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STRUCTURE OF A GALACTOMANNAN–PEPTIDE ALLERGEN FROM *TRICHOPHYTON MENTAGROPHYTES*

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SUMMARY

A galactomannan–peptide allergen isolated from the pathogenic fungus *Trichophyton mentagrophytes* has been shown to contain D-galactose (9 %), D-mannose (73 %) and protein (9 %). A molecular weight of 40 000 was suggested, indicating a basic structure of about 200 mannose units. D-Galactose occurred only in non-reducing terminal positions and in the furanose form. In addition, an equal proportion of D-mannopyranose terminal units was present. Approximately equal numbers of terminal units, chain units and branch points were found, the predominant linkages in the chain being 1→2 and 1→4, in equal proportions. N-Terminal amino acid assay indicated the presence of at least three major peptide chains ending in threonine, glycine and alanine.

INTRODUCTION

A number of yeasts and fungi have been shown[1,2] to produce polysaccharides, the most common monosaccharide constituents of which are mannose, galactose and glucose. Mannans and glucomannans have been isolated from several yeasts and in some publications[3–5] the presence of firmly attached protein has been recorded. Several yeast mannans have been studied in detail, particularly the mannan from *Saccharomyces cerevisiae*[6,7], but also one from *Saccharomyces rouxii*[5], which contained 15 % protein, and a protein-free mannan from *Candida albicans*[8]. Recently a galactomannan from *Trichophyton granulosum* has been characterised[9] and found to consist of a highly branched mannan with chiefly 1→2 and 1→6 linkages and mannopyranose and galactofuranose terminal units. The molecule contained no nitrogen, presumably owing to the alkali conditions of extraction and a prolonged treatment with trypsin.

Recent work on "Trichophytin" in these laboratories[10–12] has been concerned with the isolation and immunological properties of a purified glycopeptide from *Trichophyton mentagrophytes*. Degradation studies established that the two hypersensitivity responses elicited by the molecule, the "immediate" and "delayed" reactions, could be specifically attributed to the carbohydrate and the protein moieties respectively. The present paper concerns the more detailed chemical structure of the allergen.

MATERIALS AND METHODS

Isolation of biologically active galactomannan–peptide

Trichophyton mentagrophytes (NTC: D 281) was grown in submerged culture[13] on a medium containing glucose (4 %), "Panmede" (Paines & Byrne Ltd., 1 %) and acid-hydrolysed casein (2 %). The acetone-dried mycelium was ground in ethylene glycol (400 ml/100 g) and the extract was dialysed, Seitz-filtered and freeze-dried. 60 ml Cetrimide solution (5 %, w/v) was added to glycol extract (3 g) dissolved in 120 ml 0.5 % borate buffer (pH 7). Addition of dilute sodium hydroxide gave precipitates[14] between pH 6.0–7.6, 7.6–9.1 and 9.1–11.0 After recovery by centrifugation, these were washed with water, dissolved in 2 % acetic acid (5 ml) and the glycopeptides recovered by precipitation with ethanol followed by washing with 1 % acetic acid in ethanol, ethanol and ether. Solutions of the products were freed from the detergent by passage through Zeo-karb 225 (H^+). Yields: fraction pH 6.0–7.6, 8 mg; pH 7.6–9.1, 990 mg; pH 9.1–11.0, 40 mg. Material (1.2 g) was also recovered from the residual supernatant solution. The composition of these fractions has already been reported[12] and skin testing in sensitised guinea-pigs and infected humans established that the galactomannan–peptide precipitated between pH 7.6 and 9.1 was the most biologically active. Further chemical studies to be described concern this material. It has been shown[12] to be homogeneous in an analytical ultracentrifuge, after electrophoretic separation and after gel filtration[15] on Sephadex (Pharmacia, Uppsala). Specific rotations, $[\alpha]_D^{17}$ were measured on 1 % aqueous solutions of the galactomannan–peptide, and on the protein-free carbohydrate.

