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A β-1,3-GLUCAN HYDROLASE FROM *NICOTIANA GLUTINOSA*

I. EXTRACTION, PURIFICATION AND PHYSICAL PROPERTIES

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SUMMARY

1. A β-1,3-glucan hydrolase from the leaves of *Nicotiana glutinosa* has been purified by chromatography on DEAE-cellulose and Bio-Gel P-150, followed by isoelectric focusing. A 280-fold purification of the enzyme was achieved and the final preparation showed a single protein band on polyacrylamide gel disc electrophoresis.

2. The purified enzyme has been shown to have a mol. wt. of approx. 45 000 and a p*I* of 4.87. It is inactivated by heating at 65° for 10 min and has a pH optimum of 5.0. The K_m for *O*-carboxymethylpachyman (CM-pachyman) is 0.6 g/100 ml, 0.13 mM.

3. The purified enzyme is unstable in dilute solution in the absence of buffer salts or protein. The presence of bovine serum albumin prevents the inactivation caused by dilution, but only partially restores the activity when it is added after dilution.

INTRODUCTION

In a survey of the distribution of β-1,3-glucan hydrolases (β-1,3-glucan glucanohydrolase, EC 3.2.1.39) in a number of higher plants, CLARKE AND STONE[1] showed that the leaves of *Nicotiana glutinosa* possessed a high level of activity. This paper describes the extraction, purification and physical properties of the *N. glutinosa* enzyme, whilst the specificity and action pattern of the enzyme and inhibitor studies are described in the accompanying paper[2]. Observations relating to the enzyme in the leaf and to changes in its level in various physiological and pathological conditions are described elsewhere[3,4].

EXPERIMENTAL PROCEDURE

Plant cultivation

The *N. glutinosa* plants were grown in a greenhouse from seed and the seedlings

Abbreviation: CM-pachyman, *O*-carboxymethylpachyman.

were transferred to sand in pots. Each plant was given 50 ml of Hoagland No. 2 solution[5] twice weekly.

Preparation of leaf extracts

Leaves from fully grown plants were removed, washed with tap water, then with distilled water and blotted dry. The mid-ribs and petioles were excised and the laminae frozen at −20°. Extracts were prepared at 4° by grinding 1 part by weight of the frozen laminae with 0.5 part by weight of acid-washed sand and 1 part by volume of 0.01 M phosphate buffer (pH 7.0) containing 2.5 mM EDTA. The sand and larger particles were removed by centrifuging at 515 × *g* for 10 min, the supernatant was again centrifuged at 5750 × *g* for 30 min and the clear supernatant was dialysed overnight against 0.01 M Tris–HCl buffer (pH 8.0) at 4°.

Substrates

O-Carboxymethylpachyman. Samples were prepared from pachyman, either by the method of CLARKE AND STONE[1] or by a method based on that of KLUG AND TINSLEY[6] for the carboxymethylation of cellulose[7].

Insoluble laminarin from Laminaria hyperborea. A sample obtained from the Seaweed Research Institute (Midlothian, Scotland) was treated as previously described[8]. A further sample was obtained from Koch-Light Laboratories (Colnbrook, Bucks., England).

Other materials

Bovine serum albumin (Cohn Fraction V) was obtained from the Commonwealth Serum Laboratories (Melbourne, Victoria). A sample of chicken erythrocyte histone was a gift of Dr. C. M. Mauritzen, Melbourne University (Melbourne, Victoria). Pepsin, twice crystallised, was from Sigma Chemical Co., St. Louis, Mo.

Analyses

Protein was estimated by the biuret method[9] or by the microbiuret method[10], except in column eluates where it was estimated from absorbance at 260 and 280 nm[11] or with the Folin–phenol reagent[12]. Bovine serum albumin was used as protein standard in all cases. Total carbohydrate was estimated by the phenol-sulphuric acid method[13] with glucose as standard. Total reducing sugars were determined by the colorimetric method of NELSON[14] using the reagents of SOMOGYI[15]. The sodium concentration in column eluates was measured by flame photometry.

