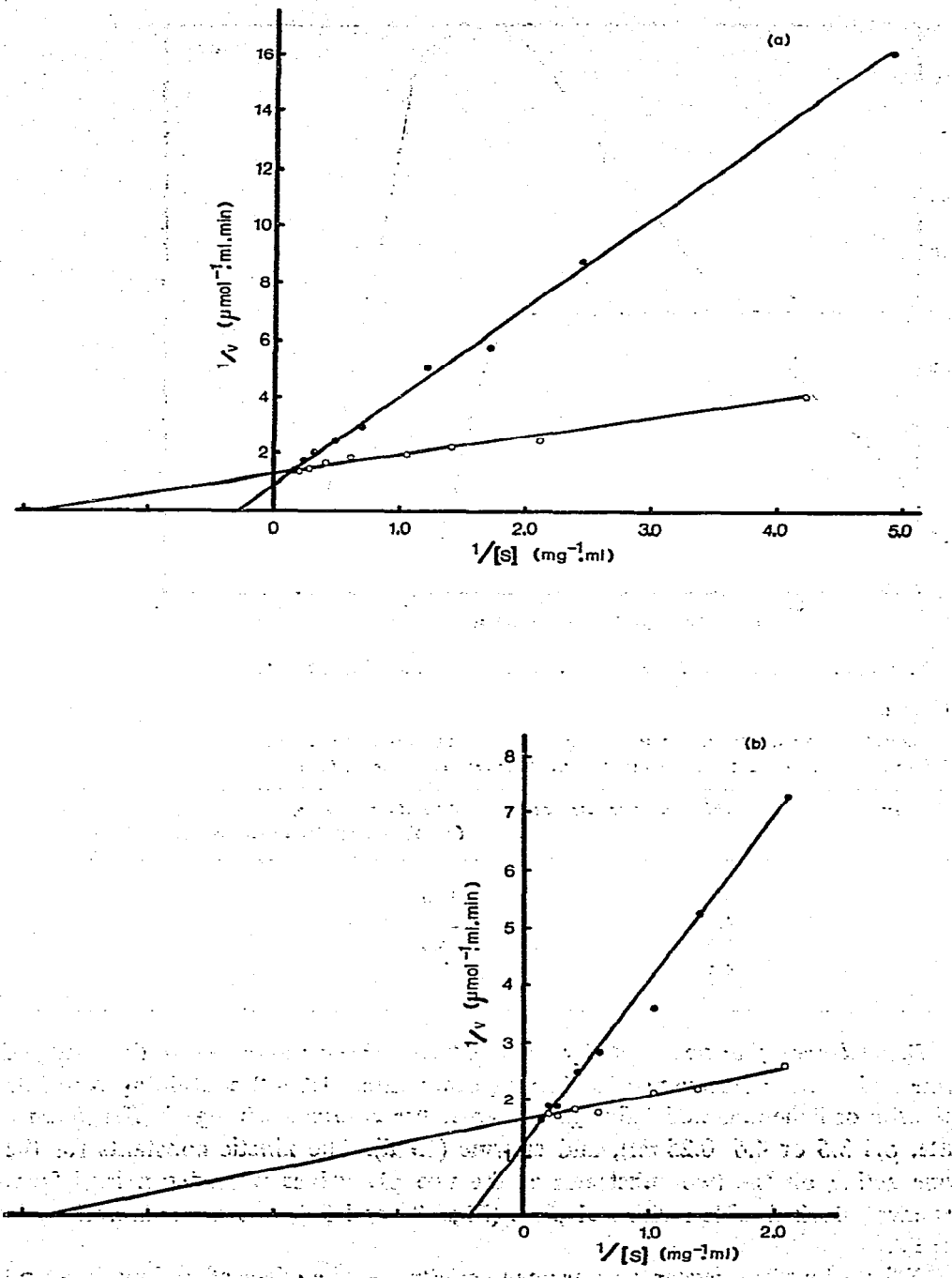


Fig. 9a. Lineweaver–Burk plot of the reciprocal of the initial rate of enzyme action (v) against the reciprocal of substrate concentration (s) for the action of *Rhizopus arrhizus* (1→3)-β-D-glucanase on laminaran (—○—○—) and lichenan (—●—●—) at pH 3.5.
b. As (a) but at pH 4.6.

