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PARTIAL PURIFICATION AND PROPERTIES OF AN ENDO-1,3-β-D-GLUCANASE FROM GERMINATED RYE*

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Key Word Index—*Secale cereale*; Gramineae; germinated rye; endo-1,3-β-D-glucanase; cereal β-D-glucans.

Abstract—An endo-1,3-β-D-glucanase from germinated rye was purified 58-fold by ion exchange chromatography on DEAE- and CM-cellulose, and by gel filtration on Bio Gel P-60. The enzyme had pH optima of 5.0 with laminarin and 5.7 with carboxymethyl pachyman and was stable up to 45°. Bovine serum albumin and certain chlorides increased the activity at low concentrations of buffer. With laminarin, the enzyme had a K_m of 0.25 mg/ml and the MW was estimated to be 24300. Isoelectric focusing indicated that the endo-1,3-β-D-glucanase was a basic protein. The properties of the enzyme are compared with those from other higher plants.

INTRODUCTION

Extracts of ungerminated rye [1], like many other higher plants [2], micro-organisms [3] and invertebrates [4] show endo-1,3-β-D-glucanase (EC 3.2.1.39) activity. Recently, the activity in rye was shown to increase markedly (more than 20-fold) during germination [5], in a similar manner to that observed previously in barley [6, 7]. The reason for this substantial increase in endo-1,3-β-D-glucanase activity during the germination of cereal grains is not precisely understood. The enzyme has only a limited ability to hydrolyse soluble cereal β-D-glucans, and this is considered to be due to the random occurrence of sequences of adjacent 1,3-linkages in the β-glucans [8]. However, recent work with the corresponding enzyme from barley and barley endosperm cell walls has shown that the enzyme can solubilize cell wall material [9], thus suggesting that the endo-1,3-β-D-glucanase may be involved in wall solubilization. In the present study, the enzyme from germinated rye was purified and characterized, in order to compare its properties with those of endo-1,3-β-D-glucanases from other plant sources.

RESULTS

Enzyme purification

Endo-1,3-β-D-glucanase was purified from an extract of germinated rye flour in an analogous manner to the procedures used for barley [10, 11]. The extract was initially passed through a pad of DEAE-cellulose equilibrated with 0.01 M Tris–HCl buffer, pH 8.5, to adsorb much of the coloured material and inactive protein. The effluent, which contained the enzymic activity, was concentrated by ultrafiltration using a PM-10 membrane, dialysed against 0.02 M sodium acetate buffer, pH 4.8 and chromatographed on a column of CM-cellulose which had been equilibrated in the same buffer. The column was eluted with a sodium chloride gradient to give the separation profile illustrated in Fig. 1. After concentration, the activity was chromatographed on a column of Bio Gel P-60, in the same buffer, to give the results shown in Fig. 2. The pooled fractions were re-chromatographed in a similar manner to remove overlapping protein peaks.

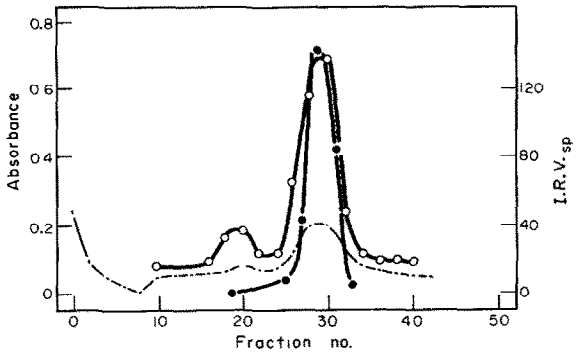

Fig. 1. CM-cellulose column chromatography of a partially purified extract of germinated rye. A linear NaCl gradient (0–0.35 M, 700 ml) was used to elute the column (30 cm × 1.5 cm). Elution of protein was followed by absorbance at 280 nm (·—·—·). Fractions (16 ml) were assayed for 1,3-β-D-glucanase activity by reductometric (O—O) and viscometric (●—●) procedures. The activities are reported as the increase in absorbance at 600 nm and as the increase in reciprocal specific viscosity ($1/\eta_{sp}$) respectively.