Enzyme assays

Enzyme activity was determined by the rate of release of reducing sugars from *O*-carboxymethylpachyman (CM-pachyman) or insoluble laminarin. In assays with CM-pachyman as substrate, 0.8 ml CM-pachyman (degree of substitution 0.3) 1% (w/v) in 0.05 M acetate buffer (pH 5.0) was incubated at 40° with 0.2 ml enzyme solution for a suitable time, after which the reaction was stopped by the addition of 1 ml of the SOMOGYI[15] alkaline copper reagent before estimation of reducing sugars. Appropriate substrate and enzyme blanks were estimated. Bovine serum albumin was usually added to the enzyme solution at a concentration of 100 or 500 μg per ml to stabilize the enzyme. Any precipitated CM-pachyman was removed by centrifu-

gation before measurement of the optical density. In the experiments on the isolation and purification of the enzyme a modified substrate solution containing 0.75% (w/v) CM-pachyman in phosphate–citrate buffer (pH 5.0) was used.

In assays with laminarin as substrate, 0.5 ml 1.6% (w/v) laminarin in 0.2 M acetate buffer (pH 5.0) was incubated with 0.3 ml water and 0.2 ml enzyme solution as described for CM-pachyman. Before use the laminarin solutions were warmed to 60° to dissolve partially precipitated laminarin. In the frontal analysis experiments assays were made with substrate solutions containing 0.6% laminarin in 0.1 M acetate buffer.

DEAE-cellulose chromatography

DEAE-cellulose powder (Whatman DE 11, W. and R. Balston Ltd., England) was prepared and the columns were packed as described in the manufacturer's handbook. In addition the powder was also washed with 1 mM EDTA before packing the columns, to remove substances absorbing at 260 nm.

Gel filtration

Bio-Gel P-150 (Bio-Rad Laboratories, Calif.) and Sephadex G-75 (Pharmacia, Uppsala, Sweden) were prepared and packed in dimethyldichlorosilane-treated glass columns as recommended in the manufacturers' handbooks.

Isoelectric focusing

The apparatus used for isoelectric focusing was the 440-ml column of LKB Instruments (LKB-Produkter AB, Sweden) which is a modification of the column described by SVENSSON[16]. The enzyme sample and carrier ampholytes, pH range 3–6 (LKB-Produkter AB, Sweden) were added to the column in a sucrose gradient using the method described by the manufacturer. Following electrolysis at 5° for 63 h, the ampholyte–sucrose solution was drained from the column and 2-ml fractions were collected. The pH gradient was determined and enzyme activities were measured with laminarin as substrate. High concentrations of ampholyte solution interfered with the Nelson–Somogyi reducing sugar estimation, but when no more than 0.05 ml of each fraction was added per ml incubation mixture little interference occurred. It was, however, necessary to clear the solution by centrifugation before reading the absorbance of the solutions.

Polyacrylamide gel electrophoresis

The polyacrylamide gel, containing 7.5% (w/v) acrylamide, 0.1% (w/v) *N,N'*-methylenebisacrylamide, 0.5% (v/v) *N,N,N',N'*-tetramethylethylenediamine, 0.05 M Tris–borate buffer (pH 8.2) and 0.125% (w/v) ammonium persulphate, was polymerized in 65 mm × 5.5 mm tubes. Enzyme samples (300 μl) containing sucrose (5%, w/v) were added and subjected to electrophoresis at 22 V/cm for 3.5 h. The protein bands were stained with Amidol Black 10B, washed and photographed by transmitted light.

Sephadex G-75 chromatography

The molecular weight of the enzyme was estimated by gel filtration on a column of Sephadex G-75 (50 cm × 2.5 cm) in 0.05 M maleate buffer (pH 6.5). Columns were

run at 4° and the samples were added in a volume of 0.5 ml. The void volume was determined using *Escherichia coli* cells (Miles-Seravac, Berks, England). The column was calibrated with bovine serum albumin, β-lactoglobulin, α-chymotrypsinogen and horse heart cytochrome *c*. The eluates, collected at a rate of 2.5 ml/20 min, were monitored by the protein method of LOWRY *et al.*[12] and by the assay of enzyme activity with laminarin as substrate.