Hydrolysis and determination of sugars

Known quantities (approx. 15 mg) of galactomannan–peptide were hydrolysed with 2 N sulphuric acid (2 ml) at 100° for 4 h. For determination of individual sugars by chromatographic methods, glucose was incorporated into the hydrolysis mixture as an internal standard. Total hexose was determined[16] by treating a solution of hydrolysate containing 40–60 μg hexose in 2 ml with 0.2 % orcinol in concentrated sulphuric acid (4 ml). Calibration was effected by similar determinations on galactose and mannose solutions in the range 10–100 μg. Portions of hydrolysate were treated with De-Acidite FF (CO_3^{2-}) to remove acid and amino acids, and aliquots containing galactose and mannose within the range 20–80 μg were applied to Whatman No. 1 paper together with calibration standards. The paper was irrigated with ethyl acetate–pyridine–water (12:5:4, v/v) and developed with aniline hydrogen phthalate. Individual hexose components were determined by elution and assay of the coloured areas[17].

Galactomannan–peptide was also treated with 0.01 N hydrochloric acid (5 mg in 1 ml) at 80° for 2 h. The solution was neutralised with silver carbonate and hexose products were examined chromatographically. The presence of D-galactose was established by assay of the hydrolysate with the D-galactose oxidase from *Polyporus circinatus*[18].

Identification of amino acid components and N-terminal end-groups

Galactomannan–peptide (15 mg) was hydrolysed with 6 N hydrochloric acid (1 ml) for 15 h at 105°. The acid was removed by distillation and the hydrolysate applied to a column of Zeo-karb 225 (H^+). After washing the column with water,

amino acids were eluted with 3 N ammonia solution and recovered by evaporation. The amino acid hydrolysate was analysed[19] by two-way separation on Whatman No. 1 paper using *n*-butanol–ethyl methyl ketone–water–17 N ammonia (5:3:1:1, v/v) followed by *n*-butanol–acetic acid–water (12:3:5).

Galactomannan–peptide (40 mg) and sodium bicarbonate (25 mg) were dissolved in water (0.4 ml) and treated with 0.8 ml 5% ethanolic FDNB[20]. Reaction was allowed to proceed for 2 h at 20° in the dark and then terminated by acidification, followed by ether extraction. The DNP-glycopeptide was precipitated from the aqueous layer with ethanol, washed with ethanol and ether and freeze-dried. DNP-terminal amino acids were obtained by hydrolysis in 12 N hydrochloric acid for 5 h at 105° followed by ether extraction. Addition of 1 N sodium bicarbonate followed by acidification and ether extraction of the aqueous layer led to the further purification of the derivatives. The residue after evaporation of the ether was heated in 17 N ammonia (1 ml) in a sealed tube for 15 h at 105°. This converted the DNP derivatives into free amino acids[21] which were recovered after evaporation and ether extraction and analysed by paper chromatography.

Degradation studies on galactomannan–peptide

Portions (15–20 mg) of galactomannan–peptide were treated with various reagents at room temperature or at 37°. After the appropriate neutralisation the reaction mixtures were applied to columns of Sephadex gel (G-50) and the eluates examined for carbohydrate and protein using the resorcinol disulphonic acid[22] and ninhydrin[23] reagents.

Periodate oxidation studies

Galactomannan–peptide (192 mg) was oxidised with 0.01 M sodium metaperiodate (500 ml) at ambient temperature for 7 days. Aliquots were examined at intervals for periodate uptake[24] and determination of oxidation products. Formic acid was determined titrimetrically, and non-volatile acid by titration after evaporation. Formaldehyde was determined using the chromotropic acid reagent[25], and ammonia using Nessler's reagent[26], calibration being carried out with a mannitol oxidation mixture and a standard ammonium chloride solution respectively.

