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PREPARATION OF RAT EPIDIDYMAL α-L-FUCOSIDASE FREE FROM OTHER GLYCOSIDASES: ITS ACTION ON ROOT-CAP SLIME FROM *Zea mays* L

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ABSTRACT

Affinity chromatography has been used in a rapid method of purification of rat epididymal α-L-fucosidase free of other glycosidases. The enzyme is suitable for analytical purposes and it has been used to investigate aspects of the structure of the fucose-containing slime synthesised by the outer root-cap cells of maize; less than 17% of the radioactive L-fucose incorporated into the slime from L-fucose-*1-t* was released by the enzyme.

INTRODUCTION

α-L-Fucosidase has been purified from *Aspergillus niger*[1], *Clostridium perfringens*[2], rat epididymis[3], and from herbivorous[4] and carnivorous[5] marine gastropods. The microbial enzymes have a specificity for (1→2)-α-L-fucosyl residues[1,2] and will not hydrolyse synthetic *p*-nitrophenyl L-fucosides, whereas the enzymes from the other sources do have this ability[3–5] and will act on a number of glycoprotein oligosaccharides[3,6] or intact blood-group substances[4]. Recently, affinity chromatography has been applied to the purification of the enzyme from human liver[7]. We have used the latter technique to purify rat epididymal α-L-fucosidase to a higher specific activity than the human enzyme; the preparation, unlike previous ones, contains no other demonstrable glycosidase activities. The enzyme has been used as a specific analytical tool to examine some of the problems concerning the structure of maize slime.

Cells of the root-cap of maize differentiate in a specific sequence from the root-cap initials[8,9], and this culminates in the outer root-cap cells synthesising a slime whose function is thought to be protective[10,11]. The slime contains polysaccharides which are synthesised by the dictyosomes of the cells[12–14] and which can be fractionated into neutral, weakly acidic, and acidic polymers[11,15]. The slime contains up to 30% of fucose[10], and the bulk of this is found in the acidic fraction[15]. Fucose is a

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comparatively unusual sugar in plants and, in maize, only trace quantities are found in water-soluble polymers extracted from the rest of the seedling[11]. Hence the presence of fucose in a polymer is a quantitative marker for the synthesis of root-cap slime. It is not known, however, whether the presence of this sugar confers any particular properties on the slime or whether the glycosidic bond is in the α or β configuration. Neither is it known whether the fucose occurs only terminally within the molecule, as in animal glycoproteins[16], or whether it also occurs at other positions within the slime polysaccharide.

EXPERIMENTAL

Purification of α-L-fucosidase. — The epididymides from adult rats (Wistar, 120–140 g) were collected, detached from the adhering fat, and stored in 30% glycerol at −20° until required[3]. Tissue (40 g) was chopped in 400 ml of a cold solution of 0.1M sodium acetate and 0.1M sodium chloride (pH 6), using an MSE Atomex Blender operated for 1 min at full speed. It was then homogenised, using a glass homogeniser with a Teflon pestle, and allowed to stand at 37° for 1 h. The homogenate was adjusted to pH 5.25 with glacial acetic acid and heated to 60° for 10 min[3]. It was cooled rapidly to 20° and centrifuged (30 min, 40,000 *g*). The supernatant solution was decanted and the pH adjusted to 6.3 with NH_4OH. Solid $(NH_4)_2SO_4$ was added to 35% saturation and the preparation left for 2 h at 4°. The solution was centrifuged (30 min, 40,000 *g*, 0°), and the supernatant solution was decanted and brought to 60% saturation of $(NH_4)_2SO_4$. The precipitate was allowed to form (18 h, 4°), collected by centrifugation (30 min, 40,000 *g*, 0°), and dissolved in 5 ml of 0.1M sodium citrate (pH 6) containing 0.02% of NaN_3 (citrate buffer)[3]. It was applied to a column (200 × 25 mm) of G-25 Sephadex and eluted with citrate buffer. The elution was monitored with an LKB 8300 Uvicord II, and only those fractions which were later shown to exhibit fucosidase activity were retained. A 2-ml column of Agarose-ε-aminocaproylfucosamine[7] (Miles Laboratories Ltd., Slough, U.K.) was equilibrated in citrate buffer, and the eluate from the G-25 Sephadex column (~20 ml) was passed through it at a speed of 36 ml/h. The column was washed with citrate buffer until the excess of protein had been removed. The α-L-fucosidase was then eluted with 1% of L-fucose[7] in citrate buffer and precipitated by the addition of solid $(NH_4)_2SO_4$ to 60% saturation (18 h, 4°). The precipitate was washed twice with 60% $(NH_4)_2SO_4$, dissolved in 2 ml of citrate buffer, and desalted on a column (90 × 10 mm) of G-25 Sephadex. The eluate was used directly; subsequent dialysis did not lead to any increase in specific activity. The affinity column was finally washed with 2M NaCl in citrate buffer before being equilibrated for re-use[7].