RESULTS

Extraction of the hydrolase from N. glutinosa leaves

A comparison was made of the activity of the hydrolase in leaf extracts prepared in the following buffers; phosphate, 0.01 and 0.25 M (pH 7.0); maleate, 0.5 M (pH 7.5); and Tris–HCl, 0.01 M (pH 8.0). All buffers contained 2.5 mM EDTA. Although there was a 50-fold variation in buffer concentration and the pH dropped in the 0.01 M buffers to as low as 5.4, the enzyme activities did not differ markedly. The total protein in the extracts was lowest in the 0.01 M phosphate buffer (pH 7.0) and this buffer was selected for routine extractions. Mercaptoethanol which has a stabilizing effect on certain autoxidizable enzymes containing sulphydryl groups[17] did not increase the activity of the hydrolase in the extracts.

Distribution of the enzyme in fractions of leaf homogenates

Table I shows the proportion of the enzyme which was extracted into the supernatant fraction, compared with the proportion remaining in the sediment, before and after washing the sediment with buffer. More than 70% of the recovered activity was found in the supernatant and a further 15% was obtained by a single washing of the sediment with buffer.

Purification of the β-1,3-glucan hydrolase from N. glutinosa leaves

DEAE-cellulose column chromatography. Preliminary experiments showed that the hydrolase was completely adsorbed by DEAE-cellulose in 0.01 M Tris–HCl buffer (pH 8.0) and was eluted in a gradient of Na_2SO_4 in the same buffer as a single sharp peak at concentrations of Na_2SO_4 between 0.025 and 0.039 M. In large scale prepa-

TABLE I

PROPORTIONS OF HYDROLASE IN DIFFERENT FRACTIONS OF A LEAF HOMOGENATE

An extract was made from 4.8 g of frozen laminae as described under EXPERIMENTAL PROCEDURE. Following centrifugation at 515 × *g* for 10 min the supernatant was recentrifuged at 2700 × *g* for 30 min. A portion of the combined sediments was resuspended in buffer and centrifuged at 2700 × *g* for 30 min giving the fractions designated "washed sediment" and "washings". Each fraction was dialysed against 0.01 M Tris–HCl buffer (pH 8.0) for 22 h and activities were assayed with CM-pachyman as substrate.

Fraction	*Activity of β-1,3-glucan hydrolase (μg reducing sugar, as glucose per 30 min per g fresh wt. of leaf)*
Total homogenate	4920
Supernatant	2820
Sediment	1168
Washed sediment	580
Washings	600

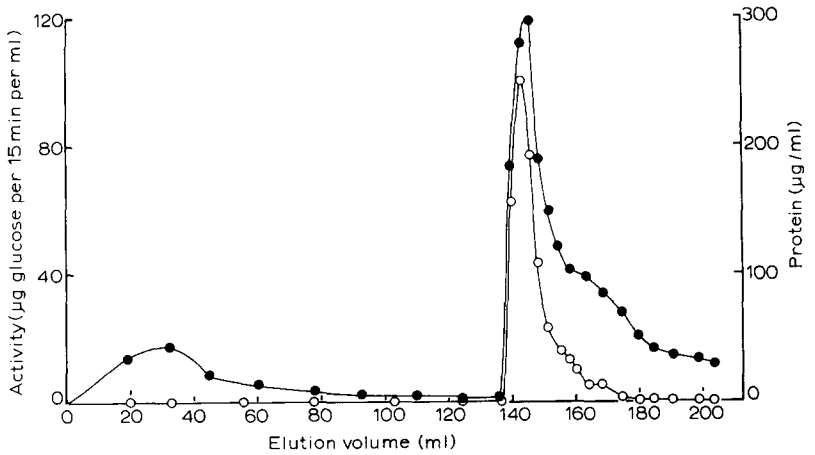


Fig. 1. Chromatography on DEAE-cellulose. A leaf extract was prepared, dialysed overnight against 0.01 M Tris–HCl buffer (pH 8.0), centrifuged and the sediment was washed. The combined supernatant and washings were added slowly to a 7 cm × 1.3 cm column of DEAE-cellulose, equilibrated with 0.01 M Tris–HCl buffer (pH 8.0). The column was washed with the same buffer until no protein was detected in the eluates and then with 0.045 M Na_2SO_4 in the same buffer. The flow rate was 10 ml/h. Fractions were assayed for protein (●—●) and for activity against CM-pachyman (○—○).

rations the enzyme was eluted in a single step with 0.045 M Na_2SO_4, with a recovery of up to 72% as illustrated in Fig. 1.