Analysis of the purified sample by electrophoresis in polyacrylamide gels at pH 4.75 indicated the presence of two major and two minor protein bands; the enzymic activity corresponded to only one of the major bands. The enzymic activity did not migrate into the gels when the electrophoresis was carried out at pH 8.9. Attempts to remove the contaminating protein material were unsuccessful and the enzyme was therefore characterized at this stage. On the basis of the specific activity assayed viscometrically, a 58-fold enrichment had been achieved.

* Part V in the series 'Studies on β-glucanases'. For Part IV see Manners, D. J., Stark, J. R., Wilson, G. and Brodie, J. Y. (1976) *Carbohydr. Res.* **49**, 383.

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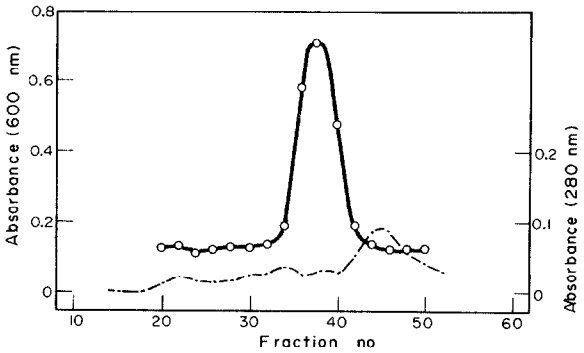

Fig. 2. Separation profile of rye endo-1,3-β-D-glucanase on Bio Gel P-60. Protein (·—·—·) was estimated in fractions (3.0 ml) eluted from the column (90 cm × 2 cm) as the absorbance at 280 nm, while activity was measured reductometrically with laminarin (O—O) and is reported as the increase in absorbance at 600 nm.

Properties of the enzyme preparation

pH *optima*. The endo-1,3-β-D-glucanase has a different pH optimum for the two substrates, laminarin and CM-pachyman (CMP). Both substrates were prepared in sodium phosphate buffers (0.12 M) at various pH values from 4.7 to 7.0. Two distinct dumbbell shaped curves were obtained, which indicated pH optima of 5.0 and 5.7 for laminarin and CMP respectively. Similar results have been reported for the barley enzyme[11]. The difference in pH optima for these two 1,3-β-D-glucans is probably due to the carboxymethyl groups in the CMP. These rather acidic groups may interact with the basic protein in such a manner that cleavage of the glucosidic linkages occurs more favourably at higher pH values.

Temperature optimum and thermal stability. For temperature optimum experiments, the enzyme was incubated with laminarin at various temperatures ranging from 30 to 55° for periods of 40 min. For heat stability experiments, the enzyme was incubated under the same conditions in the presence of 0.02 M sodium acetate buffer (pH 5.0) but in the absence of substrate. The enzyme was then cooled to 30° for 10 min before being incorporated into enzyme digests at 37°. Reductometric assays showed that the temperature optimum was 45° and the temperature of maximum heat stability using a 40 min incubation period was also 45°.

Effect of bovine serum albumin (BSA). BSA was incorporated into enzyme–laminarin digests at 3 different concentrations of sodium acetate buffer. The effect of BSA on the endo-1,3-β-D-glucanase activity (reductometric assays) is shown in Fig. 3. BSA caused a very marked stimulation of activity, especially at low concentrations of buffer. However, as the concentration of buffer was increased the stimulation effect gradually disappeared. At low buffer concentrations, it is apparent that maximum activity cannot be achieved by the addition of BSA alone. Maximum stimulation with sodium acetate occurred at 0.10 to 0.12 M. BSA is often used to stabilize enzyme activity [12] and, in several cases, has been found to increase activity [10, 13]. In the present case, the activation was dependent on the concentration of buffer and it would seem likely that these two effects will be partly dependent on the purity of the enzyme preparation being used, i.e. the amount of protein in the digest.

