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SOME PROPERTIES OF A β-1,3-GLUCANASE FROM RYE*

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Abstract—Molecular-sieve chromatography of an extract from ungerminated rye indicated the presence of enzymes which hydrolysed cellobiose, laminaribiose and the β-glucans cellodextrin, laminarin and barley β-glucan. A purified *endo*-β-1,3-glucanase was prepared from the extract by ammonium sulphate fractionation and molecular-sieve chromatography on Biogel P60. The substrate specificity and some properties of the enzyme are reported and the *in vivo* role of the enzyme is discussed.

INTRODUCTION

THE β-GLUCANASES in both ungerminated and malted barley have been extensively studied.[1,2] Little is known, however, about the β-glucanase systems of other cereals, although it is clear that enzymes of this type, particularly *endo*-β-1,3-glucanases, are almost as ubiquitous as the amylases in the plant kingdom.[3] A study of these enzymes may lead *inter alia* to a knowledge of their *in vivo* function and provide indirect information regarding the changes taking place in cereal polysaccharides during germination. For this reason, and as part of a study of the metabolism of the cereal carbohydrates, an investigation of the enzymes present in barley[4] and rye has been undertaken. The present communication reports our findings on the glucanase system of ungerminated rye and describes an investigation of an *endo*-β-1,3-glucanase (E.C. 3.2.1.6, β-1,3-glucan glucanohydrolase) from this cereal.

Previous work[3,4] has emphasized the utility of the modified β-glucans carboxymethylcellulose (CMC) and carboxymethylpachyman (CMP) for the direct assay of *endo*-β-1,4- and *endo*-β-1,3-glucanase activities. Action of endoacting enzymes on these substrates results in a rapid decrease in viscosity accompanied by only a small increase in reducing power. By analogy with the action of amylolytic enzymes on modified substrates,[5–7] exo-acting glucanases are considered to have little or no action on these substrates. *exo*-β-Glucanase activity is readily detected by the direct release of glucose from laminarin, cellodextrin or other similar substrates. Since our previous work,[4] and also more recent work,[8] has shown that *endo*-β-glucanases are a group of low molecular weight enzymes

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which may be separated by molecular-sieve chromatography on rather highly cross-linked gels, we have continued with the use of Biogel P60, a cross-linked polyacrylamide, for the separation of these enzymes.

RESULTS

Chromatography of Complete Protein Extract on Biogel P60

Freeze-dried extract of ungerminated rye (125 mg) was dissolved in 1·0 ml of 5 mM citrate buffer pH 4·6 and then fractionated by chromatography on Biogel P60. Assay of the column fractions for viscosity reducing activity and reducing power increasing activity showed the following. A high MW fraction, eluted at the void volume of the column, was active on all substrates, yielding glucose as the major product. This fraction was considered to contain a mixture of β-glucosidases or unspecific *exo*-β-glucanases capable of cleaving both β-1,3 and β-1,4-glucosidic linkages. Later column fractions, corresponding to proteins of lower MW, contained *endo*-β-glucanase activities as shown by their ability to bring about a rapid decrease in viscosity of their substrates with little release of reducing power. At least three activities of this type were present. Although these were not clearly separated, it was probable that more than one enzyme active towards CMP was present, while an intermediate fraction, active towards CMC and barley glucan, was considered to be an *endo*-β-1,4-glucanase. In view of the complex nature of the β-glucanase mixture it was considered that separation of the components into discrete fractions by chromatography on Biogel P60 alone was not feasible. Ammonium sulphate fractionation was therefore attempted as a means of affecting a preliminary separation of the activities present.

TABLE 1. SEPARATION OF RYE β-GLUCANASES BY AMMONIUM SULPHATE FRACTIONATION

Fraction	Ammonium sulphate concn. (% saturation)	Yield (g)	Specific activities* *endo*-β-1,3 Glucanase	*endo*-β-1,4-Glucanase	β-Glucosidase
F1	0–33	2·54	0·0066	0·025	36
F2	33–45	6·91	0·0046	0·016	21
F3	45–60	2·88	0·0047	0·021	130
F4	60–80	2·90	0·0026	0·049	104

* For *endo*-β-1,3-glucanase and *endo*-1,4-glucanase the specific activities are expressed as $d(1/\eta_{sp})/dt$ protein content of enzyme solution. β-Glucosidase activity is expressed as μg glucose released per hr per mg of protein.