Bio-Gel P-150 chromatography. Further purification was achieved by molecular exclusion chromatography using Bio-Gel P-150 as shown in Fig. 2.

Bulk preparation. An extract was prepared from 500 g of frozen laminae as described under EXPERIMENTAL PROCEDURE and was purified as follows: 970 ml of

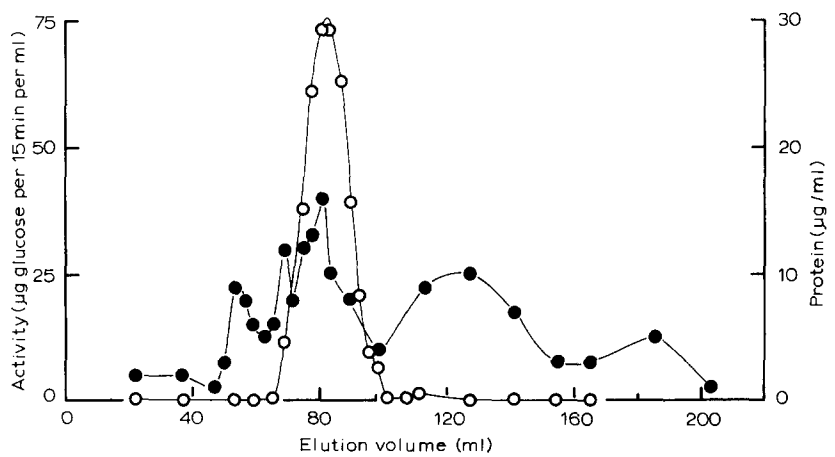


Fig. 2. Chromatography on Bio-Gel P-150. A sample of enzyme prepared by chromatography on DEAE-cellulose as described in Fig. 1, was dialysed against 0.01 M Tris–HCl buffer (pH 8.0) and freeze-dried. The sample was redissolved in water (0.24 ml) and added to a column of Bio-Gel P-150 (70 cm × 1.6 cm) previously equilibrated with 0.01 M Tris–HCl (pH 8.0). The enzyme was eluted with the same buffer and fractions were assayed for protein (●—●) and for activity against CM-pachyman (○—○).

the extract (containing 1.46 g protein) was adsorbed on a DEAE-cellulose column (37 cm × 4 cm) and the enzyme was eluted in 0.045 M Na_2SO_4 with a 50% recovery. The active fractions were dialysed against 0.01 M Tris–HCl buffer (pH 8.0) and freeze-dried. The enzyme (29.4 mg protein) was dissolved in 2.2 ml of water and chromatographed on a column of Bio-Gel P-150 (120 cm × 1.6 cm). The eluted enzyme, in a volume of 40.3 ml, contained 5.8 mg protein and 81% of the applied enzyme. The

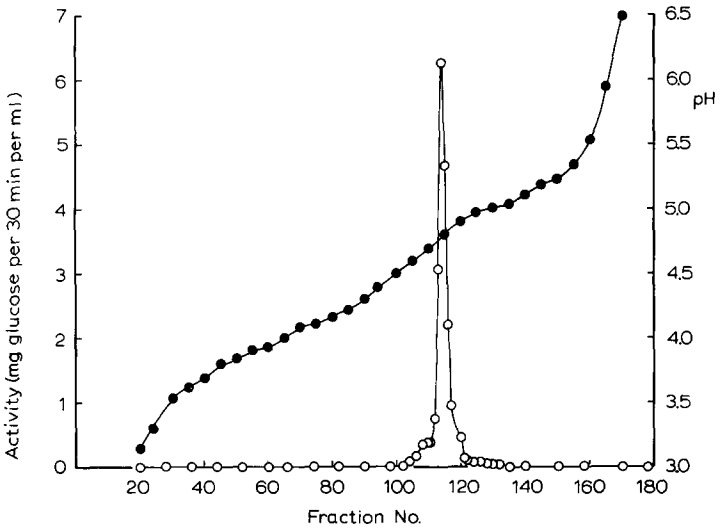


Fig. 3. Isoelectric focusing. A sample of preparation B was focused as described under EXPERIMENTAL PROCEDURE. The fractions collected from the column following electrolysis were assayed for activity against laminarin (○—○) and their pH values (●—●) were measured.

