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POLYSACCHARASE METABOLISM IN DORMANT AND GERMINATING *STYLOSANTHES HUMILIS* SEEDS

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Key Word Index—*Stylosanthes humilis*; Leguminosae; Townsville stylo; seeds; germination; polysaccharase; amylase; β-glucanase.

Abstract—The levels of certain polysaccharase and glycosidase enzymes were followed during the germination of Townsville stylo seeds. Significant levels of invertase, amylase and β-1,3-glucan hydrolase were detected, the former two markedly increasing in activity during germination. No significant activity was noted towards hemicellulose B or galactomannan. Partial purification of the amylase and β-1,3-glucan hydrolase activities was achieved by ammonium sulphate precipitation and ion-exchange chromatography on DEAE-cellulose. An exo- and endo- β-1,3-glucan hydrolase were separated on ion-exchange chromatography. β-Amylase was shown to be the major amylase activity in the ungerminated seeds.

INTRODUCTION

The seeds of *Stylosanthes humilis*, a major pasture legume in use by the beef industry in North Queensland, contain *ca* 46% protein [1], 10% lipid [2] and 40% polysaccharide [3]. The oligosaccharides sucrose [4], raffinose and stachyose [5] have been detected in significant amounts. The major polysaccharides are cellulose and a mixture of hemicelluloses [4]. There is no significant amount of galactomannan present. A xyloglucan appears to be present as one of the major polysaccharides in the seed and may function as a food reserve although it is quite different from the amyloid xyloglucans of other legume seeds which have previously been studied [6]. The *S. humulis* seed therefore appears different in its carbohydrate content from the majority of other previously studied legumes, both tropical and temperate [7].

This paper describes a study of some of the enzymes present in the dormant and germinating seed which are capable of hydrolysing well defined oligo- and polysaccharides. The partial purification and action patterns of β-1,3-glucanase and amylase activities are reported and discussed in relation to their significance in the seed.

RESULTS AND DISCUSSION

The activities were investigated of extracts of dormant and germinating seeds towards cellobiose, methyl β-D-xylopyranoside, sucrose, L-arabinan, arabinoxylan (wheat endosperm), carboxymethyl cellulose, dextran (*Leuconostoc mesenteroides* B512), galactan (larch), galactomannan (locust bean), hemicellulose B (sugar cane), laminaran (*Laminaria hyperborea*), pectin (apple), and soluble starch. Activity was measured by the increase in reducing power with time, the digests being sampled at 0 and 20 hr after which time the enzymes shown to be present were still active. No significant activity was noted towards the substrates hemicellulose B, galactomannan, galactan, carboxymethyl cellulose, dextran, methyl β-D-xylopyranoside, pectin and arabinoxylan either in dormant or germinating seeds.

β-D-mannanase and α-D-galactopyranosidase have been shown to be present and to increase during germination in legume seeds which contain galactomannans [9]. The insignificant levels of these enzymes in *S. humilis* seeds, as evidenced by the lack of hydrolysis of galactomannan by extracts of both dormant and germinating seeds, is in accordance with the chemical evidence that galactomannan is absent from the seeds.

Arabinan and cellobiose were hydrolysed to a small extent but there was no increase in this type of activity in the seeds after six days germination. The other substrates viz. soluble starch, sucrose and laminaran, were rapidly hydrolysed by the seed extract and the variation in activity with germination is shown in Fig. 1.

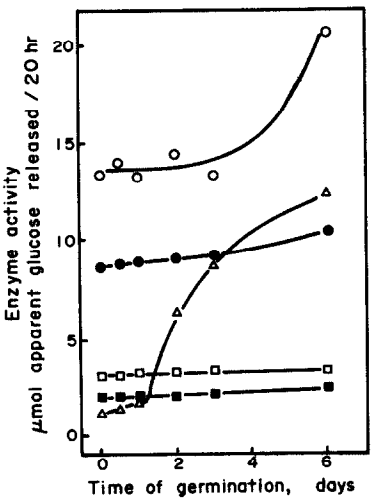

Fig. 1. Changes in the activity of amylase (○), β-1,3-glucan hydrolase (●), arabinanase (■), β-1,4-glucosidase (□) invertase (△) on germination of *S. humilis* seeds.

