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Note

Observations on the endo-(1→4)-β-D-glucanase activity of extracts of barley*

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Previous studies[2–4] have shown that extracts of barley, germinated barley, and malted barley contain a complex mixture of β-D-glucanases. The enzymes include an endo-(1→3)-β-D-glucanase[5] and endo-barley-β-D-glucanase[6]; both of these activities increase greatly during germination[2]. The barley extracts also show a low level of endo-(1→4)-β-D-glucanase activity, since reducing sugars are produced from cellodextrin, and the viscosity of CM-cellulose is decreased[2]. This activity did not change significantly during germination. Since several fungi showing CM-cellulase activity have been isolated from barley husk[7,8], it was necessary to determine the origin of the CM-cellulase, *i.e.*, endo-(1→4)-β-D-glucanase activity within the barley grain. We now conclude that a major part of this activity arises from the husk and is of fungal rather than of plant origin. A preliminary account of this work has been published[9].

RESULTS

A survey of seven cultivars of barley (Table I) showed that all contained high levels of endo-(1→3)-β-D-glucanase activity which did not vary greatly with the cultivar. By contrast, the level of endo-(1→4)-β-D-glucanase activity was relatively low, being $<1\%$ of the first-named activity, and varied considerably with the cultivar. On the basis of these results, Aramir was selected for further studies. It should be emphasised that all samples tested were of good brewing quality, and were free from visible fungal contamination.

To examine whether the endo-(1→4)-β-D-glucanase activity was located within the husk or the seed, two methods of dehusking were studied. Barley grains were dehusked with 50% sulphuric acid[10]. The dehusked grain (which was still viable, since 100% of the seeds could germinate) had lost 89% of the original endo-(1→4)-

*Studies on β-Glucanases, Part VI. For Part V, see ref. 1.

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TABLE I

RELATIVE ENDO-(1→3)- AND ENDO-(1→4)-β-D-GLUCANASE ACTIVITY OF EXTRACTS OF BARLEY

Cultivar	*Endo-(1→3)-activity*[a]		*Endo-(1→4)-activity*[b]	
	Units/ml	*Activity (%)*[c]	*Units/ml*	*Activity (%)*[c]
Golden Promise	23.4	100	0.07	100
Abacus	25.1	107	0.11	157
Aramir	28.7	123	0.20	285
Hassan	20.6	88	0.12	171
Imber	29.7	127	0.14	200
Mazurka	29.0	124	0.16	229
Maris Mink	27.3	117	0.18	257

[a]Viscometric assay using CM-pachyman. [b]Viscometric assay using CM-cellulose. [c]Relative to Golden Promise.

TABLE II

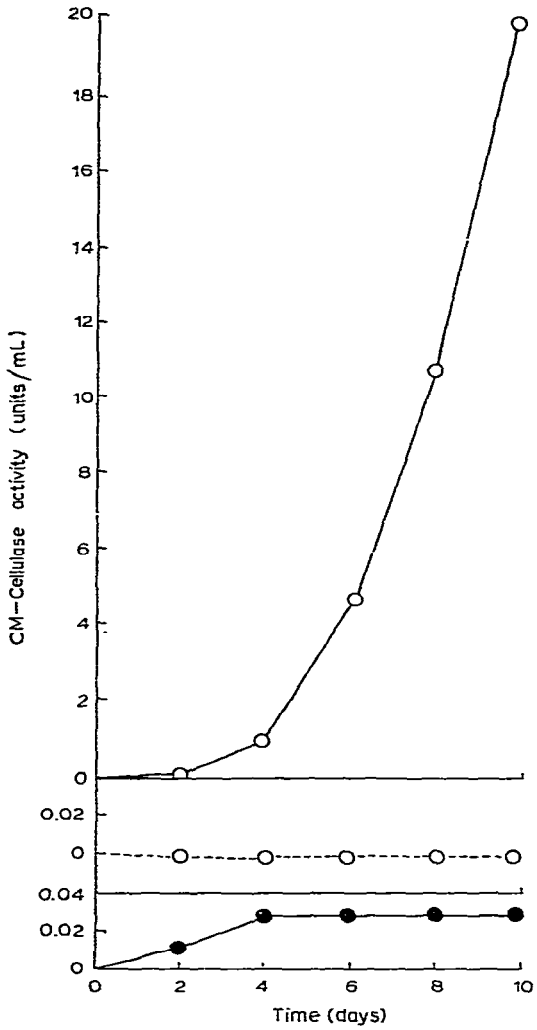
ENDO-(1→4)-β-D-GLUCANASE ACTIVITY AFTER PHYSICAL DEHUSKING OF BARLEY

Period of vigorous stirring	*CM-Cellulase Units (total)*	(%)	*CM-Pachymanase Units (total)*	(%)
First period of 10 min	4.40	25	41	6
Second period of 10 min	6.25	36	16	2
Third period of 10 min	2.24	13	101	14
Fourth period of 30 min	2.52	14	34	5
Homogenate of residue	2.21	13	527	73

β-D-glucanase activity, whereas the decrease in endo-(1→3)-β-D-glucanase activity was only 32%.