Assay of glycosidase activities. — Suitable samples of solutions of enzyme in citrate buffer were made up to 100 µl and incubated with 100 µl of substrate solution (*p*-nitrophenyl glycosides) at 37° for 1 to 60 min. The reaction was stopped by addition of 2 ml of M sodium carbonate, in which the colour was stable for at least 1 h. The absorbance was measured on a Beckman DB-GT spectrophotometer at 410 nm and

referred to a standard curve; 1 enzyme unit (e.u.) is that amount which catalysed the hydrolysis of 1 μmole of substrate per min. For the appropriate enzyme assays, substrate solutions were: 3 mM *p*-nitrophenyl α-L-fucopyranoside in 0.5M sodium citrate (pH 6.5); saturated *p*-nitrophenyl α-D-mannopyranoside in 0.5M sodium citrate (pH 5.0); saturated *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside in 0.5M sodium acetate (pH 5.0); 10mM *p*-nitrophenyl β-D-galactopyranoside in 0.5M sodium acetate (pH 3.75); 5mM *p*-nitrophenyl α-(or β)-D-glucopyranoside in 0.1M sodium citrate (pH 5.4). All solutions contained 0.02% of NaN_3, and all substrates were obtained from Koch–Light Ltd. (Colnbrook, Bucks, U.K.). Protein was measured by the method of Lowry *et al.*[17] on suitable volumes of enzyme solution, and bovine serum albumin was used as a standard.

Preparation of radioactive slime polysaccharide. — Seeds of *Zea mays* (Caldera 535) were obtained from commercial sources, and sterilised and grown as described previously[15]. Primary roots ~30 mm long were selected and the terminal 20 mm cut off with a scalpel. These were arranged on a piece of Parafilm in 6 circular groups of 10, with the roots oriented radially so that their tips touched. A solution of L-fucose-*l-t* (2 Ci/mmol; 0.5 mCi/ml) (100 μl for the roots to be harvested after 1–4 h or 200 μl for the roots to be harvested after 5 or 6 h) was applied to the tips of the roots which were then kept in a humid atmosphere at 20°. Ten roots were harvested every hour for 6 h, and transferred, with the washings (H_2O) from the piece of Parafilm, to a small vial in a total volume of ~1 ml. They were heated at 100° for 2 min, and the solution was removed from around the roots with a syringe, washing twice. The solution was dialysed 5 times against 3 l of water for 18 h in the presence of a drop of toluene, and the solution was washed out of the dialysis tubing to give a known, final volume. The radioactivity in a sample was determined by mixing with Triton/toluene scintillation fluid (6 g, of PPO, 75 mg of POPOP, 750 ml of Triton X-100, 1500 ml of toluene) and counting with a Nuclear–Chicago Unilux scintillation spectrophotometer. The total radioactivity in exported polysaccharide after various times was calculated. Slime labelled with ^{14}C-labelled sugars was obtained by feeding roots with D-glucose-*U*-^{14}C for 2.5 h[11,15]. Solutions of slime labelled with each radioactive precursor were stored at −20°.

Chromatographic procedures. — Gel-permeation chromatography was carried out as recommended by the manufacturers (Bio-Gel, Bio-Rad Labs Ltd., Bromley, Kent, U.K.; or Sephadex, Pharmacia, Uppsala, Sweden). Solutions of radioactive slime applied to the column were in water unless otherwise stated, and the fractions collected were ~1 ml. Portions (50 μl) were removed to a fresh set of tubes, and radioactivity was determined by the use of Triton/toluene scintillation fluid.