overall purification with respect to the dialysed leaf extract was 186-fold, with a recovery of 40%. This sample was used in studies on the properties of the enzyme and is referred to as Preparation A.

A sample of Preparation A was further purified by elution from DEAE-cellulose in a gradient of Na_2SO_4 (0–50 mM) in 0.01 M Tris–HCl buffer at pH 8 (Preparation B).

Isoelectric focusing. Preparation B was purified by isoelectric focusing and the results are shown in Fig. 3. A single peak with hydrolase activity was recovered in four fractions with pH's in the range of 4.848–4.906. These pooled active fractions are referred to as Preparation C.

Disc electrophoresis of enzyme preparations. Preparations A, B and C were examined by disc electrophoresis and the patterns obtained are compared in Fig. 4. The final purification steps reduced the number of detectable protein components from three to one.

Properties of the Nicotiana β-1,3-glucan hydrolase

Unless otherwise stated the enzyme used in these investigations was Preparation A.

pH optimum. The effect of pH on the activity of the enzyme was determined

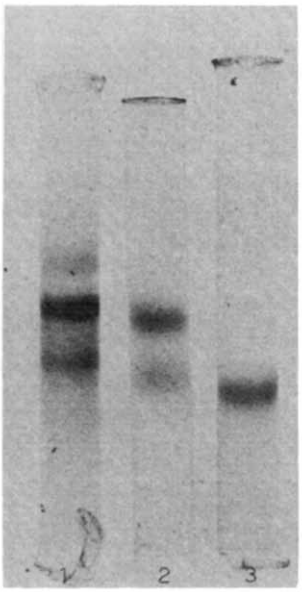
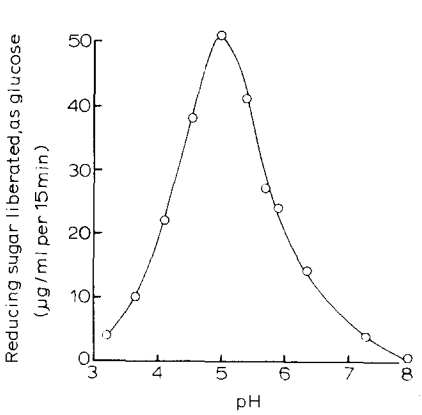


Fig. 4. Polyacrylamide gel disc electrophoresis of the purified enzyme preparations. After electrophoresis in Tris–borate buffer at pH 8.2, as described in EXPERIMENTAL PROCEDURE the protein bands were stained with Amidol Black 10B and the gels washed. 1. Preparation A purified by DEAE-cellulose and Bio-Gel P-150 chromatography. 2. Preparation B obtained from preparation A by DEAE-cellulose chromatography with gradient elution. 3. Preparation C obtained by isoelectric focusing of preparation B.



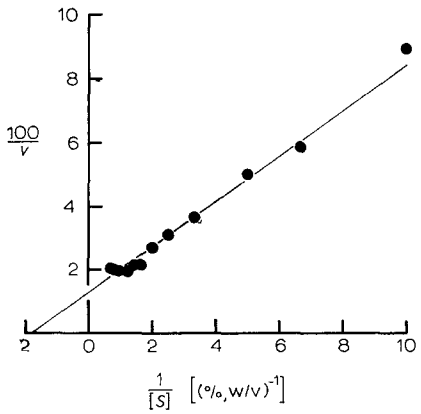


Fig. 5. pH–activity relationship. The incubation mixtures consisted of 1.2 ml 1.875% (w/v) CM-pachyman in water, 1.2 ml 0.05 M Tris–acetate buffer at each pH and 0.6 ml of appropriately diluted enzyme solution. After 15 min incubation at 40°, 1 ml of each reaction mixture was removed and heated at 100° for 5 min. The heated solutions were neutralised, diluted to 2.0 ml and the total reducing sugars determined. The pH values of the enzyme–substrate mixtures were measured after incubation.