To examine the site of the residual 11% of CM-cellulase activity, acid-dehusked seeds were dissected into endosperm and embryo, which were then extracted with buffer, and the extracts were assayed viscometrically. CM-cellulase activity could only be detected in the endosperm extract. To determine whether the residual 11% of activity was due to fungal contamination of the endosperm, acid-dehusked seeds were incubated for up to 21 days at 32° in a medium containing cellulose powder, minerals and yeast extract, penicillin, and streptomycin (see Experimental). The CM-cellulase activity of the medium was assayed at intervals of 3 days. No activity was detected. Thus, the residual activity appears to be of plant origin.

Barley grains can be dehusked by vigorous stirring, using a Waring Blendor, in the presence of buffer. This procedure provides a husk-rich fraction, which can be collected by centrifugation, but the dehusking of the grain may not be as complete as in the acid-procedure. The results in Table II show the enzymic activity of several



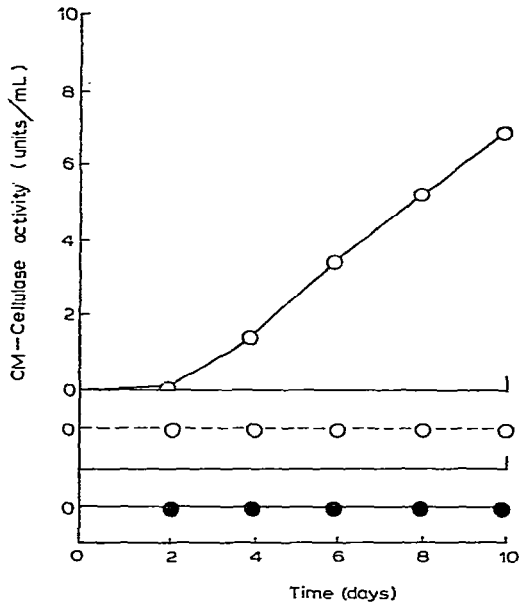


Fig. 1. Development of CM-cellulase activity during incubation of raw husk in a medium containing powdered cellulose as carbon source. Key: —○—, raw husk + streptomycin + penicillin; ---○---, raw husk + nystatin; —●—, raw husk alone.

Fig. 2. Development of CM-cellulase activity during incubation of washed husks in a medium containing powdered cellulose as carbon source. Key: see Fig. 1.

husk fractions after use of the Waring Blendor for various periods. After four periods of stirring, the CM-cellulase activity of the residual grain was ~13% of the original; by contrast, ~73% of the CM-pachymanase activity remained in the dehusked grain. These results are in good agreement with those obtained by acid-dehusking.

If the husk fractions contained cellulolytic fungi, their presence should become obvious during incubation in a medium containing powdered cellulose as carbon source, appropriate salts, and various antibiotics to suppress bacterial and/or fungal

growth. Typical results are shown in Fig. 1. During incubation for 10 days at 32°, the husk alone gave only a very slight increase in CM-cellulase activity, but in the presence of streptomycin and penicillin to prevent bacterial growth, an increase in enzymic activity could be detected after 4 days, which increased rapidly up to 10 days. This increase was completely suppressed by the fungicide nystatin. The same trend of results was obtained (Fig. 2) with husk fraction which had been washed six times with sterile water. This washing would remove much of the fungal contamination from the husk, but the washed husk still contained sufficient cellulolytic fungi to cause a production of CM-cellulase under favourable conditions.

Microbiological examination of the husk fraction by Dr. B. Flannigan showed that the major fungus present in a mixed population was *Aspergillus fumigatus*. This organism was also identified following incubation of barley grains, both with and without surface disinfection, on agar at 25° and 37°.

DISCUSSION

The present results show that extracts of one sample of Aramir barley have a low level of CM-cellulase activity, and that most of this (~88%) arises from cellulolytic fungi present in the husk. Only ~12% of the enzymic activity appears to be of plant origin, and this is present in the endosperm and not the embryo. The presence of *Aspergillus fumigatus* as the major cellulolytic fungus is somewhat unusual, since it is seldom predominant in Scottish, seed barleys, although it may be common in moist, stored, feed barley. In an earlier study[11], *Cladosporium* spp. were the commonest filamentous fungi. Since the completion of our work and the publication of a preliminary account[9], other workers[12] have reached similar conclusions on the origin of CM-cellulase in certain cultivars of Australian barley. In these experiments, the major fungal contaminants were *Aureobasidium pullulans*, *Rhizopus stolonifer*, and *Penicillium* spp. The nature and extent of any fungal contamination will clearly depend upon a number of biological factors, including the history of the grain samples, the conditions of growth and harvesting, and subsequent storage.