TABLE IV

KINETIC CONSTANTS FOR *Rhizopus arrhizus* (1→3)-β-D-GLUCANASE ACTIVITIES ACTING ON LAMINARAN AND LICHENAN

Assay pH	K_m (*mg/ml*)		V_{max}[a] (*μmole ml*$^{-1}$ *min*$^{-1}$)	
	Laminaran	*Lichenan*	*Laminaran*	*Lichenan*
3.5	0.54	3.33	0.78	1.11
4.6	0.27	2.38	0.61	0.83

[a] V_{max} values should not be compared for the different assay pH values as different enzyme solutions were used.

volume of 1.0 ml. In all experiments, the reagent was added to a mixture of buffer, serum albumin and enzyme (15 μl), with subsequent initiation of enzyme action by addition of substrate solution. All reagents were tested at a final concentration of 1.0mM. The activity was also measured in citrate buffers (final concentration 25mM) of pH 3.5 and 4.6, and compared with the activity in acetate buffers of the same pH. Appropriate blank and control digests were included to correct for any effect of the reagents used on reducing-power measurements. The following reagents were without significant effect on the enzyme activity (that is, they caused less than 10% increase or decrease): sodium chloride, calcium chloride, ferric chloride, cobalt chloride, mercuric chloride, zinc sulfate, cupric chloride, manganese(II) chloride, lead acetate, silver nitrate, cysteine hydrochloride, gluconic 1,5-lactone, ethylenedinitrilotetra(acetic acid) (EDTA), and *p*-chloromercuribenzoate. The enzyme activity was not decreased in citrate buffer. Ammonium molybdate caused almost complete inactivation.

A more-detailed examination of the inhibition by molybdate was conducted by testing the effect of the reagent on activity towards lichenan and laminaran at pH 3.5 and 4.6 (acetate buffer), by using digests of the same composition. The results are shown in Table V.

TABLE V

INHIBITION OF *Rhizopus arrhizus* β-D-GLUCANASE BY AMMONIUM MOLYBDATE (10^{-3}M)

Substrate	*Activity* (%)	
	pH 3.5	*pH 4.6*
Laminaran	7	70
Lichenan	69	100

DISCUSSION

The (1→3)-β-D-glucanase of *Rhizopus arrhizus* is an important enzyme for structural studies on (1→3)-β-D-glucans and mixed-linkage glucans of the lichenan

and cereal glucan type[5]. The unusual specificity of the enzyme, which is such that the nature of the linkage split is directed by the requirement that a 3-*O*-β-D-glycosyl-substituted glucose residue be liberated by the enzyme[2–4], may make the *Rhizopus* glucanase a useful model enzyme in our studies on the structure, specificity, and mechanism of action of glycoside hydrolases. However, as previous work[2–4,19] was carried out with unpurified enzyme, it is essential that the properties of the purified enzyme be investigated. While this work was in progress, two reports[20,21] appeared, describing partial purifications of the enzyme.

Affinity-binding techniques for the purification of β-D-glucan hydrolases have previously been reported by the author[22–25]. However, unlike other endo-β-D-glucanases investigated, the *Rhizopus* enzyme did not bind to microgranular DEAE-cellulose (Fig. 1). Chromatography on the cation-exchanger, CM-Sephadex (Fig. 2), followed by molecular-sieve chromatography on Bio-Gel P-60 (Fig. 3) gave, typically, a 165-fold purification with 24% recovery of activity (Table I). The recovery and specific activity of the enzyme were approximately the same when the purification was performed in the cold (2°) or at room temperature (20°). Although not shown in Figs. 1–3, fractions from the ion-exchanger and molecular-sieve chromatography columns were assayed for activity towards lichenan as well as laminaran. Activities towards the two substrates coincided, confirming that activity towards both substrates is due to a single enzyme. The elution volume of the enzyme on a calibrated Bio-Gel P-60 column (Fig. 5) shows that it has, as do β-D-glucan hydrolases from malted barley[26], rye[27], and a bacterial enzyme preparation[28], a rather low molecular weight (~10,000 daltons). It will, however, be necessary to confirm this figure by more-reliable physical methods when larger quantities of the enzyme are obtained.

Initial studies on the properties of the purified enzyme gave highly erratic and irreproducible results suggestive of enzyme instability. As previous work[16–18] has shown that highly purified β-D-glucan hydrolases may be stabilized by addition of inert protein such as serum albumin, the effect of incorporating this protein into enzyme digests was investigated. Human serum albumin prevented an initial rapid loss of activity of the enzyme on dilution, and markedly decreased the rate of inactivation in diluted solution at room temperature (Fig. 6). Serum albumin also stabilized the enzyme during freeze-drying and redissolving, or to freezing and thawing (Table II). Some of the experiments reported were performed by using freeze-dried enzyme, after it had been discovered that the activity could be preserved in this way. The results shown in Fig. 6, where partially inactivated enzyme was assayed in the presence of albumin, indicate that although serum albumin can increase enzyme stability, it cannot bring about re-activation.