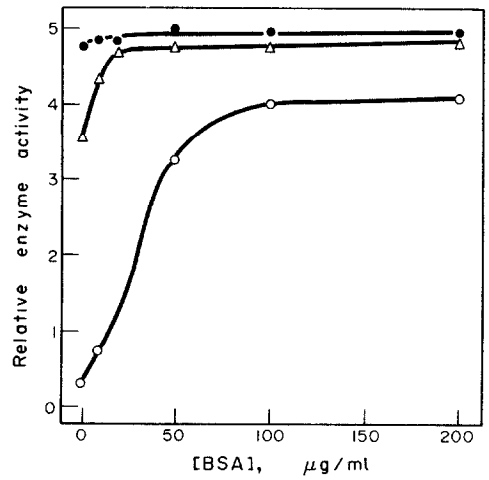

Fig. 3. Effect of BSA on enzyme activity. Increasing levels of BSA were added to digests prepared in 0.004 M (O—O), 0.024 M (△—△) and 0.064 M (●—●) NaOAc buffer at pH 5.0.

Effect of salts. The chlorides of sodium, magnesium and calcium were tested for relative activation effects on the endo-1,3-β-D-glucanase. A standard reductometric assay was used except that the buffer concentration was lowered to 0.004 M in the digest. The relative effects of the three salts are illustrated in Fig. 4. The salts with divalent ions were more effective than sodium chloride both at equal concentrations and equal ionic strengths. The activation effect with these salts disappears at higher concentrations of buffer, suggesting it is a general salt effect. Ferrell and Luchsinger [14] observed that barley endo-1,3-β-D-glucanase was also activated by a number of different salts in the absence of other salts. Manners and Wilson [10] have more recently shown that cobalt, manganese and barium may cause activation of the barley enzyme, even in the presence of 0.05 M acetate buffer. Similar results had been observed previously with these same ions causing activation of certain fungal laminarinases [3].

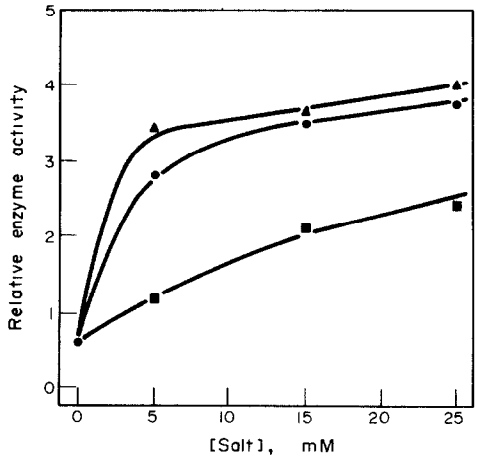

Fig. 4. Effect of salts on enzyme activity. NaCl (■—■), $CaCl_2$ (●—●) and $MgCl_2$ (▲—▲) were incorporated into digests at concentrations of 5–25 mM in the presence of 0.004 M NaOAc buffer. The activity is reported in relative units.

Hydrolysis products from β-glucans. For the germinated rye endo-1,3-β-D-glucanase, laminarin and CMP were the preferred substrates; the enzyme had no effect on CM-cellulose. With barley β-D-glucan, it caused a slow decrease in viscosity, but did not release oligosaccharide products, as indicated by paper chromatography of the enzyme digest after 24 hr. These results confirm that the enzyme is a true endo-1,3-β-D-glucanase (EC 3.2.1.39), and therefore similar to the corresponding enzymes from other higher plants which have been examined so far. However, Abeles *et al.* [15] have referred to their bean leaf endo-1,3-β-D-glucanase as laminarinase (EC 3.2.1.6) an enzyme which may hydrolyse either 1,3- or 1,4-linkages in cereal β-D-glucans although they have not, in fact, shown that the bean enzyme will act on mixed linkage substrates.