Separation of Rye β-Glucanases by Ammonium Sulphate Fractionation

Four fractions (F1–4) from an extract of rye, obtained by ammonium sulphate fractionation, were assayed for *endo*-β-1,3-glucanase, *endo*-β-1,4-glucanase and β-glucosidase activity using as substrates CMP, CMC and cellobiose respectively. The results are shown in Table 1. It is clear from these figures that this procedure yields a fraction (F1) relatively rich in *endo*-β-1,3-glucanase activity and from which the majority of the *endo*-β-1,4-glucanase activity and β-glucosidase activity have been removed. The latter activities are highest in fractions F4 and F3 respectively. For this reason, fraction F1 was chosen for further purification of *endo*-β-1,3-glucanase.

Purification of endo-β-1,3-*Glucanase from Ammonium Sulphate Fraction* F1 *by Molecular-Sieve Chromatography*

A portion of F1 (150 mg) was dissolved in 1 mM citrate buffer pH 5·0 and then fractionated on Biogel P60. The distribution of protein and *endo*-β-1,3-glucanase activity in the fractions is shown in Fig. 1. Two peaks of activity towards CMP were found, in accord with the preliminary conclusions reached by chromatography of the complete enzyme extract on Biogel P60.

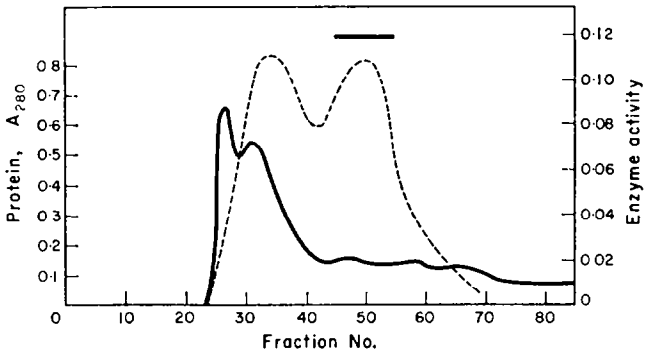

FIG. 1. ELUTION PATTERN OF ENDO-β-1,3-GLUCANASE FROM A BIOGEL P60 COLUMN.
The elution of protein is shown by the continuous line, and the enzyme by the broken line. The fractions beneath the horizontal line were combined for studies on the purified enzyme.

Qualitative Examination of Activities in Fractions from Biogel P60 *Column Chromatography of* F1

Samples (0·1 ml) from selected fractions were incubated at 37° with cellodextrin (2 mg) or with laminarin (2 mg). One drop of toluene was added to each enzyme digest to prevent microbial contamination. After 24 hr the products produced by enzyme action were examined by PC; the results are shown in Table 2. The *endo*-β-1,3-glucanase of higher MW, that with maximum activity in fraction 35 was grossly impure, being associated with a large amount of protein and β-glucosidase activity. The latter activity is maximal in fraction 27 and its presence results in the production of large amounts of glucose and transferase/reversion products. No attempt was therefore made to examine this *endo*-β-1,3-glucanase further and no definite conclusions have been reached at the present time regarding its specificity. Later column fractions, however, contain a purified *endo*-β-1,3-glucanase, clearly free from β-glucosidase, which acts on laminarin to give a series of laminarisaccharides of DP two and upwards.

Cellodextrin is attacked by the early fractions from the column yielding mainly glucose and reversion/transferase products, in agreement with the presence of unspecific β-glucosidase(s) in these fractions. Later fractions (40–45) contain a small amount of *endo*-β-1,4-glucanase, the activity detected previously, which releases mainly cellobiose and cellotriose from cellodextrin.