Fig. 6. Lineweaver–Burk plot for hydrolysis of CM-pachyman by Nicotiana β-1,3-glucan hydrolase. The enzyme was incubated at 40° and pH 5.0 for 30 min with a series of concentrations of CM-pachyman from 0.05 to 1.4% (w/v). The reaction was terminated by addition of the SOMOGYI[15] alkaline copper reagent and the total reducing sugars were measured. The reaction velocity (v) is expressed as μg reducing sugar (as glucose) liberated per 30 min per ml incubation mixture.

with CM-pachyman as substrate. The conditions used and the results obtained are shown in Fig. 5.

Estimation of K_m (CM-pachyman). Reaction velocities were measured at a series of substrate concentrations as described in Fig. 6. From the graph the K_m for the Nicotiana enzyme has been estimated to be 0.6 g/100 ml and the molar value has been calculated as follows. The degree of substitution of the CM-pachyman sample was 0.30, so that the average mol. wt. per anhydroglucose unit was 180. Assuming the degree of polymerization of pachyman to be 255 (ref. 18), the mol. wt. of the CM-pachyman was 46 000 and the K_m value 0.13 mM.

Stability. Aqueous solutions of the purified enzyme were more stable to freezing and thawing and to freeze-drying when buffer salts were present and were more stable in Tris–HCl buffers at pH 8.0 than in acetate buffers at pH 5.0.

When solutions of the enzyme were diluted, there was a rapid loss in activity with a further slow decrease over the next few hours. This loss in activity was partially restored by including bovine serum albumin (100 μg/ml) in the incubation mixture during assay. When bovine serum albumin was added to the diluting solution the loss in activity was largely prevented.

Denatured pepsin and chicken erythrocyte histone had the same effect on enzyme stability and activity as bovine serum albumin. There were small differences in the effectiveness of these three proteins and their ability to maintain enzyme activity increased as the p*I* of the protein increased.

There was little variation in the stability of the enzyme at pH values in the range 4.1–8.7, either with or without added bovine serum albumin, but the enzyme was less stable at pH 3.2.

Examination of possible changes in size or shape of the enzyme in relation to its activity. The possibility that the varying stability of the purified enzyme in water

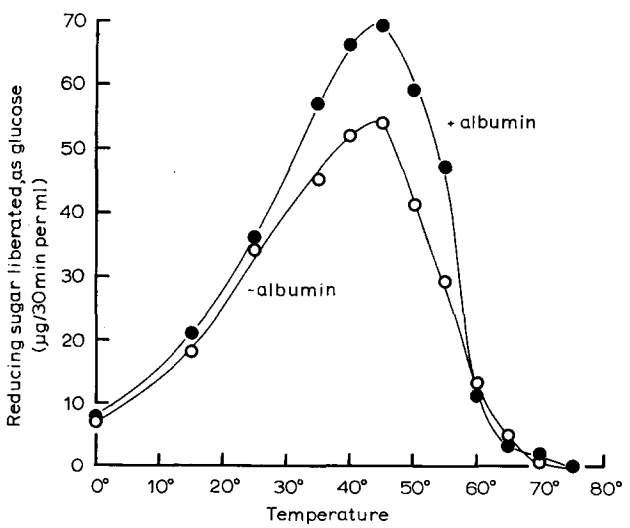


Fig. 7. Temperature–activity relationship. The enzyme was incubated with CM-pachyman substrate in the presence and absence of bovine serum albumin, for 30 min at each temperature and the reducing sugars were estimated. ○—○, no bovine serum albumin; ●—●, bovine serum albumin, 20 μg/ml.