The reaction was terminated by addition of 1 ml ethylene glycol followed by dialysis. The oxidised glycopeptide residue was recovered by freeze-drying and dissolved with an equal weight of sodium borohydride in 10 ml water[27]. The pH was maintained at 7 by the passage of carbon dioxide. After 5 h the reduced material was resolved from ions by Sephadex (G-50) column chromatography and freeze-dried.

Sugar analysis of this degraded galactomannan–peptide was performed in the same way as for the intact material. In addition, tetritol and glycerol were determined[27]. Hydrolysate solution containing 50–150 μg of sugar alcohols was applied in duplicate to Whatman No. 1 paper, together with authentic glycerol, erythritol, threitol, galactose and mannose. Irrigation was performed with ethyl acetate–pyridine–water solution (12:5:4, v/v), and marker sections were developed with silver nitrate reagent[28]. The unresolved tetritol region was cut out, eluted with water, and applied to Whatman No. 3 MM paper and subjected to ionophoresis[29] (12 V/cm) in molybdate buffer[30] (pH 5). On development with silver nitrate reagent, the mobilities of hydrolysis components were compared with those of the authentic tetritols.

Eluates (1 ml) from a further chromatogram were treated with 0.01 M sodium metaperiodate, followed after 30 min by 10% lead dithionate (1 ml). The formaldehyde yield was determined with the chromotropic acid reagent using erythritol and glycerol as calibration standards, eluted from paper and oxidised in the same way.

Methylation studies on galactomannan–peptide

Glycopeptide (490 mg) was dissolved in water (40 ml) in a 250-ml flask fitted for stirring and passage of nitrogen, and maintained at 50° on a water bath. 14 ml 30% sodium hydroxide and redistilled dimethyl sulphate (11 ml) were added[31] over 2 h, maintaining the pH as near neutral as possible. The reaction was terminated by raising the temperature to 95°, and after cooling the solution was dialysed and freeze-dried. This procedure was performed four times.

The methylated glycopeptide (270 mg, $[\alpha]_D^{17}$ + 52.5° in water (*c*, 1%) was heated under reflux in 5% methanolic hydrogen chloride (10 ml), then neutralised with silver carbonate. The methyl glycoside solution was evaporated to dryness, dissolved in 1 ml 2N H_2SO_4 and heated at 100° for 4 h. The solution, after neutralisation with barium carbonate, was evaporated to dryness and the methyl sugars were taken up in the solvent to be used for paper and cellulose column chromatography: *n*-butanol–ethanol–water–17 N ammonia (40:10:49:1, v/v).

Column chromatography on cellulose[32] was employed to resolve the methyl sugars into four major fractions containing tetra-, tri-, di- and mono-*O*-methyl components. Paper chromatography of portions of the eluate located these fractions. Ionophoresis[29] (12 V/cm) in 0.1 M borate buffer (pH 10) on Whatman No. 3 MM paper facilitated the further resolution of the mixtures of tri-*O*-methyl and di-*O*-methyl components. Areas containing different components were eluted with water, passed through Zeo-karb 225 (H^+) resin and evaporated; boric acid was removed by repeated distillation with methanol.

Demethylation was carried out with boron trichloride[33] and the products were examined by paper chromatography. Tri-*O*-methyl components were reduced with an equal weight of sodium borohydride in water for 4 h. The solution was then passed down Zeo-karb 225 (H^+) resin and evaporated to dryness several times with methanol. The methyl sugar alcohols were weighed and dissolved in 0.02 M sodium metaperiodate (0.5 mg/ml). After oxidation for 1 h, periodate uptake and formaldehyde yield were determined as already described. Oxidation products were recovered, deionised electrolytically, and examined by paper chromatography in *n*-butanol–ethanol–water–ammonia. Known standards were similarly treated and the products used to establish the identity of the unknowns.