Specificity of Purified endo-β-1,3-*Glucanase Towards β-Linked Substrates*

The specificity of the lower MW *endo*-β-1,3-glucanase obtained by molecular-sieve chromatography on Biogel P60 was examined by incubating samples of fraction 52 (0·1 ml)

with a selection of substrates (2 mg). After incubation for 24 hr in the presence of toluene to exclude microbial contamination, the digests were examined by PC. The results are shown in Table 3.

TABLE 2. PRODUCTS OF HYDROLYSIS OF LAMINARIN AND CELLODEXTRIN BY FRACTIONS FROM BIOGEL COLUMN FRACTIONATION

	Column fractions						
	26	31	40	45	50	55	60
Products from laminarin*							
Higher laminarisaccharides	++	++	+	±	+	+	+
Cellobiose	±	±	±	−	−	−	−
Laminaribiose	±	−	++	++	++	++	+
Glucose	+++	+++	+++	+++	−	−	−
Products from cellodextrin*							
Higher cellosaccharides	+++	+++	+++	+++	±	−	−
Cellobiose	−	−	++	+	−	−	−
Laminaribiose	−	−	±	−	−	−	−
Glucose	+++	+++	++	±	−	−	−

* Qualitative results obtained by PC, the intensity of the spots on chromatograms being shown by +++ heavy, ++ medium, + light, ± trace, − nil.

Some Properties of the Purified endo-β-1,3-*Glucanase*

For studies of the properties of the purified *endo*-β-1,3-glucanase, combined fractions 46–55 was used and activities were measured using CMP as substrate with a viscometric assay.

Optimum pH. Using citrate–phosphate buffers of various pH, the optimum pH of the purified enzyme was 5·2.

TABLE 3. SUBSTRATE SPECIFICITY OF PURIFIED RYE *endo*-β-1,3-GLUCANASE

Substrate	Result of enzyme treatment	Substrate	Result of enzyme treatment
Laminarin	Hydrolyzed giving laminarisaccharides without production of glucose	Cellobiose	No hydrolysis
		Laminaribiose	No hydrolysis
		Laminaritriose	Gave laminaribiose and laminaritetraose with smaller amounts of glucose and laminaripentaose
Pachyman	Hydrolyzed giving laminarisaccharides without production of glucose		
Lichenin	No hydrolysis	Laminaritetraose	Hydrolysed giving glucose, laminaribiose and laminaritriose
Barley glucan	No hydrolysis		
Oat glucan	No hydrolysis		
Cellodextrin	No hydrolysis		

Effect of added ions on endo-β-1,3-*glucanase.* Table 4 shows the effect of various possible activators or inhibitors of β-glucanases on the enzymic activity. No significant activation of the enzyme by chloride ions was observed; the sulphydryl reagent *p*-CMB had little

effect on the activity. Treatment of the enzyme with phenylmercuric nitrate resulted in almost complete loss of activity.

TABLE 4. EFFECT OF ADDED REAGENTS ON *endo*-β-1,3-GLUCANASE ACTIVITY

Treatment*	Activity (%)	Treatment*	Activity (%)
Control	100	NaCl 0·2 M	104
p-CMB, 10^{-5} M	89	Phenylmercuric nitrate, 5 mg/ml	7·5

In the case of *p*-CMB and NaCl, appropriate amounts of these reagents were preincubated with enzyme for 30 min at room temp. before addition of substrate. For phenylmercuric nitrate treatment enzyme solution was preincubated with the solid for 30 min, centrifuged than an aliquot used in standard digests.