Descending paper chromatography (Whatman No. 1) was used to separate radioactive, tritium-labelled polysaccharide from monomeric fucose-*t* released by the enzyme. Chromatograms were developed for 18 h in ethyl acetate–pyridine–water (8:2:1), and the position of fucose was determined by reference to a marker stained with aniline hydrogen phthalate[18]. The paper was cut into strips which were immersed in scintillation fluid for determination of radioactivity[10].

Purification and analysis of fucoidin. — A mixture of *Fucus* and *Ascophyllum* species was collected from the coast, and the polysaccharide was prepared by the method of Black and Dewar[19]. It was desulphated according to Danishefsky *et al.*[20] and the final sulphate content estimated[21] to be 2% (w/w).

RESULTS

Purification of α-L-fucosidase from rat epididymis. — The progress of the purification is shown in Table I. As well as the glycosidase activities indicated, the final preparation was tested for α- and β-D-glucosidase activity[22], but none could be demonstrated. The purity of the preparation was investigated by using 5% polyacrylamide–dodecyl sulphate gels[3] run at 10 mA/tube. Protein (30 μg)[3] loaded onto the gel gave a single band (mol. wt. ~54,000) after staining with Coomassie Blue[23]; this result was in agreement with the results of Carlsen and Pierce[3]. Bovine serum albumin (mol. wt. 68,000) glyceraldehyde 3-phosphate dehydrogenase (mol. wt. 36,000), and lysozyme (mol. wt. 14,300) were used as standards.

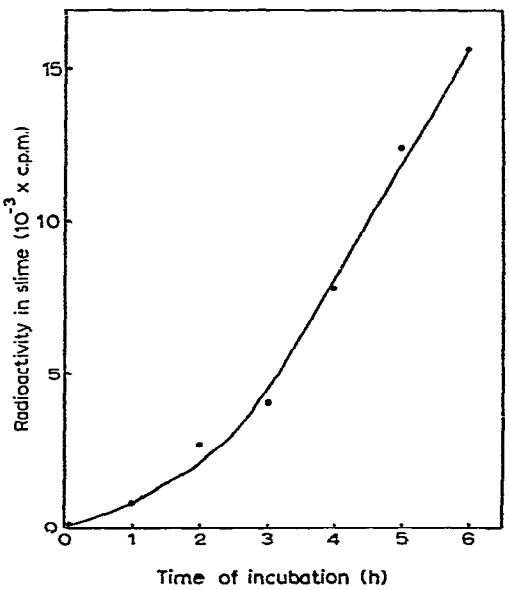

Fig. 1. Rate of incorporation of L-fucose-*1-t* into non-dialysable slime polysaccharide by maize roots. Roots were removed from incubations after different times, and the radioactivity in this material was determined (Experimental).

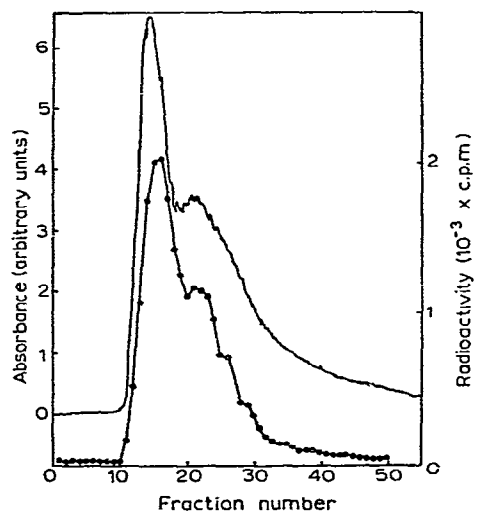

Fig. 2. Elution profile of tritium-labelled slime from a Bio-Gel P-4 column (150 × 7 mm). Solutions of slime labelled for 3–6 h (Fig. 1) were combined and freeze-dried; the product was dissolved in 1 ml of H_2O, and any insoluble material was removed by centrifugation at top speed on a bench centrifuge. The supernatant was loaded onto the column, and eluted with H_2O at 36 ml/h. The eluate was continuously monitored for absorbance at 280 nm (———). Approx 1-ml samples were collected, and 50-μl portions were taken from each for radioactivity determination (—●—).