RESULTS

The results refer to the galactomannan–peptide fraction precipitated in the pH range 7.6–9.1 by cetrimide. This had a sedimentation coefficient $s_{20,w}$ 2.21 in barbitone buffer (pH 8.6) and a specific rotation $[\alpha]_D^{17}$ +32.6° in water (*c*, 1%). After removal of 85% of the protein with ficin this value rose to +35.1°. Hexose analyses showed that the carbohydrate accounted for 80–90% of the molecule, and consisted of mannose and galactose only, in ratios varying from 7:1 to 4:1 in different cultures. Qualitative amino acid analyses showed major amounts of alanine, glycine, valine,

leucine, isoleucine, proline, serine, threonine and aspartic and glutamic acids, smaller amounts of phenylalanine and tyrosine and only traces of basic and sulphur-containing amino acids. The optical rotation of a carbohydrate-free amino acid hydrolysate was negative. On the basis of this composition the proportion of anhydro-amino acids in the galactomannan–peptide was 7–10 %. N-Terminal amino acid analysis led to the detection of glycine, alanine and threonine from dinitrophenylated derivatives. Hexose and nitrogen determinations on the material used for most of the structural investigations gave the composition D-mannose, 73 %; D-galactose, 9 %; protein, 8.8 %. The proportions of mannose and galactose in the carbohydrate moiety were, therefore, 89 % and 11 %. Hydrolysis with 0.01 N hydrochloric acid was found to liberate all the galactose but only a trace of mannose. The test with D-galactose oxidase proved positive.

Experiments to determine the stability of the galactomannan–peptide linkage have been already reported[12], since such studies were required as controls in the biological investigations. Hydrochloric acid (pH 2 at 37° for 24 h) released a negligible amount of peptide, alkali conditions (pH 9 at 37° for 24 h) produced slight degradation, while sodium borohydride had a negligible effect at pH 7, but at pH 10 the nitrogen percentage fell from 1.53 % to 1.14 %. On another material sodium periodate (0.01 N at 17° for 2 h) caused a change from 1.26 % to 1.21 % N.

Periodate oxidation studies showed a steady uptake during the first 16–24 h, followed by a slower uptake continuing for a further 4–6 days. Values of periodate uptake, formic acid and formaldehyde yields were converted to molar quantities per 162 g of carbohydrate. No ammonia was detected during oxidation. In a typical experiment an uptake of 1.36 moles sodium periodate was recorded after 24 h, rising to 1.41 moles at 96 h. Formic acid values at these times were 0.26 and 0.40 mole. Formaldehyde was produced in a yield of 0.13 mole after 4 min and did not rise subsequently.

Reduction and hydrolysis of the residue after 4 days' oxidation led to the detection of glycerol and erythritol in yields of 0.29 and 0.15 mole respectively per 162 g of carbohydrate. A separate experiment in which residues were examined after oxidation times of 4 min, 4 h, 24 h, and 4 days showed an erythritol yield which rose steadily over the 4 days, and a glycerol yield which was maximum at 24 h, and fell to 90 % of this value at 4 days. Threitol was not found in the hydrolysate. Analysis of hexoses showed a reduction from 82 % in the intact glycopeptide to 30 % after oxidation. All the galactose had been oxidised; the mannose had fallen from 73 % to 30 %, that is, from 89 % to 36.5 % of the carbohydrate moiety.

Methylation studies yielded a mixture of tetra-, tri-, di- and mono-*O*-methyl hexose derivatives which showed the following properties. M_G values refer to ionophoretic mobility in borate, and R_F values to paper chromatography in *n*-butanol–ethanol–water–ammonia.

Fraction 1 (26 % by weight of recovered methyl sugars) had $[\alpha]_{5461}^{17}$ + 32.6° in methanol (*c*, 0.5 %) and gave mannose on demethylation. It had R_F 0.80 and M_G 0.00 and was indistinguishable from 2,3,4,6-tetra-*O*-methyl mannose.