The presence of invertase in the seeds was anticipated, since sucrose is the major oligosaccharide [3] and the enzyme would also be active against the other oligosaccharides present [5]. The level of invertase increases rapidly after the first day of germination and continues to do so until day 6 at which time it is approaching a maximum. It has been established that sucrose and oligosaccharides of the raffinose family in the cotyledon of leguminous seeds are usually metabolised during the early stages of germination to provide energy for seedling development until such time as the insoluble reserves are mobilised [8]. This would also appear to be the case in *S. humilis*.

The polysaccharides in the *S. humilis* seeds (apart from cellulose) are almost entirely alkali-soluble and comprise a particularly complex mixture [4]. Xylose and arabinose are the major constituents, with decreasing amounts of glucose, galactose, rhamnose and mannose. Galacturonic acid is the major uronic acid. The xylose is at least partly present as β-1,4-D-xylopyranosyl groups, apparently associated with β-1,3- and -1,4-D-glucopyranosyl groups. The seeds contain no endo-β-1,4-D-xylanases as evidenced by the absence of enzymes which attack hemicellulose. It seems probable therefore, that the xylan in the seed is present as a cell wall component and does not function as a cell wall component and does not function as a food reserve. On this basis, it is assumed that the β-1,3-D-glucanases in the seed are responsible for breakdown of cell walls during germination, thus releasing reserves such as protein and lipid. This hypothesis is supported by the fact that the β-1,3-D-glucanases are present in the dormant seed and do not increase during germination.

The preliminary results indicated the presence of both exo- and endo-β-1,3-D-glucanases. Thus the crude extracts hydrolysed laminaran to produce glucose with smaller amounts of laminaribiose, -triose and higher oligosaccharides. Gentiobiose was also produced from branch points in the polysaccharide. The two types of enzyme action were confirmed by comparison of the action of the seed extract on laminaran and on periodate oxidised laminaran in which only the end groups have been modified by the oxidant. In both cases the total reducing power (measured as 'apparent glucose' by the Nelson–Somogyi method) greatly exceeded the true glucose produced (measured by glucose oxidase) and with the oxidised laminaran, glucose was not produced until after a 'lag' phase, during which time it is assumed that 'normal' or non-oxidised end groups were produced by endo-attack and subsequently attacked by the exo-enzyme to produce glucose.

In an ammonium sulphate fractionation of the crude extract the majority of the β-1,3-D-glucan hydrolase activity precipitated at 50–70% saturation. The measurement of the true glucose:apparent glucose ratio from the action on laminaran of this and of earlier and later fractions however showed no significant resolution of the exo- and endo-β-1,3-D-glucan hydrolase activities. The 50–70% fraction was then subjected to both isoelectric focusing and ion exchange chromatography on DEAE-cellulose.

Prior to focusing various convection stabilisers were tested at 70% (w/v) in the enzyme solution for their effect on β-1,3-D-glucan hydrolase activity. Ethylene glycol, glucitol and glycerol all produced about 50% inhibition as estimated by increase in total reducing power. However, when measured by the glucose oxidase method [10] nearly 70% inhibition was caused by ethylene glycol, thus indicating a stronger inhibition of the exo-β-1,3-D-glucan hydrolase activity. The result of the isoelectric focusing using ethylene glycol is shown in Fig. 2.