Laminaritriose and laminaritetraose were hydrolysed to yield smaller oligosaccharides and glucose. Laminaribiose was not hydrolysed, indicating that laminaritriose was the smallest substrate. With laminarin, all the oligosaccharides up to laminariheptaose were observed. This result differs from the corresponding experiments with barley [10] and tobacco [16] which produced only very small amounts of laminaritetraose in laminarin hydrolysates.

Effect of substrate concentrations on reaction velocity. The initial velocities of the enzyme were measured at increasing concentrations of a sample of laminarin with a DP of 21, in digests containing 0.125 M sodium acetate buffer pH 5.0 which were incubated at 37°. The K_m value for six experiments was estimated by the Lineweaver–Burk double reciprocal plot to be 0.24 ± 0.02 mg/ml and by the method of Eisenthal and Cornish-Bowden [17] to be 0.26 ± 0.03 mg/ml. In molar terms, the average of these two estimates yields a value of 7.3×10^{-5} M. This is somewhat smaller than the K_m observed for the barley enzyme of 2.7×10^{-4} M [10].

Molecular weight. The MW of the endo-1,3-β-D-glucanase was estimated on the basis of its elution volume from a calibrated column of Sephadex G-100. A sample of barley endo-1,3-β-D-glucanase used for comparison in this study had been prepared from the malted cereal by Dr R. W. Gordon. From Fig. 5 the MWs of the rye and barley enzymes were estimated to be about 24 300 and 21 400 respectively. Previous lower values for the MW of the barley enzyme [6, 10] may have been due to an interaction between acidic groups reported to be present in Sephadex [18] and this basic enzyme [11]. The presence of 0.1 M potassium chloride in the elution medium as recommended by Andrews [19] is considered to have overcome this problem. The rye and barley enzymes thus appear to have MWs of the same order of magnitude.

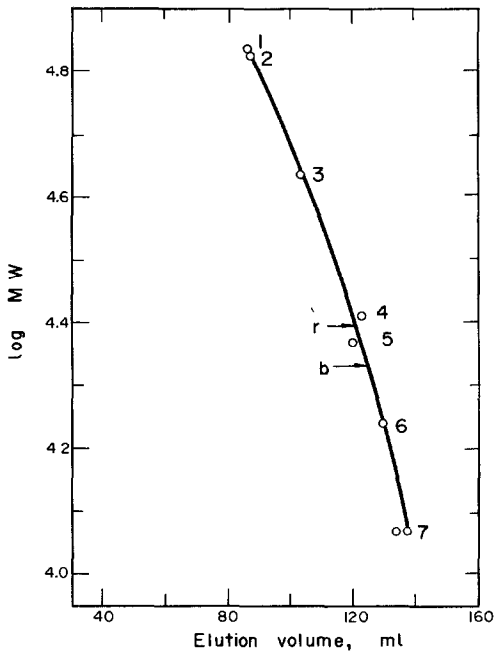

Fig. 5. A plot of log MW against elution volume from a column of Sephadex G-100 for the estimation of the MW of endo-1,3-β-D-glucanases. The arrows r and b designate the elution positions of the endo-1,3-β-D-glucanases from germinated rye and germinated barley respectively. Standard proteins: (1) human serum albumin (68 000); (2) BSA (67 000); (3) ovalbumin (43 000); (4) α-chymotrypsinogen (25 700); (5) trypsin (23 300) (6) myoglobin (17 200); (7) cytochrome-*c* (11 700).