Fraction 2 (30 % by weight) had $[\alpha]_{5461}^{17}$ + 35.7° in methanol (*c*, 0.5 %) and gave mannose only on demethylation. It was partially resolved on paper chromatography into components having R_F 0.70 and 0.66. M_G values of 0.00 and 0.40 facilitated ionophoretic separation into components giving characteristic colours, red and

brown respectively, with aniline hydrogen phthalate. Ionophoresis of authentic 2,3,6-, 2,4,6-, 2,3,4- and 3,4,6-tri-*O*-methyl mannoses gave M_G values of 0.00, 0.00, 0.04 and 0.40, respectively. The former three were red on paper with aniline hydrogen phthalate, the 3,4,6-isomer was brown. A component of M_G 0.04 was not found, so that 2,3,4-tri-*O*-methyl mannose was excluded. In the presence of 5 % methanolic hydrogen chloride at ambient temperature evidence of furanoside formation was not obtained for either isomer, but this does not exclude 2,3,6-tri-*O*-methyl mannose which, unlike the glucose derivative, does not show this property[34]. Periodate oxidation of the tri-*O*-methyl sugar alcohols yielded formaldehyde in the case of the M_G 0.4 reduction product, but only a trace was produced from the other isomer. This would exclude 2,3,4-tri-*O*-methyl mannose in the M_G 0.0 fraction, and indicate 3,4,6-tri-*O*-methyl mannose as the M_G 0.4 component. Each fraction showed a periodate uptake approximating to 1 mole/mole, indicating the absence of 2,4,6-tri-*O*-methyl mannose as a major constituent. Chromatography of the deionised oxidation products from authentic and unknown tri-*O*-methyl mannitol derivatives showed different patterns of reducing compounds which served to confirm the identity of the two ionophoretic components. The M_G 0.4 component was thus established as 3,4,6-tri-*O*-methyl mannose, and the M_G 0.0 component was concluded to be 2,3,6-tri-*O*-methyl mannose, (though a small proportion of the 2,4,6-isomer was not excluded).

Fraction 3 (28 % by weight) contained a mixture of di-*O*-methyl sugars. Two materials of R_F 0.57 and 0.51 were partially resolved on paper chromatography, but ionophoresis effected a resolution into at least four components, M_G: 0.13, 0.19, 0.53 and 0.58. Fraction 4 (16 % by weight) was an unexamined mixture of monomethyl sugars, R_F: 0.34–0.37.

The proportions by weight of these D-mannose derivatives account for the mannan, but must be adjusted to express percentages in the galactomannan. The values for tetra-, tri-, di-, mono-*O*-methyl mannose proportions then become 23:27:25:14.

DISCUSSION

The yield and constitution of the "Trichophytin" galactomannan–peptide were found to show some variation in different cultures, but all samples were constant in their biological properties. The chief variation was in the proportion of D-galactose, which, in two samples analysed, accounted for 9 % and 20 % of the glycopeptide.

Analysis of the peptide moiety indicated the presence of ten major amino acids (predominantly L) of the neutral, hydroxy and acidic types, smaller amounts of the basic and aromatic types, and a negligible proportion of sulphur-containing units. Evidence for a limited number of peptide chains is given by the finding of three N-terminal amino acids, while the ability of proteolytic enzymes to remove 80–90 % of the amino acids[12] indicated a predominance of peptide rather than carbohydrate–amino acid linkages. Assuming a molecular weight of 40 000, 4000 would be accounted for by a 10 % proportion of protein. The presence of ten predominant amino acids of average anhydro molecular weight 98 allows of four of each type of unit. If each N-terminal unit represented a single chain, then three peptides of 13–14 units would be indicated.

After treatment with sodium periodate, slight degradation of the protein was apparent; this has been frequently observed in mucopolysaccharide chemistry[35]. Sodium borohydride also caused some peptide cleavage, in both the oxidised and

intact glycopeptides, but this was obviated by performing the reaction at pH 7. Incubations with dilute alkali (pH 10) and acid (pH 2) at 37° had little effect. After methylation, amino acid chromatograms lacked the hydroxy amino acid components, suggesting that the hydroxyl groups were unsubstituted and not involved in the carbohydrate–peptide bond. No definite conclusion can therefore be adduced on the nature of the carbohydrate–peptide linkage, but its considerable stability is to be noted.