The β-1,3-D-glucan hydrolase activity was determined both by Nelson–Somogyi and glucose oxidase methods and shown to elute from the column at pH 5.5. No other peak was detected. However when fractions at pH 9–12 were combined, dialysed against 0.1 M sodium phosphate buffer (pH 6.5) and then incubated with laminaran, paper chromatography showed that glucose and traces of gentiobiose, laminiaribiose and higher oligosaccharides were produced. Evidently therefore there is other

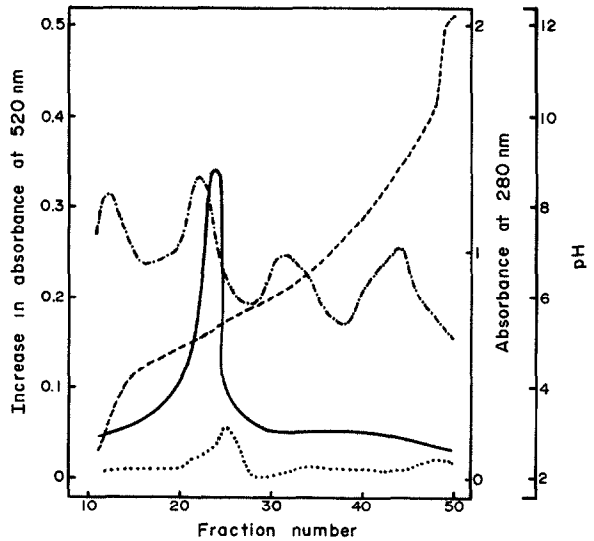

Fig. 2. Isoelectric focusing of 50–70% ammonium sulphate fraction; ----- pH; -·-·-·- protein (280 nm); ——— amylase; · · · · · β-1,3-glucan hydrolase.

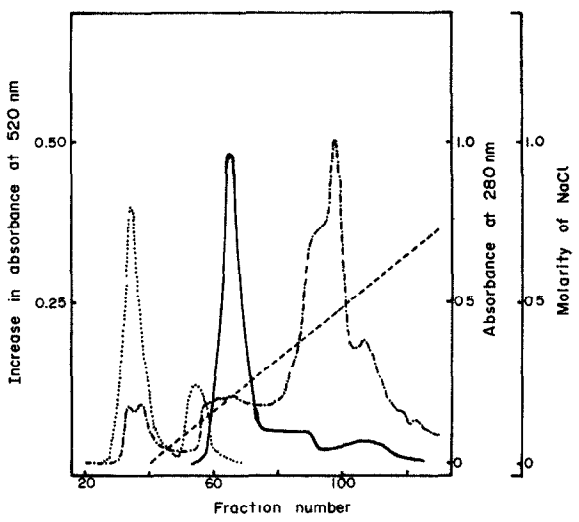

Fig. 3. Ion exchange chromatography on DEAE cellulose of 50–70% ammonium sulphate fraction; ---- molarity of sodium chloride; -·-·-·- protein (280 nm); ——— amylase; · · · · β-1,3-glucan hydrolase.

β-1,3-D-glucanase activity present due to enzyme(s) of high pI. The failure to detect activity in the fractions before dialysis may be due to the pH effect of the ampholytes in the assay digests.

The main β-1,3-D-glucan hydrolase activity (fractions 25–28, pI = 5.5) gave a true glucose:apparent glucose ratio of 1 when incubated with laminaran, thus indicating that it is an exo-β-1,3-D-glucan hydrolase free from endo activity. Glucose was the only product detected on PC. The pH optimum of the enzyme was pH 6 and its activity fell rapidly outside the range pH 5.5–6.5.

Following ion exchange chromatography of the 50–70% ammonium sulphate fraction at pH 6.2 with a linear 0–1 M sodium chloride gradient, two peaks of β-1,3-D-glucan hydrolase activity were detected (Fig. 3). Examination of the action of these two activities on laminaran by PC revelaed that peak A released a homologous series of oligosaccharides with β-1,3-D-glucopyranosyl linkages and no glucose was detected, whereas peak B yielded only glucose. It appears therefore that ungerminated *S. humilis* seeds contain two β-1,3-D-glucan hydrolases, one being endo- and the other exo-.