TABLE I

PURIFICATION OF RAT EPIDIDYMAL α-L-FUCOSIDASE[a]

Purification stage	*Volume (ml)*	*Protein (mg)*	*Enzyme activity (total units)*				*Specific activity of α-L-fucosidase (units/mg)*	*Recovery of fucosidase (%)*
			Fucosidase	*Mannosidase*	*Galactosidase*	*Glucosaminidase*		
Homogenate after heating at 37°	275	1420	60.7	70.1	77.0	81.1	0.04	100
Supernatant after heating at 60°	260	754	57.2	52.0	44.2	65.0	0.08	94
35–60% Ammonium sulphate fraction after desalting on G-25 Sephadex	17	175	20.8	34.4	20.4	20.4	0.12	34
Fraction retained by affinity column after G-25 Sephadex gel-filtration	3	0.57	3.68	0	0	0	6.45	6.1

[a]The enzyme was purified and assayed as described in the Experimental Section; protein was measured by the method of Lowry[17].

Incorporation of L-*fucose*-1-t *into polysaccharide by maize roots.* — The time course of incorporation of radioactive L-fucose into maize slime is shown in Fig. 1. After a lag of 2–3 h, the rate of output of label in polysaccharide was linear over the period tested. Solutions of slime exported after 3–6 h were combined and freeze-dried. Kirby and Roberts[27] have shown that fucose is not broken down or converted into any other sugar by the root-tissue, and we have confirmed this finding.

Bio-Gel P-4 gel-filtration of L-*fucose*-1-t-*labelled slime.* — In order that slime of reasonably constant molecular size could be treated with enzyme, and to be certain that no monomeric, radioactive L-fucose contaminated the solution, the radioactive polysaccharide was purified by gel filtration. Solutions of radioactive slime voided columns of Bio-Gels P-4, P-100, and P-200, indicating a very large, apparent molecular size in solution.

The elution profile of a solution of radioactive slime from a column (150 × 7 mm) of Bio-Gel P-4 is shown in Fig. 2. Fractions were stored individually and fractions 15 and 16 were used in further experiments.

Time course of enzymic hydrolysis of L-*fucose*-t *from maize slime.* — A sample (900 µl) of fraction 15 from the Bio-Gel P-4 column was mixed with solid sodium citrate to give a final concentration of 0.1M (pH 6), and to this was added a solution of α-L-fucosidase (100 µl, 0.13 e.u. in citrate buffer). The enzyme had a specific activity of 6.45 e.u./mg and a protein concentration of 0.19 mg/ml. The mixture (which contained 0.02% of NaN_3) was maintained in a stoppered tube at 37° for periods up to 334 h. Samples of 20 µl (~600 c.p.m.) were removed periodically, and the released fucose was separated from the polymer by paper chromatography. The radioactivity corresponding to polymer and monomer was determined at different times (Fig. 3).

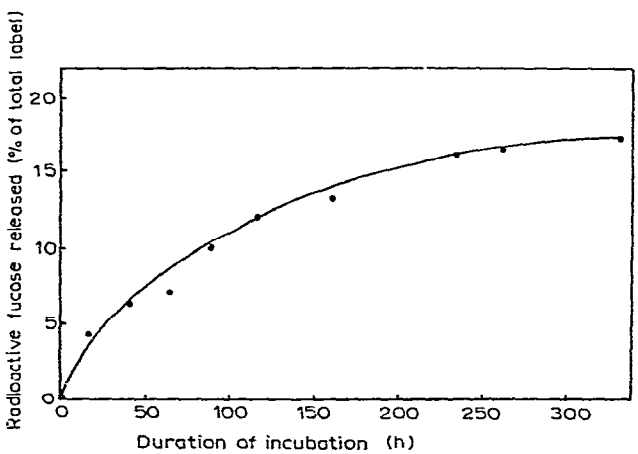

Fig. 3. Rate of enzymic hydrolysis of L-fucose-*t* from slime labelled with this precursor. Samples were removed at different times from an incubation containing buffered α-L-fucosidase and tritium-labelled slime. The radioactivities in polymer and monomer were determined and expressed as % of fucose released (Experimental).

The released radioactivity approached a maximum of 17% after 334 h. The time course was exactly similar to one obtained earlier with the material from fraction 16 (Fig. 2), although this incubation was smaller in volume and could not be continued for so long.