The presence of D-galactose was confirmed by the specific reaction with D-galactose oxidase, while its preferential release on mild hydrolysis suggested terminal positions and the furanose form. The immediate release of 0.13 mole of formaldehyde on periodate oxidation suggested that the galactose units (11 % of the carbohydrate) were responsible, and the finding of arabinose after reduction and hydrolysis of the 4-min oxidation product confirmed that galactofuranose with a free C-6 hydroxyl was present. The absence of methyl ethers of galactose in the products of methylation was accounted for by the effect of wide variations of pH, and a temperature of 40° during the procedure. Cleavage of the furanoside linkages would be followed by loss through dialysis.

The isolation of 23 % of the methylated carbohydrate as 2,3,4,6-tetra-*O*-methyl D-mannopyranose was evidence of terminal mannose units, but, in view of the loss of galactose end groups, this must include 11 % of former penultimate mannose units, leaving 12 % actual terminal mannose residues. Conclusions may be drawn on the interior of the carbohydrate from the proportion (27 %) of the tri-*O*-methyl fraction and from the detection of erythritol from 15 % of the hexose units. Such units must be 1→4 linked, and would give rise to 2,3,6-tri-*O*-methyl mannose in the methylation procedure. The remaining 12 % of the tri-*O*-methyl fraction is the 3,4,6-isomer produced from units linked 1→2.

At 24 h, 26 % of the units are oxidised with the liberation of formic acid; overoxidation may account for part of this figure and would explain the continuous increase from 0.26 mole at 24 h to 0.40 mole at 4 days. Formic acid would be produced from the 12 % of non-reducing terminal pyranose residues, and further amounts would arise from overoxidation of the malondialdehyde structure[36], produced in the terminal furanose units. The glycerol yield (0.29 mole) is the combination of yields from 1→2 linked units and terminal mannopyranose and galactofuranose. However, overoxidation precluded the formation of the full theoretical yield and explains the fall in glycerol obtained from products of oxidation after 24 h.

Yields of the di- and mono-*O*-methyl fractions (25 % and 14 %) together account for 39 % of the carbohydrate, which is to be correlated with the percentage of hexose residues resistant to periodate oxidation (36 %). Branch points in the mannose chain include units to which protein is attached. The galactomannan–peptide of the postulated molecular weight 40 000 would contain a proportion of mannose accounting for about 32 000, that is, 200 mannose units. Peptides are thus only attached to 3 in 200 units; and, on a similar basis, galactofuranose units (11 % of the carbohydrate) are attached as end-groups to 22 in 200 units.

In the mannan itself, the proportions of terminal, 1→2 linked, 1→4 linked and branch units are 1.9:1:1.25:3.25. A similar mannan in which the corresponding ratios are 1.65:1:0.18:1.9 has been isolated from *Candida albicans*[8]. The excess of branch points is partly the effect of protein attachments, which, besides giving rise to additional "branch" points may also sterically hinder methylation, on which the ratios

are based. Allowing for these effects, the ratio of terminal:non-branched:branched units in the mannan becomes approximately 1:1:1 as in the yeast mannans[5–7].

Certain differences are noted between the polysaccharides of *Trichophyton mentagrophytes* and *Trichophyton granulosum* despite the close relationship of these strains. In the latter, a smaller degree of branching was found, and the predominant linkages were 1→2 and 1→6. The polysaccharides were, however, similar in having terminal galactofuranose units, a feature found also in galactocarylose[37] and Pneumococcus type 34 polysaccharide[38]. The difference in the protein content of the two materials may be explained by the extraction procedures; it was, however, the aim of this work to establish in more detail the structure of the reactive sites, both carbohydrate and protein, concerned in the hypersensitivity responses.

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