DISCUSSION

It has been shown that ungerminated rye, like barley,[9] contains a complex system of enzymes for the degradation of β-glucans. Fractionation of these by molecular-sieve chromatography shows the presence of at least one glucose liberating enzyme (this may be a β-glucosidase or an *exo*-β-glucanase; no effort has been made to distinguish between these possibilities). At least three β-glucanases are present and one of these has been obtained in a purified form by molecular-sieve chromatography, after a preliminary ammonium sulphate fraction. The enzyme is specific for β-1,3-linked polysaccharides and without action on β-1,4-linked polysaccharides or mixed linkage polysaccharides of the cereal glucan and lichenin type. Action on laminarin yields laminaribiose as the smallest oligosaccharide indicating that the smallest possible substrate for the enzyme is laminaritetraose. Incubation with laminaritriose, which at a low concentration is not hydrolysed, at a concentration of 2% results in the formation of higher and lower oligosaccharides including glucose. It is likely that this is the result of transferase action although some hydrolysis cannot be ruled out. That hydrolysis to yield glucose may take place is indicated by the action of the enzyme on laminaritetraose where small amounts of the monosaccharide, apparently without the formation of oligosaccharides of DP higher than the tetrasaccharide was detected. The enzyme may therefore be analogous to α-amylase in its ability to liberate glucose at a slow rate from lower oligosaccharides in which the possibility of random *endo*-action has been eliminated. The transferase activity mentioned above is a further analogy between the rye enzyme and α-amylase.

The lack of any significant inhibition by *p*-CMB indicates that the enzyme does not have a sulphydryl group in its active site. The inability to detect a chloride activation of the rye enzyme is similar to the barley enzyme[4] and supports our view that reports of the activation of enzymes of this type by sodium chloride are incorrect, and are most probably the result of interference with the assay procedure used. Phenyl mercuric nitrate was used by Preece and Hoggan[10] to inactivate *exo*-β-glucanase activity and was reported to have no effect on *endo*-acting enzymes. That this selective inhibition is not generally applicable is evident from the near-complete inhibition of the *endo*-β-1,3-glucanase. However, it may be that the inability to achieve selective inhibition is dependent on the purity of the preparation since Preece and Hoggan[10] used the inhibitor for treatment of unpurified enzyme preparations. In such cases the contaminating protein may help to prevent inactivation of the *endo*-activity.

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The similarity both of the glucanase complexes and the purified *endo*-β-1,3-glucanases from barley and rye will be apparent by comparison of the present results with those we have reported earlier.[4] Although we have not accurately determined the MW of the purified rye *endo*-β-1,3-glucanase, the similarity is carried to that also. The position of elution of the rye enzyme on Biogel P60 indicates that, like the barley enzyme, this also has a relatively low MW. In view of the ease of purification of the enzyme it too may be suitable for amino acid sequence determination in the near future.

The discovery of *endo*-β-1,3 glucanases which are without action on the major glucans in extracts of the cereals, as well as in tissue cultures of barley cells,[11] suggests this enzyme must have an *in vivo* role. However the major structural polysaccharides in cereals are considered to be cellulose, pentosans and mixed-linkage glucans, none of which are substrates for this type of enzyme. The only plant β-1,3-linked glucan commonly known is callose[12] which occurs in the pores of the sieve plates of the phloem. Taiz and Jones[13] have suggested, on the basis of histochemical studies, that barley aleurone cell walls contain a β-1,3-glucan which is digested by a specific β-1,3-glucanase. Related observations on the aleurone cell walls of wheat by Fulcher *et al.*[14] do not support this view. In this latter cereal, these authors obtained evidence for the presence of a ferulic acid—carbohydrate complex in the cell walls, and since the carbohydrate is PAS-positive, it cannot be a β-1,3-glucan.

The walls of endosperm cells contain a mixture of polysaccharides, including cellulose, hemicelluloses, and β-glucan. Part of this glucan is extractable with warm water (40–60°), and contains about 70% of β-1,4- and 30% of β-1,3-glucosidic linkages.[1,15] The remaining glucan is insoluble and of unknown constitution, but is solubilized during germination by the action of hydrolytic enzymes on the cell walls. It is possible that the less soluble glucan contains a higher proportion of β-1,3-glucosidic linkages, and that the *in vivo* role of the *endo*-β-1,3-glucanase is to facilitate the solubilisation of the cell wall glucan. This would then allow the amylases to reach the starch reserves in the endosperm.