Whereas enzymic activity in the unpurified enzyme preparation with laminaran as substrate was greatest at about pH 4.5 (hence the conditions chosen for assay of enzyme activity; see METHODS), activity in the purified preparation was optimal at a much lower pH (approximately 3.5), with a shoulder at pH 4.5 (Fig. 7a). A similar situation was observed with lichenan as substrate, although the relative activities at the two pH values differed from that towards laminaran at the same pH values. When

activity towards laminaran was measured in the absence of albumin, or in the presence of albumin after pre-incubation without albumin, activity was only present in the higher-pH region of the biphasic pH-activity curve (Fig. 7b). This is clearly emphasized in Fig. 7c, where the ratio of the activities towards laminaran in the presence and absence of albumin is shown as a function of pH. The biphasic pH–activity curve and the preferential loss of one part of it in the absence of albumin (Figs. 7a–c) indicate the presence in the purified enzyme preparation of two distinct activities, which have been referred to as "pH 3.5 activity" and "pH 4.6 activity".

The "pH 4.6 activity" shows a normal dependance of activity on temperature, having optimum activity at about 55°, and being irreversibly inactivated above 50° (Fig. 8b). The "pH 3.5 activity" showed only low activity above 60°, but was not irreversibly inactivated at such temperatures (Fig. 8a). After heating for 30 min at 74°, followed by assay at 37°, 55% of the activity remained. Although the differences in heat-stability behavior provide, at first sight, evidence that "pH 3.5 activity" and "pH 4.6 activity" are distinct, this is not so. It is the pH at which heat treatment is performed which determines the amount of activity lost during heat treatment (Table III). Thus, on heat treatment at pH 3.5, neither form of the enzyme is irreversibly inactivated. Possibly a conformational change takes place which prevents effective enzyme–substrate binding, but on cooling the enzyme returns to its native conformation. During heat treatment at pH 4.6, activity is lost, largely irreversibly. Although the same conformational change would be proposed to occur during heating at the higher pH, the native conformation clearly is not regained after this treatment.

A number of reagents known to activate or inactivate other glycoside hydrolases[29] were without significant effect on the enzyme activity, although this lack of effect may be due, in part, to the protective action of serum albumin. However, the lack of inhibition by calcium-sequestering agents (EDTA, citrate, and phosphate) and by mercuric chloride, was confirmed in the absence of albumin. Thus the *Rhizopus* enzyme differs from other β-D-glucan hydrolases[26], in either not requiring calcium, or in binding this cation very firmly. The purified enzyme is strongly inhibited by ammonium molybdate, a reagent previously observed to inhibit several other carbohydrases[30–32], by an as-yet unknown mechanism. The greater effect of this reagent on pH 3.5 activity than on pH 4.6 activity (Table V) provides further evidence that these two activities are distinct.

The kinetic constants for the two (pH 3.5 and 4.6) activities, with laminaran and lichenan as substrates, were determined from Lineweaver–Burk plots (Figs. 9a and 9b) and are summarized in Table IV. The differences in the K_m values for the two substrates (a factor of 6–9) is not great. This may be largely explained on the basis of the "effective" substrate concentrations in laminaran and lichenan solutions of the same weight concentrations, the former having a considerably higher proportion of susceptible linkages. It may be concluded that the enzyme has about the same affinity for the region which it binds in the two types of polysaccharides. The maximum velocity of enzyme action is 35–40% greater on lichenan than on laminaran. As the majority of the linkages split in the former substrate are β-D-(1→4), rather than

β-D-(1→3) as in laminaran, it does seem that β-D-(1→4) linkages are more easily cleaved by the enzyme than are β-D-(1→3) linkages. Most of the experiments and assays reported have not been performed under saturating substrate conditions, particularly in the case of lichenan. This would be impossible because of high substrate blanks and, with lichenan, viscosity problems.

There remains the question as to whether there are, indeed, two enzymic species in the preparation, as indicated by the pH–activity, pH–stability studies, and by the differential inhibition by ammonium molybdate. If, there are two forms, they are very closely related, one possibly being formed from the other, for example by partial unfolding or protease action during isolation. Modification of the tertiary structures of enzymes during isolation, giving enzymes having different susceptibilities to non-competitive inhibitors, has been reported previously[33,34]. The alternative possibility, proteolysis, is well exemplified by the proteolytic modification of D-fructose 1,6-diphosphatase during isolation[35,36], resulting in changes in properties, including optimum pH. Several species of *Rhizopus* produce powerful acid proteases[37–39]. Proteolysis of the unfolded, or partly-unfolded, enzyme by a heat-stable protease active at pH 4.6 but not at pH 3.5, could well explain the striking differences between the heat stability properties at these two pH values. It is hoped that the question will be answered by further studies in progress.

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