and buffer solutions, the loss of activity on dilution and the stabilizing effect of bovine serum albumin could be related to conformational changes in the size or shape of the enzyme molecule was investigated. Concentration-dependant differences in size and shape of proteins can be detected by changes in elution volume using the method of frontal analysis on molecular exclusion media[19]. Elution volumes for the Nicotiana enzyme were measured by this method on Sephadex G-75 at a range of concentrations (1:40 to 1:5000 dilutions of Preparation A) in two buffers, 0.01 M Tris–HCl (pH 8.0) and 0.01 M acetate (pH 5.0). Elution volumes were also measured in the

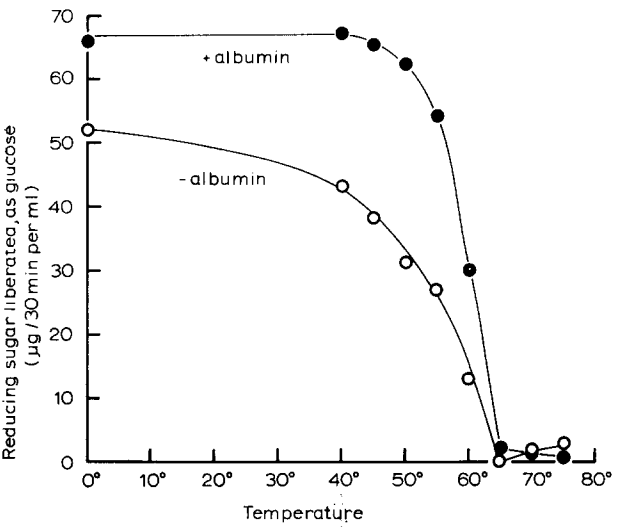


Fig. 8. Temperature stability. Samples of the enzyme, with and without bovine serum albumin were held at each temperature for 10 min prior to assay against CM-pachyman. ○—○, no bovine serum albumin; ●—●, bovine serum albumin, 100 μg/ml.

presence and absence of bovine serum albumin. No significant changes in elution volumes were detected under these conditions, indicating that detectable concentration-dependent changes (such as polymerization) were not responsible for the changes in enzyme activity and that no detectable differences in size or shape occurred in the two buffers or on addition of bovine serum albumin.

Effect of temperature on reaction velocity and stability. The effect of temperature on the activity of the enzyme was investigated in the range of 0–75°, both in the presence and absence of bovine serum albumin (20 μg/ml incubation mixture). The results are shown in Fig. 7, and the stability of the enzyme at temperatures between 40° and 75° is shown in Fig. 8. Under the conditions of the experiment the enzyme was completely inactivated at 65° in 10 min. The presence of bovine serum albumin did not alter the temperature for optimum activity or the temperature stability of the enzyme.

Molecular weight estimation by gel filtration. Chromatography was performed as described in EXPERIMENTAL PROCEDURE and showed that the Nicotiana enzyme had an exclusion volume of 134 ml and was eluted between bovine serum albumin (109 ml) and β-lactoglobulin (152 ml). The molecular weight estimated from the calibration curve was approx. 45 000.

DISCUSSION

The β-1,3-glucan hydrolase from *N. glutinosa* leaves has been obtained in an electrophoretically homogeneous state. The enzyme has characteristics which are similar to those of other plant β-1,3-glucan hydrolases for which data are available[20]. All have pH optima near pH 5.0 and are inactivated by heating to temperatures above 55–60°. The K_m for a wheat β-1,3-glucan hydrolase[21] acting on laminarin was 0.35% (w/v) which is of the same order as that for the Nicotiana enzyme with CM-pachyman as substrate.

The only other plant β-1,3-glucan hydrolase reported to have been purified to a state of homogeneity is one from the leaves of red kidney bean (*Phaseolus vulgaris*)[22]. This enzyme is apparently smaller than the Nicotiana hydrolase, since its molecular weight was 34 000 as determined by ultracentrifugation or between 11 500 and 12 500 when determined by gel filtration, amino acid analysis and antigen-antibody reactions. Its isoelectric point was reported to be near pH 11 as compared with the value of 4.9 determined for the Nicotiana enzyme.

The purified Nicotiana hydrolase has also been examined from the point of view of its action pattern, specificity and inhibitor behaviour and the results are described in the accompanying paper[2].

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