A different preparation of slime labelled with L-fucose-*t* was treated with 0.17 e.u. of α-L-fucosidase in a total volume of 300 μl. The mixture was left at 37° for 5 weeks. At the end of this time, the radioactivity in polymer and monomeric fucose was determined, and the label in the liberated fucose comprised 8.9% of the total. The mixture (100 μl) was then treated with 100 μl (0.2 e.u.) of another preparation of α-L-fucosidase in the same citrate buffer. After incubation for a further 7 days, the label in the liberated fucose was 8.8% of the total. Hence the release of fucose in the first incubation has been limited by the nature of the substrate and not by feedback inhibition of the enzyme by fucose[7] nor inactivation of the enzyme. The protein present in the slime did not interfere with the action of the fucosidase since preincubation of the slime with pronase (100 μg, 37°) for periods up to 10 days did not give any increase in release of fucose when the pronase was destroyed by boiling and the sample reincubated with the fucosidase.

Effects of α-L-fucosidase on slime containing ^{14}C-labelled sugars. — Slime which had been prepared by feeding D-glucose-*U*-^{14}C to maize roots[11,15] was purified on a column of Bio-Gel P-4 by elution with 0.1M citrate buffer (pH 6). A fraction (1 ml) of the eluate containing 11,000 c.p.m. was treated with 300 μl (0.39 e.u.) of purified α-L-fucosidase (specific activity, 6.45 e.u./mg; protein concentration, 0.19 mg/ml). The mixture was kept at 37° for 260 h, boiled for 2 min to inactivate the enzyme, and passed down a column of Bio-Gel P-4 to separate the substances of low molecular weight released by the enzyme. Too little radioactivity was present in the substances which did not void the column to allow further examination. The material which was eluted in the void volume was recovered and applied to a P-100 column. It was also too large to be included by this column, and therefore the purified fucosidase did not bring about any substantial degradation of the molecular species in the slime.

The experiment was repeated using more concentrated, purified slime, in order to examine the nature of the material included by the column. However, a control incubation which contained only 0.1M citrate buffer (pH 6.0) and no fucosidase also showed a small percentage (9%) of radioactivity in the included volume after 7 days at 37°. The materials were hydrolysed and the distribution of radioactivity between the monomers of included material from incubations in the presence and absence of fucosidase was the same, and this was very similar to the pattern of radioactivity labelling found in the material which had voided the column.

Effect of α-L-fucosidase on desulphated fucoidin. — Two accurately weighed samples of desulphated fucoidin (~1 mg) were taken and one was treated with 300 μl (0.39 e.u.) of α-L-fucosidase in citrate buffer at 37° for 288 h. The other was dissolved in 72% (w/v) H_2SO_4, hydrolysed[24] in 4% (w/w) H_2SO_4, and neutralised with $BaCO_3$. Both samples were dried, and the alditol acetates were prepared and analysed[11] by g.l.c. with *myo*-inositol as internal standard. The results showed that

the fucosidase released 48% of the amount of fucose which was released from the same amount of fucoidin by complete hydrolysis with acid. It is reasonable to assume that branch points and residual sulphate esters prevented complete degradation from taking place. The structure of fucoidin is generally thought to consist of a chain of L-fucose monomers joined by α-(1→2) links, carrying sulphate groups at C-3 and branches of L-fucose monomers at C-4 or C-3[30].

DISCUSSION

Robinson and Thorpe[7] were concerned with using the affinity-chromatography purification of α-L-fucosidase more for clinical diagnostic purposes than for preparation of the enzyme, and consequently did not give quantitative details of their purification. The final specific activity of their enzyme was 0.7 e.u./mg. Our value of 6.45 e.u./mg was not the highest that we have obtained with various preparations, but we did not achieve the specific activity (12.8 e.u./mg) obtained by Carlsen and Pierce[3]. The advantage of our purification was that we were unable to demonstrate the presence of any activity of the other glycosidases known to occur in this tissue[3,22] and which could not be completely removed by other methods[3]. In addition, the method took only 3 days to yield pure fucosidase; a pure enzyme, even if it means poor recovery, is an absolute requirement if it is to fulfil its potential as an analytical tool. It may be that it will be possible to improve the recovery during the affinity chromatography by closer attention to the conditions which we did not attempt to optimise.

Slime labelled with L-fucose-*t* was taken for treatment with α-L-fucosidase once the rate of incorporation of label into exported polysaccharide had become constant. Non-radioactive fucose was omitted from the medium in the hope that this would preserve as high as possible a specific activity in the exported polymer. Maize roots are reported[25] to synthesise slime with a periodicity of approx. 3 h, but this was not observed in our experiments since the roots were not synchronised.