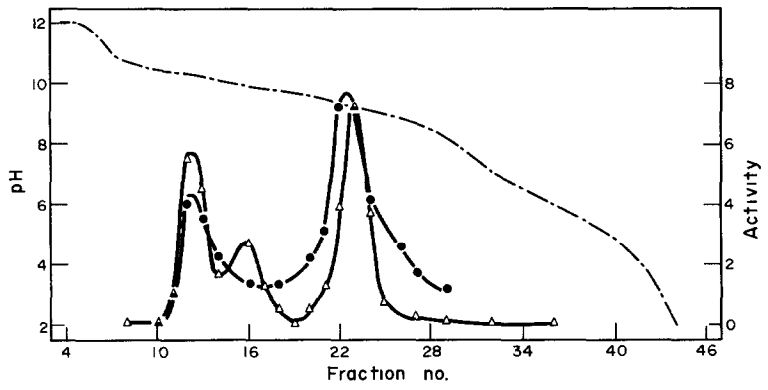

Fig. 6. Isoelectric focusing profile of rye endo-1,3-β-D-glucanase. The pH (·—·—·) and activities as estimated viscometrically (●—●) and reductometrically (△—△) were as shown. The activities for both methods of determination are expressed in relative units.

Table 1. Characteristics of endo-1,3-β-D-glucanases from higher plants

	pH optimum Lam	pH optimum CMP	Temp. optimum °C	Temp. stability* °C	K_m (laminarin) mg/ml	MW	pI	Major hydrolysis products	Ref.
Germinated barley	4.6		—	50, 2 hr	0.92	—	—	oligosacch + G	21
Malted barley		5.4	—	—	—	9000	—	oligosacch + G	6
Malted barley	5.0		37, 1 h	—	—	12000	—	oligosacch + G (G_4, trace only)	10
Germinated barley	5.0	5.8	—	60, 40 min	—	—	9.8	—	11
Malted barley	—	—	—	—	—	21400	—	—	—
Barley, wheat, oats, potato, hyacinth bulb	5		—	—	—	—	—	—	22
Rye	5.2		—	—	—	—	—	oligosacch + G	1
Germinated rye	5.0	5.7	45, 40 min	45, 40 min	0.25	24300	9.2, 10.3	oligosacch + G	—
Bean leaves	5		50 —	—	—	12000 34000	*ca* 11	—	15
Tobacco leaves		5.0	45, 30 min	40, 10 min	†	45000	4.87	oligosacch + G (G_4, trace only)	16, 23
Tobacco leaf cells	5.0		—	40, 20 min	2.2	—	basic	—	20
Grape vine	5–6		—	30, 10 min	—	—	—	—	2

* Temp stability—little or no loss in activity in the period indicated.
† 6 mg/ml towards CMP.

Isoelectric points. The isoelectric focusing profile of the endo-1,3-β-D-glucanase is shown in Fig. 6. The results demonstrate that the enzyme is a basic protein, as had been suggested from its behaviour on DEAE-cellulose and during electrophoresis at pH 8.9. It had two components with pI values of 9.2 and 10.3. Endo-1,3-β-D-glucanase from bean leaves [15], tobacco leaf cells [20], and barley [11] has also been found to be basic protein. If the original extract of germinated rye was allowed to stand at 4° for several days before being fractionated, a large number of isoelectrically different fractions appeared. One of the two forms found in Fig. 6 may therefore have been derived from the other but this possibility has not yet been investigated further.

DISCUSSION

A survey of the literature (Table 1) shows that although enzymes acting on 1,3-β-D-glucans are present in many higher plants, relatively few have been adequately characterized. As far as the various properties are concerned, the pH optimum of the enzyme from germinated rye towards laminarin of 5.0 is typical of most higher plants, and the optimum pH value with CM-pachyman is generally similar to the enzymes from germinated barley [11] and tobacco leaves [16].

Information on the effect of temperature is more limited. Optimum temperature values ranging from 37 to 50° have been reported (Table 1), but since these depend on the actual period of incubation which may be variable or unspecified, strict comparison is not possible. However, these plant enzymes, as a group, do not show marked temperature stability, and the enzyme from germinated rye would appear to be less stable than its counterpart in germinated barley.