It is well known that particular endo-β-D-glucanase preparations from cereals are heterogeneous, and that the various component enzymes can be separated by appropriate protein-fractionation techniques. In preliminary experiments, we have shown that extracts of barley husk contain a peak of CM-cellulase activity which has, on gel filtration on Biogel P-60, an elution volume similar to that produced by a pure culture of *A. fumigatus*. In isoelectric focusing, barley husk shows at least two peaks of enzymic activity. Rabbit antisera to the CM-cellulase preparation from *A. fumigatus* was also made (unpublished) and used in immunoprecipitation studies with extracts of barley husk. Isoelectric focusing of the supernatant solution after immunoprecipitation contained only one of the two original peaks of enzymic activity. It therefore seems probable that the antisera to the *A. fumigatus* enzyme had reacted with one of the enzymes present in barley husk.

The present investigation was confined mainly to CM-cellulase activity, and

therefore poses the question as to whether many of the other polysaccharases in barley extracts are of plant or microbial origin. In the case of endo-(1→3)-β-D-glucanase activity, the major part (~70%) of the activity in one sample of *Aramir* barley is of true plant origin, but activity arising from the husk could contribute to the total extract. In more-recent studies[13] of the α-L-arabinofuranosidase activity, it has been shown that part arises from the husk, and part from the seed, and that the two enzyme proteins differ in certain properties, for example, electrophoretic mobility, interaction with concanavalin A, and their biosynthetic response to gibberellic acid.

In future studies of cereal polysaccharide-degrading enzymes, it will be advisable to use dehusked grain as the starting material, as, for example, in recent work on limit dextrinase[14], to avoid any ambiguity on the biological origin of the enzyme(s) under investigation. An immunological approach to the purification and characterization of enzymes is likely to be important in problems of this type. For example, the cellulolytic complex of *Trichoderma reesei* QM 9414 has recently been separated into CM-cellulase and cellobiohydrolase activities, which have been located immunochemically[15], whilst a new amylase from barley has been purified by immunoaffinity chromatography[16,17].

EXPERIMENTAL

Enzyme assays. — Endo-(1→4)-β-D-glucanase activity was measured viscometrically in digests containing 1.0 ml of 1% CM-cellulose solution, 0.5 ml of 0.2M ammonium acetate buffer (pH 5.0), and 1.0 ml of enzyme preparation. The flow time at 37° was measured at intervals, and the reciprocal specific viscosity calculated. One unit of enzyme activity is defined as the amount of enzyme which gave a rate of increase of the reciprocal viscosity of 0.1 under these conditions. The buffer has a flow time of 22.70 s in the viscometer used.

Endo-(1→3)-β-D-glucanase activity was measured in similar digests containing 1.0 ml of 1% CM-pachyman as substrate.

Extraction of barley. — Barley flour (20 g) was extracted with 60 ml of 0.2M ammonium acetate buffer (pH 5.0) at room temperature for 3 h. After centrifugation, the extracts were freeze-dried and then dissolved in 10 ml of water for enzyme assays.

Dehusking experiments. — For physical dehusking, 100 g of Aramir barley was stirred with 200 ml of 0.2M ammonium acetate for 10 min. After centrifugation, the supernatant solution was freeze-dried, the residue was dissolved in 20 ml of water, and the solution was clarified by centrifugation and assayed for enzymic activity. The barley was stirred with the buffer for two further periods of 10 min and one of 30 min before the residual grain was homogenised.

Incubation of parts of the grain in a culture medium. — The raw-husk fraction, washed-husk material, and acid-dehusked seeds were incubated in the medium described by Flannigan and Sellars[9], which contained powdered cellulose as the carbon source. The aqueous medium contained inorganic salts, yeast extract (Oxoid powder; 50 mg), and Whatman cellulose powder (10 g) per litre. In addition, nystatin

(Mycostatin from the Sigma Chemical Co. Ltd., Poole; 400 p.p.m.) and streptomycin sulphate and sodium benzyl penicillin (both from Glaxo Laboratories Ltd., Greenford; 800 p.p.m.) were added as inhibitors of fungal and bacterial growth[18].

The raw-husk fraction (10 ml), prepared by stirring the seeds in buffer for 1 h as described above, was added to 100 ml of medium at 32°, and samples (1 ml) were removed at intervals for the viscometric assay.

The washed-husk material was prepared from seeds which had been washed six times in sterile water, to remove loose, fungal contamination.

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