The starch content of *S. humulis* (< 2% of dry weight) is significantly less than in those Leguminosae dependent on starch for their primary food reserve. The possibility of an increase in the level of starch in the seeds or shoots during germination was not investigated and cannot be discounted. Such synthesis of starch has been observed in germinating seeds of the galactomannan storing *Trigonella foenum-graecum* [10]. If such an increase in starch did occur it would be compatible with the increase in amylase activity demonstrated during the later stages of germination. Prelimfnary investigation of the activity of the crude extract of ungerminated seeds on amylopectin showed that the main product was maltose and that smaller amounts of glucose, maltotriose and higher oligosaccharides of the series were produced.

Like the β-1,3-D-glucan hydrolase activity, the majority of the amylase activity was precipitated between 50 and 70% ammonium sulphate saturation. Following isoelectric focusing of this fraction the only significant activity against starch was obtained in fractions 22–26 with a peak at pH 5.3 (Fig. 2). The products from digestion of this peak with amylopectin were maltose and a trace of glucose, maltotriose and oligosaccharides of DP 4 and above. The main amylase activity of these fractions is therefore β-amylase which must be contaminated with a trace of either α-amylase or debranching activity.

Ion exchange chromatography of the 50–70% ammonium sulphate fraction on DEAE cellulose yielded only one amylase peak as determined by the activity of the fractions towards amylopectin. This activity was eluted from the column by 0.1 M sodium chloride and was free from β-1,3-D-glucan hydrolase activity. The only low MW product, of action on amylopectin was maltose. The β-amylase had therefore been separated from any trace of the α-amylase or debranching activity which had been present in the crude extract and the isoelectric focusing fraction. It must be concluded that β-amylase is the main enzyme responsible for the hydrolysis of starch at least until the fourth day of germination of *S. humilis* seeds. The increased amylase activity generated after the fourth day of germination (Fig. 1) may be mainly due to α-amylase since there is a sharp rise in the activity of the crude extracts against amylopectin β-limit dextrin.

EXPERIMENTAL

Plant material. The *S. humilis* seeds were harvested and then mechanically dehusked. The dehusked seeds were scarified by mechanical abrasion in a sandpaper-lined rotating cylinder and stored in a dry container at room temperature.

Starch content. A suspension of finely ground seeds (2 g) in H_2O (100 ml) was boiled for 10 min, heated to 130° for 90 min under pressure then filtered on Whatman No. 1 paper while warm. A sample of the extract (0.05 ml) was incubated at 50° for 2 hr with 0.02 M Na citrate buffer (pH 5.5, 0.95 ml) containing amyloglucosidase (19 mg, Rhizopus sp.) and barley α-amylase (1.9 mg) and subsequently analysed for D-glucose with glucose oxidase [11].

Periodate oxidised laminaran. A solution of laminaran (73 mg) in 0.1 M $NaIO_4$ (50 ml) was kept at 4° in the dark for 72 hr. Ethylene glycol (2.5 g) was next added, the solution dialysed against distilled H_2O and then concentrated to a final concentration of 1% (w/v) polysaccharide.

Paper chromatography. Incubation mixtures were deproteinised with trichloroacetic acid (1%) and then deionised with a mixture (1:1) of Amberlite resins IR-45 (OH^-) and IR-20 (H^+). Descending PC was carried out on Whatman No. 1 paper using EtOAc–pyridine–H_2O (10:4:3). Reducing sugars were detected using the alkaline $AgNO_3$ [12] reagent.

Germination. Scarified seeds (1–20 g) were soaked in 1% NaClO solution for 15 min then in water for 24 hr. The seeds which had imbibed water and swollen were selected for germination. These were germinated in the dark on moistened Whatman No. 1 filter paper in sterile petri dishes. To obtain the results summarised in Fig. 1, 100 germinated seeds with similar shoot length were selected for each experiment.

Enzyme extraction. The seeds were cooled (4°) and shoots removed and discarded. All subsequent operations were at 4°. The seeds were ground in a mortar and pestle and then homogenised in a Potter–Elvejhem homogeniser with 10 vol. of ice-cold 0.1 M Na phosphate buffer (pH 6.5). The homogenate was centrifuged (16000 g, 30 min, 4°) and the supernatant then dialysed against the extraction buffer for 24 hr.