If the slime were globular, the fact that it was excluded by Bio-Gel P-200 would imply that it had a molecular weight in excess of 200,000. Floyd and Ohlrogge[26] have used methods based on the turbidity of solutions of slime isolated from nodal roots of maize to estimate the molecular weight as 9×10^7. It is difficult to imagine that this value represents the size of a single molecule of slime, but it confirms that a polysaccharide which is gel-like in its normal state can display extensive intermolecular associations.

When the slime labelled with L-fucose-*l*-*t* was separated on a Bio-Gel P-4 column, the radioactivity elution-profile corresponded exactly with a trace measuring the absorbance at 280 nm. Since the absorption of sugars is negligible at this wavelength, this result implies that the polysaccharide and the 30% of protein that the slime contains[24] are in close association, and suggests that the slime, in common with other extracellular polymers which have a lubricatory function[16], might be a glycoprotein.

The conclusions which can be drawn from the various experiments in which purified slime was treated with pure α-L-fucosidase can be taken together. A difficulty arises over interpretation of the results since the absolute specificity of the enzyme is not known. In particular, we do not know whether the enzyme is exclusively an exo-fucosidase or whether it also has endofucosidase activity; this problem arises since synthetic substrates (*p*-nitrophenyl α-L-fucopyranoside) and naturally occurring glycoproteins or glycoprotein fragments carry L-fucose in terminal positions linked only through C-1 of the L-fucose. Carlsen and Pierce[3] examined the release of fucose from the LH-β peptide (the hormone-specific chain of bovine luteinising hormone) in the presence of α-L-fucosidase and found that all the fucose had been released after 200 h under these conditions. Some intact glycoproteins, however, are not susceptible to fucosidase[4,6]. The time course of release of L-fucose-*t* from radioactive slime reached a plateau after 334 h; at which time, ~17% of the radioactive L-fucose in the polymer had been removed. Lower percentages of L-fucose were liberated from some radioactive preparations of slime which had been labelled to a different extent by incubation with L-fucose-*t*. This result confirms the demonstration by Kirby and Roberts[27] that the fucose is in the L configuration, and the specificity of the enzyme[28] indicates that at least some α-links are present. The remaining 83% of the fucose must be linked through glycosidic bonds that cannot be split by the enzyme, either because the glycosidic link is β or because the fucose is in such a position within the molecule that the enzyme cannot attack it. This situation must also be true of the intact glycoproteins which are resistant to the enzyme[4,6] probably due to the tertiary structure of the molecule in solution. The purified fucosidase was capable of releasing at least 48% of the fucose monomers from a fucoidin preparation from which most of the sulphate residues had been removed. Therefore, a homo-oligomer of α-L-fucose residues with L-fucose at the non-reducing end is susceptible to the enzyme, probably by successive removal of residues from this end.

If the enzyme removes only the external 17% of the fucose residues of the slime it is not surprising that, using ^{14}C-labelled slime in which the fucose residues comprise only 12% of the total label[15], no difference could be detected between material released in the presence and absence of fucosidase.

When the products of a digestion of L-fucose-*t*-labelled slime and α-L-fucosidase were separated by paper chromatography, no indication was found of radioactive material other than at the origin (polymeric material) or in the position of fucose. Hence, no dimers of fucose were released. Neither was the polymer substantially degraded in size by the enzyme.

In all the glycoproteins that contain fucose and in which the structure of the carbohydrate moiety has been examined, fucose occurs terminally[16]. This is also the position occupied by this sugar in the fucoxyloglucan isolated and characterised from the medium of suspension-cultured sycamore cells[29]. The α-L-fucosidase from rat epididymis was capable of removing all the terminal fucose residues from the LH-β peptide[3], although not from some intact glycoproteins[6]. Therefore, unless the slime has such a complex three-dimensional structure as to mask 83% of its terminal fucose

units, a large proportion of the fucose residues of maize slime are not at the non reducing ends of branches. We have previously presented evidence which indicated that the slime contained polysaccharides in which the fucose was present with other sugars as neutral blocks attached to a polyuronic acid backbone[11,15]. A large proportion of the galacturonic acid residues were glycosidically linked to glucose[24]. The results obtained with pure α-L-fucosidase are consistent with these ideas.

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