EXPERIMENTAL

Enzyme preparations. Rye (var. Lavats patonia) was milled in a hammer mill and the flour extracted with 100 mM acetate buffer pH 5·0 (3 l./kg of flour) for 5 hr at room temp. After centrifugation to remove grain residues, starch, etc. protein was precipitated from the extract by the addition of $(NH_4)_2SO_4$ to 80% saturation. The precipitated protein was collected by centrifugation, dissolved in 100 mM acetate buffer pH 5·0 and dialysed for 48 hr against a slow stream of running H_2O. After removal of insoluble material by centrifugation, the solution was freeze dried. Alternatively protein extracts from rye flour prepared by extraction with acetate buffer as described above were fractionated by the addition of ammonium sulphate. The protein precipitating between 0–33, 33–45, 45–60 and 60–80% saturation was collected by centrifugation, dialyzed and freeze dried. The yields of the four fractions are given in Table 1.

Substrates. Barley β-glucan and oat β-glucan were prepared by $(NH_4)_2SO_4$ fractionation of extracts of the appropriate cereal as described by Preece and MacKenzie.[16] Lichenin[9] from *Cetraria islandica*, soluble laminarin[4] from *Laminaria digitata*, and sodium carboxymethylcellulose[9] (CMC) (Cellofas B, medium viscosity) supplied by Imperial Chemical Industries Ltd., were the samples used in earlier studies. Sodium carboxymethylpachyman (CMP) was prepared from pachyman (the β-1,3-glucan from the fungus *Poria cocos*[17] and kindly provided by Prof. W. J. Whelan) by the method of Clarke and Stone.[3] Laminarisaccharides were prepared from laminarin by partial acid hydrolysis, followed by charcoal-Celite chromatog-

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raphy and preparative PC. Cellobiose was a commercial sample whose purity was checked by PC. Cellodextrin was prepared by Dr. D. H. Hutson, by the partial acid hydrolysis of cellulose.[18]

Analytical methods. Chromatography. Descending PCs were developed in EtOAc–pyridine–H_2O (10:4:3) at room temp. using Whatman No. 1 paper. Reducing sugars were detected with the alkaline $AgNO_3$.[19] *Reducing sugar measurements.* These were determined quantitatively by the colorimetric adaptation[20] of the Somogyi method.[21] *Protein contents.* In column fractions these were determined from the absorption at 280 nm; in enzyme preparations, a colorimetric method[22] was used.

Enzyme activities. endo-β-Glucanase activities: Digests contained substrate solution (1% barley glucan, 1% CMC or 2% CMP, 1·0 ml), buffer (200 mM citrate pH 5·3; 0·5 ml) and enzyme solution in a total vol. of 2·5 ml. A sample (2·0 ml) was used for viscometry in a miniature M4 ostwald viscometer immersed in a H_2O bath at 42·5°. The flow time was measured at intervals; the reciprocal specific viscosity was plotted against time and the slope of the line, $d(1/\eta_{sp})/dt$ measured. The rate of increase of reciprocal specific viscosity was taken as a measure of enzyme activity. H_2O had a flow time of 18·0 sec in the viscometer used.

Reducing power increasing activities. Activities towards the various substrates (cellobiose, laminaribiose, cellodextrin, laminarin, barley β-glucan or lichenin) based on reducing power measurements which, as has been discussed above, may be due to *exo*-β-glucanase, β-glucosidase or *endo*-β-glucanase, was determined by incubating substrate in sodium citrate buffer pH 4·6 with a suitable amount of enzyme solution. The amount of substrate present in the digest varied between 0·25 mg/ml and 4·0 mg/ml depending on its reducing power, but in all cases was such as to give maximum enzyme velocity. After incubation for suitable lengths of time at 37° (30 min–4 hr) samples were removed for determination of reducing power released. Enzyme activities towards the various substrates are expressed in terms of the release of reducing power (as glucose equivalents) per hr.

Molecular-sieve chromatography. This was carried out at 2° using a column (70 × 2·5 cm) of Biogel P60 (100–150 mesh, Bio-Rad Laboratories, Inc.) eluted with 5 or 1 mM sodium citrate buffer pH 4·6 or 5·0 at a rate of 5 ml/hr. Fractions of 3 ml were collected automatically.

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