Enzyme assays. Determination of polysaccharase and glycosidase activities was by incubation of the enzyme solution (0.4 ml, *ca* 5–10 mg protein) with 2 ml of the appropriate substrate solution (0.5% polysaccharide or 5 mM oligasaccharide in 0.05 M Na citrate–phosphate buffer (pH 5.6). The digests were incubated under toluene at 37° and duplicate aliquots (0.3 ml) removed at appropriate intervals and assayed for increased in reducing power by the Nelson–Somogyi method [13] against substrate and enzyme blanks. Protein was estimated by the Hartree modification of the Lowry method [14].

Enzyme fractionation. All steps were carried out at 4°. Dormant seeds (10 g) were ground in a mortar and pestle and then homogenised with 0.2 M Na phosphate buffer (pH 6.2, 100 ml). Following the extraction the suspension was centrifuged (16000 *g*, 30 min) and the supernatant (80 ml) dialysed against the buffer for 24 hr. Solid $(NH_4)_2SO_4$ was slowly added with constant stirring to bring the solution to 50% saturation. After equilibration (1 hr) and removal of the precipitate by centrifugation (10000 *g*, 20 min), more ammonium sulphate was added to 70% saturation. The resultant 50–70% ppt. was redissolved in 0.02 M Na phosphate buffer (pH 6.2, 20 ml) and dialysed against several changes of the same buffer for 48 hr. The solution was applied to a DEAE cellulose column (2.6 × 30 cm) equilibrated with the same buffer and eluted with buffer (70 ml) followed by a linear NaCl gradient (0–1 M) in the same buffer (500 ml). Fractions (4 ml) were collected and analysed for protein (280 nm), laminaranase and starch hydrolase activities.

Isoelectric focusing. The 50–70% $(NH_4)_2SO_4$ sample was fractionated by isoelectric focusing on an LKB 110 ml column at 4°. The pH gradient (pH 3–10) was obtained using LKB

ampholine carriers (1%) and stabilised with 70% ethylene glycol. Focusing was carried out at 200 V (4 hr) followed by 475 V (112 hr). Fractions (1.5 ml) were collected following focusing and pH, protein (280 nm) and enzyme activities were measured for each fraction. The ampholytes interfered with the reducing sugar estimation and it was necessary to centrifuge the solution prior to measuring absorbance of the final solution in the Nelson–Somogyi analysis. No interference with glucose oxidase assay was observed. Active fractions were combined and dialysed (4°, 260 hr) against 0.1 M sodium phosphate buffer (pH 6.5) prior to characterisation.

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REFERENCES

1. Playne, M. J., McLeod, M. N. and Dekker, R. F. H. (1972) *J. Sci. Fd Agric.* **23**, 925.
2. Dekker, R. F. H. Private communication.
3. Pang Way, C. and Richards, G. N. (1971) *Australian J. Chem.* **24**, 1041.
4. Pang Way, C. (1976) M.Sc. Thesis, James Cook University of North Queensland.
5. Lehmann, K. L. and McIlroy, R. J. (1967) *Nature* **216**, 1044.
6. Kooiman, P. (1957) *Nature* **179**, 107.
7. Bailey, R. W. (1971) *Chemotaxonomy of the Leguminosae* (Harborne, J. B., Boulter, D. and Turner, B. L. eds). Academic Press, New York.
8. McCleary, B. V. and Matheson, N. K. (1974) *Phytochemistry* **13**, 1747.
9. McCleary, B. V. and Matheson, N. K. (1975) *Phytochemistry* **14**, 1187.
10. Reid, J. S. G. (1971) *Planta* **100**, 131.
11. Dekker, R. F. H. and Richards, G. N. (1971) *J. Sci. Fd Agric.* **22**, 441.
12. Trevelyan, W. E., Proctor, D. P. and Harrison, J. S. (1950) *Nature* **166**, 444.
13. Nelson, N. (1944) *J. Biol. Chem.* **153**, 375.
14. Hartree, E. F. (1972) *Anal. Biochem.* **48**, 122.