There are only a few reports of K_m values with laminarin, and the enzyme from germinated rye has the lowest value of those listed. In all cases, enzyme action leads to the production of the homologous series of laminarisaccharides, although the amount of glucose and laminaritetraose is lessened with certain enzymes. In general, the enzymes show a high degree of specificity for 1,3-β-D-glucans, and their action on cereal β-D-glucans which contain both 1,3- and 1,4-linkages cannot be considered as substantial.

In terms of physical properties, most of the enzymes are basic proteins of relatively low MW. The actual values for the enzymes from germinated barley and bean leaves [15] have not been unequivocally established. Different techniques have given different results, or, in the case of gel filtration measurements, there is some evidence of anomalous interactions between the column support and the basic proteins. However, the apparent MW value of 24300 for the germinated rye enzyme indicates that it could be amenable to primary structural analysis, at a later date, if this result can be confirmed by other physico-chemical measurements.

EXPERIMENTAL

Enzyme source. A sample of rye (var. *Lavats patonia*) was surface sterilized by immersion in 10% aq. NaClO for 10 min. The grain was rinsed several times with distilled H_2O, steeped for 16 hr and then germinated on moist filter paper at 17° for 6 days. The germinated grain was then frozen, freeze-dried and milled to a flour in a Casella mill. The flour (30 g) was extracted × 2 by stirring with successive vols (100 ml) of aq. 2%NaCl. After centrifugation, the supernatant was filtered through glass wool, and dialysed against 0.01 M Tris-HCl buffer, pH 8.5 at 4°. The dialysate was concentrated by ultrafiltration over a PM-10 membrane.

Substrates. Insoluble laminarin (sample BB2), and another sample were laboratory samples prepared from *Laminaria cloustoni*. Barley β-D-glucan was prepared following the procedure of Bass and Meredith [24] for β-polyglucoside. Small amounts of α-glucan were removed by digestion with purified amyloglucosidase. Laminarisaccharides were prepared by partial acid hydrolysis of pachyman [25]. The oligosaccharides were separated by PC after salts and glucose had been removed with a charcoal Celite column. Carboxymethyl pachyman (CMP) was prepared by the method of Clarke and Stone [2].

Analytical methods. Reducing sugar estimation. Reducing sugars were estimated quantitatively by the Nelson–Somogyi colorimetric method using the reagent formulation described by Robyt and Whelan [26].

Protein content In column fractions, eluted protein was indicated by the absorbance at 280 nm. For quantitative estimation of protein, the Miller modification [27] of the Lowry *et al.* [28] method was used.

Electrophoresis: This was carried out at pH 8.9 and 4.75 following the procedures described by Davis [29] and MacGregor and Meredith [30] respectively. Isoelectric focusing was accomplished with an LKB 8100 Ampholine Electrofocusing apparatus (110 ml column) using primarily a 9–11 Ampholine following the procedure recommended by the manufacturers. The density gradient was produced with sorbitol. Electrofocusing was carried out at 4° under a potential difference of 600 V until the current was constant (72–90 hr).

Assay of enzyme activity: For reductometric assays, the standard digest contained 0.40 ml of laminarin (0.1%) in 0.2 M NaOAc buffer, pH 5.0 and 0.10 ml of a suitably diluted enzyme. Incubation was normally at 37°. The viscometric assay involved mixing 2.0 ml of CMP (0.66%) in 0.2 M NaOAc buffer pH 5.5 with 0.5 ml of enzyme. The digest was rapidly mixed and 2.0 ml transferred to an Ostwald type viscometer equilibrated at 37°. The viscometric activity was measured as the increase in reciprocal specific viscosity ($1/\eta_{sp}$) with time.

Estimation of MW: A Sephadex G-100 column (93 × 1.5 cm) was eluted with 0.02 M NaOAc buffer pH 4.8 containing 0.1 M KCl. The column was eluted at a flow rate of 30 ml/hr at room temp. Samples of 1.0 ml were loaded while 2.0 ml fractions were collected. The standard proteins were prepared at 5 mg/ml and detected in effluent fractions by absorbance at 280 nm.

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